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# Therapeutic Response in Human Cancers: The Dual Role of STAT Signalling

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# Therapeutic response in human cancers: The dual role of STAT signalling
# THESIS FOR DOCTORAL DEGREE (PhD)

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*To my beloved husband Klementy,*

*To my wonderful daughter Anja,*

*To my dearest parents Viktor Kolosenko and Kateryna Kolosenko*

But it ain't about how hard you hit. It's about how hard you can get hit and keep moving forward. How much you can take and keep moving forward. That's how winning is done

S. Stallone

If it wasn't hard, everyone would do it. It's the hard what makes it great.

T. Hanks

# Abstract

STAT proteins were discovered as the mediators of interferon (IFN) signalling in response to viral infections. Later, it has become evident that STATs are activated by many stimuli and that they exert multifaceted effects by regulating gene transcription. Of seven members of the STAT family, my thesis dealt with STAT1, STAT2, and STAT3 and the genes regulated by transcriptional complexes containing them. STAT1 and STAT3 were believed to regulate the opposing functions, the former acting as a tumour suppressor and the latter being an oncogene.

Type I IFNs have been used for the treatment of infectious diseases and some types of cancer. In **Paper I**, we investigated pathways involved in the IFN-induced apoptosis in a myeloma cell line, and what role the STAT1 phosphorylation plays in this model. We used chemical inhibitors of pSTAT1, Akt, mTOR, and cells with a dominant-negative mutant form of STAT1 to evaluate which pathways are essential for the pro-apoptotic effect of IFNα. We have found that pSTAT1 is important, but cooperation with other signalling pathways is necessary to maximize the pro-apoptotic effect of IFNα.

In **paper II,** we used multicellular spheroids (3D culture) as a model to study a gene signature associated with drug resistance. We have found that STAT1, STAT2, and IRF9, as well as IFN-stimulated genes (ISGs), have increased expression in this drug resistance model. Moreover, a similar gene signature is induced in cells cultured for a prolonged time with no trace of IFN detected. The expression of ISGs was not STAT1 dependent but was controlled by STAT2 and IRF9. Overexpression of IRF9 alone was sufficient to drive the transcription of the ISGs and to induce drug resistance in the cells. Therefore, IRF9-induced gene signature can be explored as a marker for therapy response in cancer.

In **Paper III**, we studied the role of the constitutively activated STAT3 in the sensitivity of multiple myeloma cells to the Hsp90 inhibitors treatment. We used a panel of cell lines categorised by different basal levels of the pTyr705STAT3 and of CD45 and found that the sensitivity of myeloma cells to an Hsp90 inhibitor correlated with the presence of pSTAT3. Using samples from multiple myeloma patients, we have demonstrated that it is the pSTAT3+CD45+ cell population that undergoes apoptosis in response to the Hsp90 inhibitor treatment. Thus, pSTAT3/CD45 can be used as a stratification marker for the use of these drugs.

In **Paper IV,** we attempted to develop new inhibitors targeting STAT3. After the screening campaign, we have chosen several inhibitors that preferentially affect the viability in STAT3-dependent cell lines. The inhibitors have different effects on the phosphorylation of STAT3 and STAT1, but regardless of that, all the compounds interfere with the STAT3-driven gene transcription. One of the compounds, KI16, preferentially inhibits the phosphorylation of STAT3 over STAT1. It also docks well to the SH2-domain of STAT3 and has a potential to be developed as a STAT3-targeting drug. Other compounds act through a different mechanism(s), but are also plausible for chemical modifications and development, both as drugs and as molecular probes to identify novel targets important for the full oncogenic function of STAT3.

Taken together, our findings demonstrate that the roles of STAT1 and STAT3 in cancer are not strictly determined, but are highly context-dependent. It appears that the IFN/STAT1 signalling can be both pro-apoptotic and pro-survival, whereas the oncogenic JAK/STAT3 axis can be targeted to induce cancer cell death.

# List of Scientific Papers

I. Arulampalam, V., **Kolosenko, I.,** Hjortsberg, L., Bjorklund, A.C., Grander, D. and Tamm, K.P. (2011) Activation of STAT1 is required for interferon-alpha-mediated cell death. *Experimental cell research*, **317**, 9-19.

II. **Kolosenko, I.,** Fryknas, M., Forsberg, S., Johnsson, P., Cheon, H., Holvey-Bates, E.G., Edsbacker, E., Pellegrini, P., Rassoolzadeh, H., Brnjic, S., Larsson, R., Stark, GR., Grander, D., Linder, S., Tamm, K.P.* and De Milito, A*. (2015) Cell crowding induces interferon regulatory factor 9, which confers resistance to chemotherapeutic drugs. *International journal of cancer.* **136**, E51-61.

III. Lin, H*., **Kolosenko, I***., Bjorklund, A.C., Protsyuk, D., Osterborg, A., Grander, D. and Tamm, K.P. (2013) An activated JAK/STAT3 pathway and CD45 expression are associated with sensitivity to Hsp90 inhibitors in multiple myeloma. *Experimental cell research*, **319**, 600-611.

IV. **Kolosenko, I.,** Yu, Y.,* Busker, S.,* Page, B., Liu, J., Reese, A., Volk, N., Haraldsson, M., Lundbäck, T., Tamm K.P. and Grander D. (2016) Development and characterization of novel STAT inhibitors. *Manuscript in preparation.*

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Akcakaya, P., Ekelund, S., **Kolosenko, I.,** Caramuta, S., Ozata, D.M., Xie, H., Lindforss, U., Olivecrona, H. and Lui, W.O. (2011) miR-185 and miR-133b deregulation is associated with overall survival and metastasis in colorectal cancer. *International journal of oncology*, **39**, 311-318.

# Contents

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 17-DMAG | 17-Desmethoxy-17-N,N-dimethylaminoethylamino-geldanamycin |
| 17-AAG | 17-Allylamino-17-demethoxygeldanamycin |
| ABC | ATP-binding cassette |
| ABL | Abelson murine leukaemia viral oncogene homolog 1 |
| ADP | Adenosine diphosphate |
| AIDS | Acquired immune deficiency syndrome |
| AKT(PKB) | Protein kinase B |
| AML | Acute myeloid leukaemia |
| AP1 | Activator protein 1 |
| ATP | Adenosine triphosphate |
| BAFF | B-cell activating factor |
| BCL3 | B-cell lymphoma 3-encoded protein gene |
| BCR | B-cell receptor |
| BMSC | Bone marrow stroma cell |
| C/EBP | CCAAT-enhancer-binding protein |
| CD45 | Cluster of differentiation |
| cDNA | complementary DNA |
| CML | Chronic myeloid leukaemia |
| CNTF | Ciliary neurotrophic factor |
| cPARP1 | cleaved poly-(ADP-ribose) polymerase |
| CRP | C-reactive protein |
| CSC | Cancer stem cells |
| DNA | Deoxyribonucleic acid |
| DR5 | Death receptor 5 |
| dsRNA | Double-stranded RNA |
| ECM | Extracellular matrix |
| EGF-R | Epidermal growth factor receptor |
| EMSA | Electrophoretic mobility shift assay |
| EMT | Epithelial–mesenchymal transition |
| EPO | Erythropoietin |
| ER | Oestrogen receptor |
| ER | Endoplasmic reticulum |
| Erk | Extracellular signal-regulated kinase |
| ESC | Embryonic stem cells |
| FDA | Food and Drug Administration |
| FGF | Fibroblast growth factors |
| FISH | Fluorescence in situ hybridisation |
| FOXP3 | Forkhead box P3 |
| G-CSF | Granulocyte-colony stimulating factor |
| GABA | gamma-Aminobutyric acid |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GAS | Gamma interferon activation site |
| gp | Glycoprotein |
| GPCR | G-protein-coupled receptor |

| | |
|---|---|
| Grp (HSP90B1) | Heat Shock Protein 90 kDa Beta Family Member 1 |
| GSEA | Gene-set enrichment analysis |
| HER2 | Human epidermal growth factor receptor 2 |
| Hsp90 | Heat shock protein 90 kDa |
| Hsp90-I | Hsp90 inhibitors |
| hTERT | Telomerase reverse transcriptase |
| IC50 | Half maximal inhibitory concentration |
| IFI27 | Interferon alpha-inducible protein 27 |
| IFITM1 | Interferon-induced transmembrane protein 1 |
| IFN | Interferon |
| IFNAR | Interferon-α/β receptor |
| IGF-R | Insulin-like growth factor 1 receptor |
| IHC | Immunohistochemistry |
| IL6 | Interleukin 6 |
| IRDS | Interferon-related DNA damage signature |
| IRF9 | Interferon regulatory factor 9 |
| IRS | insulin receptor substrate |
| ISGF3 | Interferon-stimulated gene factor 3 |
| ISGs | Interferon-stimulated genes |
| ISRE | interferon-stimulated response element |
| JAK | Janus-associated kinase |
| JNK | c-Jun N-terminal kinases |
| JUNB | Transcription factor jun-B gene |
| LIF | Leukaemia inhibitory factor |
| MAPK | Mitogen-activated protein kinases |
| MCS | Multicellular spheroids |
| MDM2 | Mouse double minute 2 homolog |
| MEK/MAPKK | Mitogen-activated protein kinase kinase |
| MGUS | Monoclonal gammopathy of undetermined significance |
| MHC | Major histocompatibility complex |
| mitoSTAT3 | Mitochondrial STAT3 |
| MM | Multiple myeloma |
| MnSOD | Manganese superoxide dismutase |
| mRNA | Messenger RNA |
| mTOR | mammalian target of rapamycin |
| MUC | Mucin |
| NF-κB | Nuclear factor-κB |
| NK | Natural killer cells |
| NMDAR | N-methyl-D-aspartate receptor |
| OAS1 | 2′-5′-oligoadenylate synthetase 1 |
| ODN | Oligodeoxynucleotide |
| PAIN | Pan-assay interference compounds |
| PD-L | Programmed death-ligand |
| PDGF-R | Beta-type platelet-derived growth factor receptor |
| PGC1 | Peroxisome proliferator-activated receptor-γ coactivator |

| | |
|---|---|
| PI3K | Phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PIAS | Protein inhibitor of activated STAT |
| pS, pSer | phospho Serine |
| pTyr | phospho-Tyrosine |
| Pyr6 | Pyridone 6, JAK inhibitor I |
| qRT-PCR | Reverse transcription polymerase chain reaction / Real-time polymerase chain reaction |
| RA | Rheumatoid arthritis |
| Rb | Retinoblastoma protein |
| RNA | Ribonucleic acid |
| RNAi (siRNA) | RNA interference (short inhibitory RNA) |
| Rnase L | Ribonuclease L |
| ROR | RAR-related orphan receptor |
| RTK | Receptor tyrosine kinase |
| S1PR | Sphingosine-1-phosphate receptor |
| SH2 domain | Src Homology 2 |
| shRNA | Short-hairpin RNA |
| SIE | STAT-inducible element |
| SLC | Solute carrier |
| SNP | Single nucleotide polymorphism |
| SOCS | Suppressor of cytokine signalling |
| SRC | Sarcoma proto-oncogene tyrosine-protein kinase |
| SSM | Smouldering multiple myeloma |
| STAT | Signal transducer and activator of transcription |
| STAT3C | Constitutively active STAT3 |
| STATTIC | STAT inhibitory compound |
| TAD | Trans-activating domain |
| TC45/TCPTP | T-cell protein tyrosine phosphatase |
| TGF | Transforming growth factor |
| Th | T helper cell |
| TK | Tyrosine kinase |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| TRAIL | TNF-related apoptosis-inducing ligand |
| TRAP | Tumour necrosis factor-associated protein 1 |
| Tregs | Regulatory T- cells |
| TRIM15 | Tripartite motif containing 15 gene |
| Tyk2 | Tyrosine-protein kinase 2 |
| VEGF | Vascular endothelial growth factor |

# 1 INTRODUCTION

In this section, I address the main concepts that are relevant for the papers included in the thesis.

## 1.1 CANCER AS A PAYMENT FOR MULTI-CELLULARITY AND LONGEVITY

Cancer is not a single disease, but a collective name for hundreds of diseases drastically different in their topology, clinical course, therapy response, and outcome.[1] From the evolutionary point of view, cancer is a by-product and a consequence of the multicellular organisms development that have complex regulatory mechanisms of cell fate.[2] Despite the intricate mechanisms of cell cycle and cell death regulation in higher animals, stochastically occurring mistakes do happen during the cell division. Normally, they get corrected by a DNA repair system, or a cell carrying a defect is eliminated.[3] However, when odds are not in one's favour, a cell with the damaged genomic information survives and passes the defect to the offspring cells. It creates a vicious circle of cell divisions that produce a lot of cells with an unrepaired DNA damage. The probability of a mistake leading to oncogenic transformation is proportional to the cell number and the lifespan of an organism (hence, the number of divisions).

However, not all mutations lead to cancer development. The human genome is estimated to contain 6 billion base pairs. About one mismatch is generated per one cell division. That accounts for the estimated mutation frequency of about $10^{-5}$-$10^{-7}$ per gene.[4] Of note, the mutation frequency is not uniform across the genome and even throughout a chromosome.[5] Firstly, it highly depends on a gene expression. Secondly, a gene location within a chromosome plays a role, as the genes at the chromosome ends tend to be more prone to mutation accumulation. It might be explained by nucleotide depletion and a shorter time these genes are in a complex with a DNA-polymerase and mismatch repair proteins.[6,7]

Advances in sequencing have allowed the estimation of the mutation rate in absolute values. Y-chromosomes from two individuals with a common ancestor born 200 years ago and 13 generations of faithful Y-chromosome passing from fathers to sons were sequenced. That summed up to about one mutation/ $3x10^7$ base pairs and resulted in an estimate of about 100-200 new mutations per generation.[8] Thus, the mutation frequency is much higher than the frequency of cancer development when DNA repair systems and the immune system detection are in place.

### 1.1.1 The acquired capabilities of cancer cells

The common features shared by the majority of cancers regardless of their origin have been called the 'hallmarks of cancer'.[9] As the knowledge on the molecular basis of cancer deepened, the view of cancer hallmarks also progressed and new hallmarks have been added.[10] Interestingly, following the publication of both reviews on the hallmarks of cancer, an avalanche of papers appeared suggesting yet new hallmarks of cancer. (e.g., cancer stem cells or intra-tumour pH).[11,12] This demonstrates that we still have a lot to learn regarding how cancer cells function.

According to the most general and the most simplistic theory, all cancers can be described by the following acquired capabilities (hallmarks) that distinguish them from normal cells:

- sustaining proliferative signalling;

- evading growth suppressors;
- resisting cell death;
- enabling replicative immortality;
- inducing angiogenesis;
- activating invasion and metastasis.

Sustaining proliferative signalling and evading growth suppressors.

Under normal conditions, a balance between proliferative and anti-proliferative signals directs the fate of a cell depending on the requirements of the environment. Genes involved in the regulation of proliferation in normal cells (proto-oncogenes) become oncogenes upon gain-of-function mutations, amplification, or chromosomal re-arrangements that lead to a continuous proliferative signalling. For example, HER2 is a receptor Tyrosine kinase that is weakly expressed in normal epithelial cells but is overexpressed in several types of cancer.[13] Ligands do not usually bind HER2 directly but they recognise specific sites at HER1, HER3, or HER4. HER2 functions as a co-receptor and exhibits a kinase activity only in a complex with other HER family members.[14] HER2-containing complex is preferred by many ligands because of its slow internalisation rate and, therefore, a prolonged response.[15] Overexpressed HER2 spontaneously forms heterodimers with other HER-receptors, that results in the activation of the pro-survival PI3K/Akt and MAPK pathways without an external stimulus.[16] Cells harbouring such a defect would normally be detected and eliminated through the p53-regulated cell death, but the cells with mutations in p53 alongside HER2 amplification will evade apoptosis and survive.

This example demonstrates that (1) proto-oncogenes (e.g., HER2) can become oncogenes due to genetic changes; (2) tumour suppressor genes (e.g., p53) can become inactivated (through mutations, promoter methylation, etc.); (3) at least two genetic events are usually necessary to initiate uncontrollable cell proliferation and survival (at least in epithelial tissues).[16]

Resisting cell death. Damaged or infected cells are normally eliminated through a programmed cell death. Despite the accumulated defects, transformed cells are able to avoid apoptosis and to prolong their lifespan. The tumour-suppressor p53 is the main inducer of the cell death upon stress: It stops the cell cycle and regulates apoptosis through both transcriptional and non-transcriptional mechanisms.[17] Concurrently to p53 inactivation, the apoptosis evasion is ensured through the upregulation of anti-apoptotic proteins (e.g., Bcl2, Mcl1)[18] or inhibitors of apoptosis (e.g., survivin).[19]

Enabling replicative immortality. Following a certain number of cell divisions, non-transformed cells enter a quiescent state and eventually undergo apoptosis. This phenomenon ('Hayfliks limit') has been correlated with the length of the telomeres, small signalling segments of DNA that become shortened with every cell division. Upon critical telomere shortening, a cell gets quiescent, enters a crisis state and undergoes apoptosis.[20] A telomerase complex that restores the telomeres after each cell division is normally active in the early embryogenesis, and adult cells do not express it. To overcome the Hayflicks limit, the cells might overexpress a telomerase thus becoming virtually immortal. Apart from that, telomeres can get stabilised by special telomere-binding proteins. Overcoming the Hayfliks limit is yet another strategy of cancer cells to evade physiological death.[21]

Inducing angiogenesis. Cancer cells, even more so than normal cells, depend on the nutrients and metabolites that are transported by the vasculature.[22] During embryogenesis, blood

vessels partially form *de novo,* and partially sprout into the growing tissues in response to pro-angiogenic stimuli. Thus, the vasculature develops as an organism grows, and in a fully-grown organism blood vessels sprout only on demand (e.g., upon injuries) in response to the short-termed activation of pro-angiogenic factors. As both proto-oncogenes and tumour-suppressor genes regulate normal cell division, the balance of pro- and anti-angiogenic factors determines angiogenesis.[20] A tumour is a growing organ that quickly gets hypoxic.[23] That, together with an oncogenic stress, induces the expression of angiogenesis activators of (e.g., MMP-9,[24] VEGF,[25] TGFβ[26]) and triggers 'angiogenic switch'.[27] An unbalance in the expression of angiogenesis-regulating factors leads to the development of the disorganised vasculature with non-hierarchical, dilated, and highly permeable vessels.[28]

Activating invasion and metastases. The terminal stage of tumour progression is the dissemination of tumour cells and the development of metastases that eventually kill a cancer patient. Dissemination is believed to begin with the epithelial-to-mesenchymal transition (EMT) in individual tumour cells. They upregulate mesenchymal transcription factors (e.g., Slug, Snail, Twist), become growth arrested, lose intercellular adhesion, and increase cell motility.[29] Tumour cells together with the cells of the microenvironment actively remodel extracellular matrix (e.g., through secretion of the extracellular proteases of MMP family) to provide an access to the bloodstream.[30,31] The abnormally fenestrated epithelium of tumour vessels facilitates the intravasation and dissemination of tumour cells.[32] When in the blood stream, tumour cells require additional mechanisms of survival and protection from immune recognition. It has been shown that context-specific activity of PI3K/Akt pathway is instrumental for the survival of cancer cells both in the circulatory system and in a new site.[33] Also, a distinct CD47 expression on disseminating cancer cells (that is absent in the primary tumour cells) was shown to protect tumour cells from the reactive lymphocytes.[34] It is proposed that seeding occurs in specific niches of distant organs that have receptive microenvironment conditions allowing tumour cells to reside therein.[35] Upon reaching such a niche, cells undergo mesenchymal-to-epithelial transition that is required to overcome a growth arrest.[36] After some dormancy period, disseminated cells establish new microenvironment conditions suitable for their subsequent proliferation and growth.[37,38]

Current treatment regimens are not efficient in eliminating metastases for several reasons. Firstly, resolution of the detection methods does not allow to detect a tumour at the stage when no disseminated cells are present.[39] There is a view suggesting that tumour cell dissemination is not a late process (as was thought before), but might occur at early stages; thus, it is not possible to eliminate all cancer cells by the surgical removal of the primary tumour.[40] Secondly, cells that have undergone EMT are rather resistant to treatment, that allows them to survive outside the primary tumour.[41] Thirdly, a mutation profile of metastases only partially overlaps with that of the primary tumour cells; therefore, the treatment cannot be tailored according to the original tumour mutation status.[42] Finally, we might lack sufficient knowledge and methods to detect metastases before disseminated cells overcome dormancy and start proliferating.

It should be noted that apart from the purely genetic defects discussed above (i.e., aberrations in the protein coding genes) there are multiple additional layers of transcriptional, translational, post-translation regulations not mentioned here (e.g., epigenetic regulation, alternative splicing, miRNA regulation, lncRNA, SNPs, etc.) Also, it cannot be stressed enough that each cancer type has a unique pathogenesis where a complex interplay between different molecules orchestrates the final phenotype.

### 1.1.2 Cancer stem cells

> The simplest view appears to me undoubtedly to be that in an early stage of embryonic development more cells are produced than are required for building up the part concerned, so that there remains unappropriated a quantity of cells--it may be very few in number--which, owing to their embryonic character, are endowed with a marked capacity for proliferation… The only point on which I lay stress is that the real cause of the subsequent tumour is to be sought in a fault or irregularity of the embryonic rudiment. (Julius Cohnheim, 1889)

Similarities between ontogenesis and oncogenesis were noticed years ago. That gave rise to the cancer stem cell (CSC) theory of cancer initiation and progression. This view is akin to normal development and tissue regeneration, suggesting that there are few pluripotent cells within each tissue that can give rise to all differentiated cells within an organ and at the same time maintain themselves. Tissue-specific stem cells originate from even less specialised omnipotent cells that can give rise to virtually any cell type, while also sustaining their own pool through an asymmetric division. An intricate constellation of growth factors present in the environment[43] tightly regulates each stage of division and differentiation.

The analogy between normal and cancer stem cells was drawn from the observation that stem cells, in order to divide, temporarily activate proto-oncogenes and then return to the quiescent state until a further need. Research has found that not all tumour cells are equally tumourigenic when xenografted into immunodeficient mice.[44] Taken together, these notions gave rise to a cancer theory claiming that there are, at least, two functional classes of cells within a tumour: one that could have lost its tumour-initiating capacity and forms the tumour mass and the other that can give rise to new tumours.

The origin of cancer stem cells is debatable. They could, potentially, emerge from normal stem cells since they possess many of their features (e.g., resistance to apoptosis),[45] which might be the case in some cancers.[46] Since the mutation rate is highly dependent on the number of replications, and stem cells were estimated to have ≈100-fold lower mutation frequency than somatic cells. Therefore, the probability of a stem cell becoming a cancer stem cell is rather low.[47] An alternative theory suggests that after the malignant transformation, certain cells de-differentiate, gain the features of stem cells, and then divide asymmetrically to produce tumour mass.[48] Also, cells with cancer stem cell-like properties can be induced by, for example, STAT3-mediated inflammation,[49] as well as other factors in the existing tumour.[50] It demonstrates that the pool of CSCs and orthogonal differentiation of cancer cells might not be terminal.[51]

CSCs are the most resistant to any external stress and, therefore, they survive during the treatment and initiate a new tumour which then develops clonally.[52] Presumably, the treatments at our disposal cannot reach and affect cancer stem cells, that may explain tumour recurrence.[53] Therefore, CSCs targeting has high potential in anti-cancer treatment regardless of the cancer origin. Also, it is believed that CSCs might maintain their own microenvironment within a tumour that allows them to survive. Studying and manipulating the conditions of cancer stem cells micro-niche can also be a treatment strategy by itself.[54]

Overall, the existence of therapy-resistant cells adds to the complexity of tumour cells targeting. Identification of this cell population for research purposes is a tedious task, especially when we do not know exactly what we are seeking. However, as the knowledge about the biology

of CSCs accumulates, there are more and more studies that develop systems for anti-CSCs drugs screening.[55]

To summarise, there is profound evidence that cancer is a disease of the genome even though most cancer types are not inheritable. Diverse mechanisms regulate neoplastic transformation in different tissues, and the combinations of genetic alterations are impossible to count. The development of deep-sequencing methods allowed to look more closely at the mutational landscape in several cancer types, but the functional studies are still to be done to distinguish the meaningful information from the genetic noise. Overall, it is not doubted now that there will not be a single treatment to cure cancer, but there will be therapies and combinations of therapies to treat individual cancers.

## 1.2 INTERFERONS (IFN)

IFNs are a group of ancient cytokines exerting pleiotropic effects in an organism. They are naturally produced by cells in response to the danger-sensing patterns (e.g., infection). The discovery and the first phenomenological description of IFNs date back to 1957 when Isaacs and Lindenmann discovered the phenomenon of 'interference' with secondary viral infection is prevented within hours and even days after the primary infection.[56,57] It was clear that a soluble factor was involved, since the treatment of the cells with inactivated virus led to the protection from a secondary infection with a replication-competent virus. This factor had extreme potency, as it was applied as a component of a crude cell extract and still had remarkable efficiency. It was not until about 20 years later that interferons were purified, and their effects were studied in more detail.[58,59] It became apparent that interferon is not a single substance but comprises several types, later denominated as type I, II, and III interferons.

Type I IFN includes IFN$\alpha_{1\text{-}12}$, IFNβ, IFNε, IFNκ, and IFNω; type II—only IFNγ, and type III—IFN$\lambda_{1\text{-}4}$. The difference among the types of IFNs lies in the receptors they bind to, in the activating stimuli and, partially, the mediators of signal transduction.[60] In the papers included in this thesis, we mainly worked with IFN$\alpha_{2a}$, and only a little with IFNβ (in paper II) and IFNγ (paper IV), therefore, I will mainly focus on type I IFNs.

### 1.2.1 IFN-induced signalling

Type I IFNs signal through the cognate IFNα/β receptor that consists of IFNAR1 and IFNAR2 chains. Unlike many growth factor receptors, the IFN receptor complex does not have the kinase activity. Instead, IFNAR2 is constitutively associated with JAK1 in its cytoplasmic domain. Following IFN binding to the external domain of the receptor, the receptor subunits dimerise allowing JAK1 to phosphorylate the IFNAR1-bound kinase Tyk2. Next, JAK1 and Tyk2 get further activated by cross-phosphorylation and they, in turn, phosphorylate the intracellular part of the receptor complex to create docking sites for the SH2-domains of STATs and other signalling proteins.[61]

Type I IFNs induce phosphorylation and homo- and hetero-dimerisation of virtually all STAT proteins, depending on the cellular context. The main transcription factor induced by IFNα is

ISGF3 that is a triple complex of pSTAT1, pSTAT2, and a DNA-binding protein IRF9. This complex binds to interferon-sensitive response elements (ISRE) in the promoters of target genes thus regulating their transcription.[62] Apart from ISGF3, IFN also induces homo- and heterodimers of phosphorylated STATs that regulate the expression of a separate gene set through binding to the gamma-activated sequence (GAS).

IFNγ binding of to its receptor complex leads to a similar cascade of events. The receptor-associated JAK1 and JAK2 cross-phosphorylate and activate cytoplasmic STAT1. Phosphorylated STAT1 homodimer is the main IFNγ-induced transcription factor that binds to the GAS elements in the gene promoters.[63] Also, there are reports that, in special cases, IFNγ can induce ISGF3 complex,[64] phosphorylation of STAT3, and interaction with other transcription factors, thus providing a multifaceted response and a prerequisite for the signalling crosstalk.[65]

There is some overlap between type I IFN- and type II IFN-induced genes. For example, IFITM1 can be induced by either as its promoter contains both ISRE and GAS binding sites. There is also a subset of the genes induced exclusively by one or another (e.g., OAS2 promoter has ISRE, but no GAS, therefore, it can be induced only by type I IFNs).[66]

### 1.2.2 Interferon-stimulated genes

IFNs induce 500-2,000 genes depending on the cell type, treatment duration and the dosage.[67] Knowledge about the functions of different ISGs is not homogenous. The exact mechanistic effects of all ISG products on viral clearance remain to be determined. However, some of the genes are relatively well-studied. Below, I will focus on the functions of some ISGs that are under study in this thesis.

One of the best characterised genes induced by IFN stimulation is 2'-5'- oligoadenylate syntase (OAS 1-3 and RNase L). The function of these enzymes is to polymerise ATP into 2'-5'-oligoadenylates. Proteins of OAS family members are almost non-detectable under normal conditions, but their expression is rapidly induced by viral dsRNA. 2'-5'-oligoadenylate binds to the monomeric RNase L causing its dimerisation and activation. In turn, this leads to the enzymatic degradation of the viral and host RNAs into small ssRNAs.[68,69] The products of RNase L can be further sensed by the cells and induce further production of IFN.[70]

The mechanism of action of an ISG product IFITM1 is less clearly understood. It has been shown that IFITM1 protein can localise close to the intracellular vesicles, forming exosomes, the Golgi apparatus, and the plasma membrane.[71] Unlike OAS, it is expressed in the cells at a basal level and it is involved into cell adhesion. IFNs further induce IFITM1 that provides protection from a wide range of viruses.[72] IFITM1 has also been shown to induce p53-dependent senescence in the cells.[73] In tumours, the role of this protein and other IFITM family members is not as clear. Elevated levels of IFITM1 expression have been associated both with drug resistance and treatment response in different systems.[74-77] There is also a report that shows that increased expression of IFITM1 leads to aggressive tumour phenotype.[78]

IFI27 was first cloned from the breast cancer cell line MCF7 and was immediately associated with tumourigenesis.[79] Further investigations mostly confirmed the initial finding, as IFI27 (or p27) was identified by multiple gene expression arrays in conjunction with aggressive forms of cancer, treatment resistance, and the regulation of the epithelial-mesenchymal transition in various cancers.[80-82]

### 1.2.3 Alternative signalling pathways induced by type I IFNs

The JAK/STAT pathway was discovered and first characterised as the main mediator of the IFN-induced response. Currently, there is abundant evidence that IFNs also stimulate other pathways. The PI3K pathway was shown to be activated alongside with the JAK/STAT signalling and was found essential for the adequate transcriptional response. From the mechanistic point of view, it has been found that type I IFNs induce phosphorylation of IRS1 and IRS2 adaptor proteins that bind the activator subunit of PI3K p85 through the SH2 domains.[83,84] p85 activation induces the catalytic subunit of PI3K p110 followed by the signal propagation to AKT, mTOR and, eventually, to several transcription factors (e.g., NF-κB, AP1, etc.) Simultaneously, the negative feedback regulation of PI3K is activated that efficiently shuts down the IFN signal.[85,86] To summarize, IFNα induces JAK-dependent phosphorylation of the IRS proteins that trigger the PI3K signalling cascade. This process is independent of the STAT phosphorylation.[87]

Some reports show that IFNγ also induces PI3K without the involvement of the phosphorylated IRS proteins. The PI3K pathway activity is required for the phosphorylation of STAT1 on Ser727 that is performed by a PI3K-activated kinase PKCδ and is necessary for the full transcriptional activity of the STAT1 dimer.[88,89]

A separate arm of the PI3K signalling goes through mTOR. This protein is rapidly phosphorylated downstream of the PI3K, which activates it and leads to the phosphorylation of p70S6 kinase.[90] p70 targets S6 subunit of ribosomes, thus stimulating translation through the 5′-terminal oligopyrimidine tract. Additionally, mTOR/p70S6K activation leads to the deactivating phosphorylation of the transcriptional repressor 4EBP1 on several serine and threonine residues. This event leads to the release of the 4EBP1 from the inhibitory ElF4E protein and initiates translation.[91]

Ras/MAPK pathway is activated by the type I IFNs as well.[92] The adaptor protein CRKL is phosphorylated by Tyk2, which leads to the phosphorylation of a small GTPase RAP1. It further signals to MAPKKK and, ultimately, to p38, which is of particular importance for the antiviral defence.[93,94] It has been shown that p38 participates in the phosphorylation of STAT1 on Ser727,[95] but this fact was later debated.[96] The activities of the MEK/Erk and the JNK arms were also registered in response to IFNs. As was earlier shown by the study from our lab, JNK is important for the activation of the mitochondrial proteins Bak and Bax that participate in the IFNα-induced apoptosis.[97]

### 1.2.4 The biological effects and clinical use of IFNs

IFNs appeared early in the evolution of vertebrates, that indicates their importance in homeostasis. The main function, as was discussed above, is the control and eradication of infections. IFNs execute the first non-specific line of the defence against pathogens by inducing the expression of ISGs. Apart from the direct antiviral effect, IFNs also stimulate the immune system by activating a number of the immune cells (e.g., natural killer cells (NK cells) and macrophages) and by enhancing the antigen presentation through the upregulation of MHC class I expression. [98,99] NK cells activation was found to be a prerequisite for efficient anti-tumour immunity.[100]. It has also been noticed that IFNs induce a cell cycle arrest and, as a consequence, inhibit cell proliferation by

prolonging almost all phases of the cell cycle.[101,102] Moreover, IFNs have a direct pro-apoptotic effect on cancer cells independently of the cell cycle arrest.[103,104] effect on cancer cells by inducing their cell death independently of cell cycle arrest. Due to these effects, recombinant IFNs were tried in the treatment of viral and oncological diseases.

In 1986, the FDA approved IFN for the treatment of hairy cell leukaemia, and later for multiple sclerosis, follicular lymphoma, chronic hepatitis B and C infection, and AIDS-related Kaposi sarcoma. IFNs were also evaluated for the treatment of other types of cancer, but their wide-spread usage was limited by the narrow therapeutic window. The side-effects of the IFN treatment, such as muscle and back pain, depression, anorexia, congestion, and flu-like symptoms, are common for almost 50% of the patients. The mechanisms of these effects are poorly understood; therefore, there is a limited opportunity to relieve them.[105,106] However, when used in sufficiently high doses, the IFN treatment is very beneficial for the patients.[103,107] Significant side effects in the absence of predictive biomarkers make oncologists reluctant to use IFNs in their practice.[108,109] Overall, considering all cancer types, the gain of IFN treatment is rather moderate due to dose limitations, but there is a high potential which might be realised by novel intra-tumour delivery methods and the development of robust criteria for the clinical benefit of this treatment.

## 1.3 IL6 SIGNALLING IN CANCER

Originally, interleukin-6 (IL6) was discovered as a soluble factor produced by T-cells, which stimulated B-cells to terminal differentiation and immunoglobulin production.[110] IL6 is secreted mainly by the immune cells (lymphocytes, monocytes, macrophages, etc.) as well as some non-immune cells (fibroblasts, endothelial cells, keratinocytes, etc.). The IL6 production can be stimulated by some cytokines and growth factors (IL1, TNF, PDGF, etc.) as well as viral and bacterial infections. IL6 signalling starts from the binding of IL6 to its receptor. Immune cells usually express a transmembrane IL6-R, whereas other cells mostly express a soluble form of the receptor (sIL6-R) produced either by the proteolytic cleavage of the transmembrane receptor or by the alternative splicing of the IL6-R gene. There is no catalytic domain in the IL6-R, therefore IL6 binding to either transmembrane or soluble receptors is followed by the association with another transmembrane receptor subunit gp130. The signalling triggered by IL6 binding to its membrane receptor is commonly referred to as ‘a classical pathway’, whereas IL6 binding to the sIL6-R is referred to as ‘a trans-signalling pathway’. The difference between the two pathways is in their sensitivity and even in the ultimate effect. [111,112]

After the formation of the IL6/IL6-R/gp130 or the IL6/sIL6-R/gp130 complex, several signalling pathways are induced: Ras/MAPK triggers the cascade leading to the transcription of acute phase genes while JAKs recruitment leads to the activation of STATs and PI3K, both stimulating the expression of the genes involved in complex immune reactions.[113]

Dysregulation of the IL6 pathway leading to sustained signalling has been associated with several diseases. Firstly, it leads to autoimmune diseases in the nervous, cardiovascular, and respiratory systems and causes the immune-mediated kidney and colon diseases.[106] Secondly, increased production and continued IL6 signalling contribute to various steps in cancer progression through the cell proliferation induction, mediation of the tumour cells-stroma interactions, the epithelial-mesenchymal transition stimulation, angiogenesis, and drug resistance.[114,115] As an example, mammospheres of normal breast epithelium exposed to IL6 showed signs of transformation and increased proliferation.[116] Also, constitutive expression of IL6 in a non-invasive

MCF7 cell line led to the downregulation of E-cadherin, and the upregulation of vimentin, N-cadherin, Snail and Twist, and promoted increased metastasising capacity of the cells.[117]

The first drugs targeting the IL6 pathway were the humanised anti-IL6 antibodies. The trials of tocilizumab, one of the anti-IL6 drugs, showed the efficiency of the IL6 blockage in active rheumatoid arthritis (RA) even as a monotherapy,[118] and even higher efficiency in combination with anti-inflammatory drugs. Other biologics tested for the inhibition of the IL6 signalling include modified anti-IL6 antibodies olokizumab,[119] sirukumab,[120] siltuximab;[121] anti-IL6R antibodies such as sarilumab;[122] and drugs targeting IL6-IL6-R complex like gp130-Fc.[123].[124] The blood levels of C-reactive protein (CRP) are used to assess the effect of the drugs in auto—inflammatory diseases.[125] Overall, IL6 targeting was successful in the treatment of RA, which encouraged investigating the anti-IL6 drugs in cancer.

Several IL6-targeting drugs were in clinical trials for the castration-resistant prostate cancer, the metastatic colon cancer, and some other advanced solid tumours. Mostly, the side-effects were mild and tolerable. However, the clinical response was not significantly different compared to placebo.[126] Similarly, the addition of an IL6-targeting drug to bortezomib in the treatment of multiple myelomas did not improve the clinical outcome of patients.[127] CRP levels were prominently downregulated under the treatment; however, this did not correlate with the anti-tumour efficacy of the drug, unlike in the anti-inflammatory effect in RA.[127]

In summary, biological drugs against IL6 are efficiently used in the treatment of some autoimmune disorders. The use of anti-IL6 drugs in cancer has not been successful yet. A possible reason is the dose limitations which do not allow efficient inhibition of IL6 signalling in tumours. Another reason might be the substitution of the IL6 signalling by other cytokines from the IL6 family. Currently, the efforts are directed towards the development of a non-antibody drug to decrease the immunogenicity and to improve the affinity to IL6. While clinical testing is rather straightforward in RA, testing anti-IL6 drugs in cancer is more challenging and time-consuming.

## 1.4 SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION (STATS)

The family of signal transducers and activators of transcription comprises seven family members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6, each encoded by a different gene. This group of transcription factors was discovered in conjunction with the studies on the IFN-induced signalling as it was found that they were the main mediators of the IFN signal to the nucleus. Later, it became apparent that many of the cytokines and growth factors also passed their signals through STATs. Moreover, STATs are in the centre of the Tyrosine kinase activity within a cell; therefore, they mediate multiple fundamental processes in cell homoeostasis.[128]

All seven STATs share the same five-domain structure: an N-terminal domain, a coil-coiled domain, a DNA-binding domain, an SH2 domain, and a C-terminal domain (illustrated in Figure 1). The STATs are similarly activated: upon the external signalling molecule binding to the corresponding receptor, STATs get phosphorylated in the cytoplasm by JAKs, SRC, or other tyrosine kinases. A crucial phosphorylation position is a tyrosine residue located within the transactivation domain. Some STATs (STAT1, STAT3, STAT4, STAT5a, and STAT5b) have an additional Serine phosphorylation site located in the close proximity to this Tyrosine residue. The primary event in the STAT-activation is the Tyrosine phosphorylation, whereas Serine

phosphorylation is secondary. It was shown that the Serine phosphorylation is required for the full transcriptional activity of STATs and for some of their non-transcriptional functions.[129]

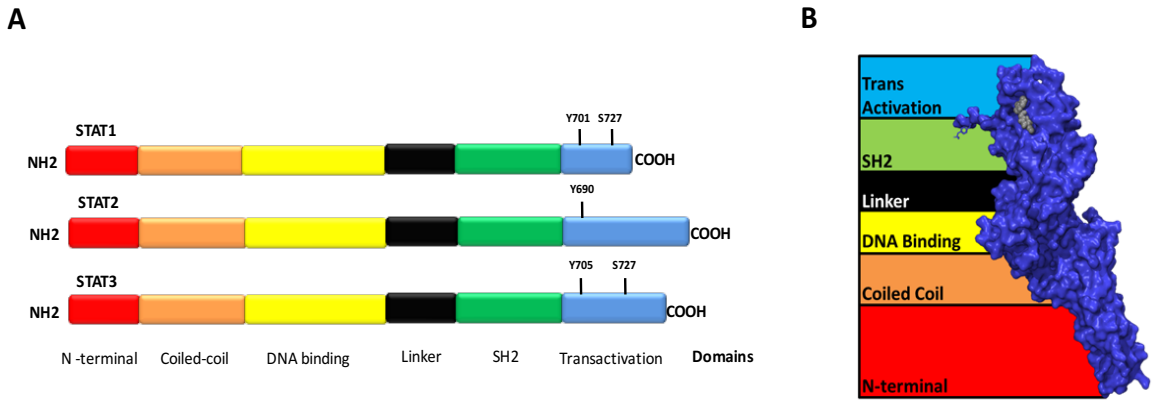


Figure 1. (A) The general domain structures of STAT1, STAT2 and STAT3. Phosphorylation sites in the transactivation domain are marked (B) The crystal structure of a STAT3 protein, domains are designated (the courtesy of B.Page)

Once activated, STATs form homo- and heterodimers through the reciprocal interactions between phosphor -Tyrosine residues and the SH2 domains. In the dimerised form, STATs expose their nuclear localisation signals and are actively transported through the nuclear pore complex by importins. In the nucleoplasm, the STAT dimers recognise their binding motifs at DNA in the gene promoters, thus regulating the gene transcription. Nuclear phosphatases (e.g., TC45) dephosphorylate STATs released from DNA, and they are returned to the cytoplasm by exportins.[130] Also, STATs induce the transcription of their negative regulators suppressors of cytokine signalling (SOCS)[131] and other cytoplasmic phosphatases, thus providing a dynamic mechanism for the feedback regulation.

Nuclear import of STAT3,[132,133] STAT5,[134,135] and STAT6[136] does not depend on their phosphorylation status, causing these proteins to shuttle continuously between the nucleus and the cytoplasm. Recently, it has been shown that unphosphorylated STATs are not latent factors as was thought before, but instead, they have a unique transcriptional activity or function as part of a larger transcriptional complex.[137]

Although there is a high similarity between the amino acid sequences of the STATs, each of them mediates different functions depending on the context. Despite the fact that the general activation principle is shared by all STATs, the degree of specificity is conferred by the activating kinases, interactions with specific importins and transcription factors, and differential affinity to DNA sequences. Below, I will try to outline briefly some specific functions of STATs and their involvement in cancer and other diseases.

### 1.4.1 STAT1

Activation of STAT1 by IFNs leads to a massive multi-level immune response directed towards clearance of viral infection.[138] STAT1 deficiency causes increased susceptibility to pathogens due to abrogated IFN signalling as well as through independent mechanisms.[139-141] Also, mice lacking STAT1 are more prone to tumour development than wild-type animals, suggesting a

tumour suppressive role of STAT1. The role of STAT1 in cancer is described in detail below in the corresponding section.

### 1.4.2 STAT2

STAT2 was discovered as a participant in the IFN-induced signal transduction which remained its only known function for decades. Type I IFNs activate it through the phosphorylation on a Tyrosine 680 residue. Afterwards, STAT2 forms heterodimers with other STATs. The roles of STAT2 homodimers and some of the heterodimers are not well understood. The well-studied complex containing STAT2 is ISGF3 (a triple complex of IRF9, STAT1, and STAT2). STAT1 and IRF9 provide DNA binding of the ISGF3 complex, whereas STAT2 recruits transcriptional enhancers. As described above, ISGF3 mainly regulates antiviral effects of type I IFNs through regulation of the genes containing ISRE in their promoters.[142] It has been found that type I IFNs can propagate the signal also in the absence of STAT1 through an alternative complex containing a STAT2-homodimer and IRF9.[143,144] Since the DNA-binding affinity of this complex is considerably lower than that of ISGF3, it was suggested that elevated levels of STAT2 and IRF9 or both might be required.[145] It was also shown that the STAT2-IRF9 complex provides a prolonged transcription of the the ISGs that is particularly important for eliminating certain pathogens, such as L. pneumophila, Dengue virus, paramyxovirus, and so forth.[146,147] On the other hand, the STAT2-IRF9 complex might act as a reserve mechanism of the innate immune system in case viruses interfere with the function of STAT1. Recently, it was demonstrated that Zika virus expresses a ubiquitin ligase specifically targeting STAT2 and, in this way, preventing the formation of the normal IFN-induced virus clearance.[148] Overall, STAT2 appears to play an important role in mediating immune response, not only through the classical ISGF3 complex.

The direct role of STAT2 (outside its involvement in the function of the immune cells) in tumour development and tumour progression is less clear. Some evidence suggests that STAT2 might be involved in the malignant transformation by regulating the production of IL6. This conclusion was drawn from the experiments on the STAT2-null mice that had decreased serum levels of IL6 comparing to the wild-type animals. The STAT2-null mice were also protected from the formation of colon tumours.[149] On the other hand, increased STAT2 staining was more frequently detected in cervical cancer specimens than in the non-cancerous and pre-cancerous lesions.[150] Some studies also point out at the involvement of STAT2 in drug resistance through the regulation of ISGs.[151] Taken together, the role of STAT2 in cancer remains controversial and is most probably cell-context dependent.

### 1.4.3 STAT3

STAT3 was first discovered as a factor interacting with the promoters of acute-phase response genes upon the IL6 stimulation of hepatocytes. It was later shown that it had a structure similar to one of the earlier described transcription factor STAT1.[152] Apart from IL6, STAT3 is activated in response to other cytokines that signal through the gp130 receptor (e.g., IL1, IL5, LIF), IFNs (mainly type I), and multiple growth factors (e.g., EGF, HGF, FGF), as illustrated in Figure 2.[153] The STAT3-regulated genes were found not to be restricted to acute-phase response regulators but turned out to cover a wide range of genes important for development, survival, and homoeostasis of an organism.[154] Moreover, due to its infamous involvement in the pathogenesis of cancer and autoimmune disorders, STAT3 became one of the most studied STAT family members.

Immune system

The STAT3 transcriptional function in the immune cells is, probably, best described as a double-edged sword as it is involved in both immune suppression and immune activation. Under normal conditions, STAT3 is the main transcription factor that responds to epithelial G-CSF during emergency granulopoiesis (e.g., in response to infections).[155] It directly regulates CEBPβ and c-myc expression in the granulocyte precursors in the bone marrow.[156,157] It was also observed that STAT3 plays a crucial role in the development of dendritic cells: It inhibits their maturation.[158-160] In phagocytes, STAT3 limits the inflammatory response through downregulation of the signalling from TLRs: Mice lacking STAT3 in the myeloid compartment spontaneously develop enterocolitis.[161] In the adaptive immunity, STAT3 is also involved throughout. For example, it is required for the early stages of pro-B-cells development and for the IgG production in mature B-cells.[161] T-cells also require STAT3 for survival (independently of the regulation of Bcl2) and also for the differentiation of Th17 lineage through the transcriptional regulation of RORγ and RORα,[162] IL23R and IL17R.[163]

Given the important role the immune system plays in cancer, the role of STAT3 in the malignant transformation and the tumour progression is also intricate. It was proposed that IL6/STAT3 signalling contributes to the induction of the immune tolerance. For example, STAT3$\beta$-bearing tumours were significantly diminished in their size comparing to STAT3 wild-type tumours in the immune-competent mice. Also, STAT3 knockdown in the tumour cell compartment led to the immune cells infiltration into the tumour site and a massive production of pro-inflammatory cytokines and chemokines that induce the anti-tumour immune response.[164] STAT3 deletion in a different subset of the immune cells led to an increase in the anti-cancer immunity, pointing out that the constitutive STAT3 activity in some immune compartments may contribute to the local suppression of the anti-tumour immunity.[165]

It was also demonstrated that a signalling circuit between tumour cells and the cells of the microenvironment leads to continuous expression of growth factors and immunosuppressive cytokines that not only activate STAT3 during their signalling, but also their expression is directly regulated by STAT3. For example, myeloid cells-derived VEGF binds to its receptor and induces STAT3 phosphorylation and dimerisation followed by nuclear translocation of STAT3 and its binding to the promoter of VEGF.[166] Similarly, IL6 signalling through the gp130 receptor utilises STAT3 and regulates its own expression.[167] Apart from the increased secretion of immunosuppressive factors, it was shown that increased STAT3 activity interferes with the maturation of dendritic cells at the tumour site, thus making them incapable of efficiently presenting tumour-cell antigens.[168] Also, STAT3-regulated expression of IL10 and TGF$\beta$ in the immune cells leads to an increase in the population of Treg cells within a tumour that provides an additional level of immune tolerance.[169-171] Taken together, the paracrine interaction between tumour and immune cells in the stroma reciprocally regulate constitutive signalling activity necessary for the tumour cells proliferation and the suppression of the immune-mediated cell death.

On the other hand, it is also well-documented that STAT3 promotes inflammation in epithelial tissues, thus supporting tumourigenesis.[172] One of the described mechanisms involves interaction with the RelA subunit of NF-kB that leads to the repression of its transcriptional activity and inhibition of the Th1-mediated anti-tumour immune response. Other mechanisms include stimulation of IL6 expression by NF-kB, strong upregulation of the pro-tumourigenic genes by a STAT3-NF-kB complex (when STAT3 and NF-kB binding sites are adjacent in a promoter) and others that include multi-layered interactions with NF-kB.[173-175]

To summarise, STAT3 is an important transcription regulator in almost all subsets of immune cells. It participates in both immune suppression and immune activation, depending on the context. Its activity is controlled under normal conditions to provide optimal immune response, but cancer cells take advantage of the immune cells in the tumour site by engaging them in a continuous STAT3-dependent circuit of signalling. Inhibition of STAT3 is believed to be beneficial not only to induce apoptosis directly in cancer cells addicted to it, but also to inhibit the STAT3-mediated inflammation or to induce anti-tumour immune response through the modulation of the resident immune cells the activity (a 'bystander effect').[176]

Reproduction and early development

For a long time, physiological functions of STAT3 were difficult to study using knockout mice since ablation of STAT3 led to early embryonic lethality (unlike any other STAT members). Careful investigation of the phenotypes upon conditional STAT3 deletion in different compartments of the female reproductive system revealed that STAT3 is expressed throughout from oocytes to the cells of the uterus. The leptin/STAT3 signalling was found to be involved into oocyte polarisation.[177] Surprisingly, though, oocyte depletion of STAT3 did not affect the fertilisation rate in mice. Conversely, the conditional deletion of STAT3 from the stromal compartment of uteri led to the reduced litter size due to abnormal placenta formation and the increased frequency of fetal resorptions.[178] IL6 and STAT3 were also detected in spermatozoa, and the STAT3 inhibitor V impaired their motility.[179] Since spermatozoa were mostly transcriptionally inactive and also because STAT3 localised outside their nuclei, it was assumed that STAT3 is important for the regulation of the motility through a non-transcriptional mechanism.[180] Later, it was found that STAT3 regulated the mitochondrial activity of spermatozoa through a non-transcriptional mechanism.[179]

Apart from its function in germ cells, STAT3 was found to be involved in the maintenance and differentiation control of stem cells.[181,182] It gets phosphorylated by LIF in the four-cell embryo developmental stage and maintains the inner cell mass cell lineages through the transcriptional regulation of OCT4 and NANOG.[183] In the context of the stem cell regulation during embryogenesis, STAT3 is indispensable.

Mammary gland involution

Another physiological function of STAT3 is the induction of the caspase-independent, lysosome—mediated cell death in response to LIF and OSM during mammary gland involution after lactation.[184,185] Conditional deletion of STAT3 in the mammary epithelium causes significant delays in post-lactation regression, especially when abolished at the initiation stage of the involution.[186] It was shown that during the first 24h of involution, Prolactin/STAT5 signalling ceases while the LIF/STAT3 cascade increases its intensity. Proposed mechanisms of the STAT3 involvement in the lysosome-mediated cell death encompass activation of cathepsins expression, repression of serine protease inhibitor 2A (Sip2a), and also a less understood process of the phenotypic switch in the mammary epithelium cells from the secretion to the non-professional phagocytosis of milk fat globules.[185] The latter provides triglycerides that are hydrolysed to free fatty acids in lysosome-like vacuoles resulting in permeabilisation of their membranes and leakage of the cathepsins into the cytoplasm that concludes the cell death process.[187] Transcriptional analysis of the STAT3 target genes in the mammary epithelium showed upregulation of cathepsin B and cathepsin L, as well as of the pro-

inflammatory genes that are believed to contribute to the phenotype switch of the epithelial cells.[188,189] Taken together, STAT3 appears to play an important role in the induction of cell death in the mammary gland. Supposedly, a similar mechanism might be involved in other hormone-dependent cases of cell death, which remains to be investigated further.

Cardiovascular system

STAT proteins were found to play important roles in the function of the heart. All STATs are expressed in the heart; dysregulation of each STAT family member leads to a distinct phenotype that can be correlated to specific cardiomyopathies.[190-192] Physiological and pathological remodelling of the myocardium in response to some stress (e.g., pregnancy, exercise, infarction, infections, etc.) initially have similar mechanisms. However, some types of stress lead to irreversible remodelling that is destructive for the function of the heart. STAT3 appears to contribute to both these processes and is believed to coordinate the fine balance between the normal and the pathological remodelling by regulating the expression of secreted factors that allow communication between different cell types.[191,193] The cardiomyocyte-specific knock-in experiments showed that STAT3 regulates the expression of a pro-angiogenic factor VEGFA that promotes vasculogenesis and increases vascular permeability.[194] VEGFA is induced in the heart in response to stress by, for example, the ischemia-induced cytokines IL6 and EPO. G-CSF leads to the STAT3-dependent survival of the cardiomyocytes and increases capillary density during the myocardial infarction through mediation of the cardiomyocyte—endotheliocyte paracrine communication.[195] The cardiomyocyte-restricted STAT3 knockout showed that male animals have an increased rate of cardiac fibrosis and the heart failure towards the older age.[196] Female mice developed heart failure during pregnancy: in response to uncontrolled oxygen stress (normally relieved by PGC1α and MnSOD in a STAT3-dependent manner), cathepsin D was released from the cells, thus inducing the cell death and the degradation of capillaries.[197-199]

STAT3 is also involved in the paracrine communication between cardiomyocytes and cardiac fibroblasts that is important for maintaining the optimal structure and condition of the extracellular matrix (ECM). STAT3 downregulates the expression of ECM components by cardiomyocytes that is necessary for efficient vascularisation after injury.[200] At the same time, it also promotes survival, proliferation, and the ECM synthesis in fibroblasts that lead to fibrosis if not properly regulated.[200,201]

Overall, STAT3 appears to play a complex role in maintaining heart homoeostasis and response to injury. Importantly, an increase in STAT3 levels in the heart triggers hypertrophy and inflammation,[202] whereas diminishing STAT3 expression below certain threshold leads to heart failure—a phenotype to be aware of upon treatment of cancer patients with targeted therapies.[203]

Central Nervous System (CNS)

STAT3 expression in adult CNS is relatively low compared to other systems. The highest expression is observed during early embryogenesis and it gradually declines during the development and maturation.[204] STAT3 expression in the adult brain is mostly restricted to neural stem cells where it regulates their proliferation and neuronal differentiation in response to glia-secreted cytokines (e.g., IL15, leptin, CNTF, LIF, etc.). Importantly, neurogenesis and neurite outgrowth in adults are dependent on the JAK/STAT3 signalling and cannot occur in the absence of it.[205,206] Additionally, STAT3 is expressed in the gonadotropin-releasing neurones in the hypothalamus where they are

involved in the expression of sex hormones. Inhibition of the LIF/CNTF/STAT3 signalling leads to hampered reproductive development and sexual behaviour in rodents.[207]

JAK2 and STAT3 were found to be important for the hippocampal synaptic plasticity.[208] Using JAK inhibitors, it was shown that the JAK2/STAT3 pathway regulates the expression of certain neurotransmitter receptors (e.g., GABA,[209] NMDA,[210] muscarinic acetylcholine[146]). Additionally, STAT3 regulates NMDAR-dependent long-term depression in the post-synaptic density, in this way also contributing to synaptic plasticity. For this function STAT3 seems to act independently of its transcriptional function.[208,210]

Since STAT3 is involved in the regenerative processes in the brain, its activity is transient. As in other systems, defects in its activity regulation lead to pathological conditions such as brain inflammation and impaired neural and glial survival. Consequently, abnormal STAT3 signalling is frequently detected in the CNS diseases of different aetiology (e.g., brain cancer,[211] epilepsy,[212] Alzheimer's disease).[213]

Non-transcriptional functions of STAT3

Most of the studies on the function of STAT3 investigated its transcriptional effects on gene expression (similarly to other STAT proteins) that occurs through direct binding of the tyrosine—phosphorylated STAT3 dimers to the promoters of target genes. In recent years, it was found that STAT3 also regulates gene expression in an un-phosphorylated form (U-STAT3) and even without a direct contact with chromatin. For example, U-STAT3 was found to be transported to the nucleus in a complex with NF-kB that regulates expression of certain genes.[214,215]

An example of an outside-of-the-nucleus function of STAT3 is its activity in mitochondria (mitoSTAT3).[216] It was found that STAT3 depletion in cardiomyocytes led to impaired function of complex I and II of the electron transport chain. It resulted in cell damage through excessive production of ROS.[217]

Also, there is an indication that mitoSTAT3 is involved in the metabolic rewiring of cells by skewing their energy production towards glycolysis early in cancer development.[218] MitoSTAT3 was found to be Ser727 phosphorylated independently of Tyr705 phosphorylation, and its deregulation was found to be involved in the oncogenic transformation in several cancer types.[181,216]

Overall, accumulating evidence suggests that non-nuclear STAT3 plays an important role in cellular respiration and metabolism. Therefore, design of STAT3 inhibitors for cancer therapy cannot be solely focused on the function of STAT3 as a transcription regulator.[219]

STAT3 in cancer

Since overexpression and activating mutations of multiple growth factors or their receptors or both are common in cancer, constitutively activated STAT3 is frequently detected in several cancer types. Considered a 'non-classical' oncogene, STAT3 regulates the expression of genes contributing to tumour progression at different levels.[220] The target genes include anti-apoptotic, pro-survival, pro-proliferation, and pro-invasion genes, many of which are recognised oncogenes.[221] Since these genes are important for the very basic cell functioning; they are regulated by STAT3 in conjunction with other transcription factors, thus providing relative specificity of the response to different stimuli. STAT3-dependent regulation of the targets named above is well-studied in different systems. I will focus on the recently identified STAT3 functions implicated in carcinogenesis.

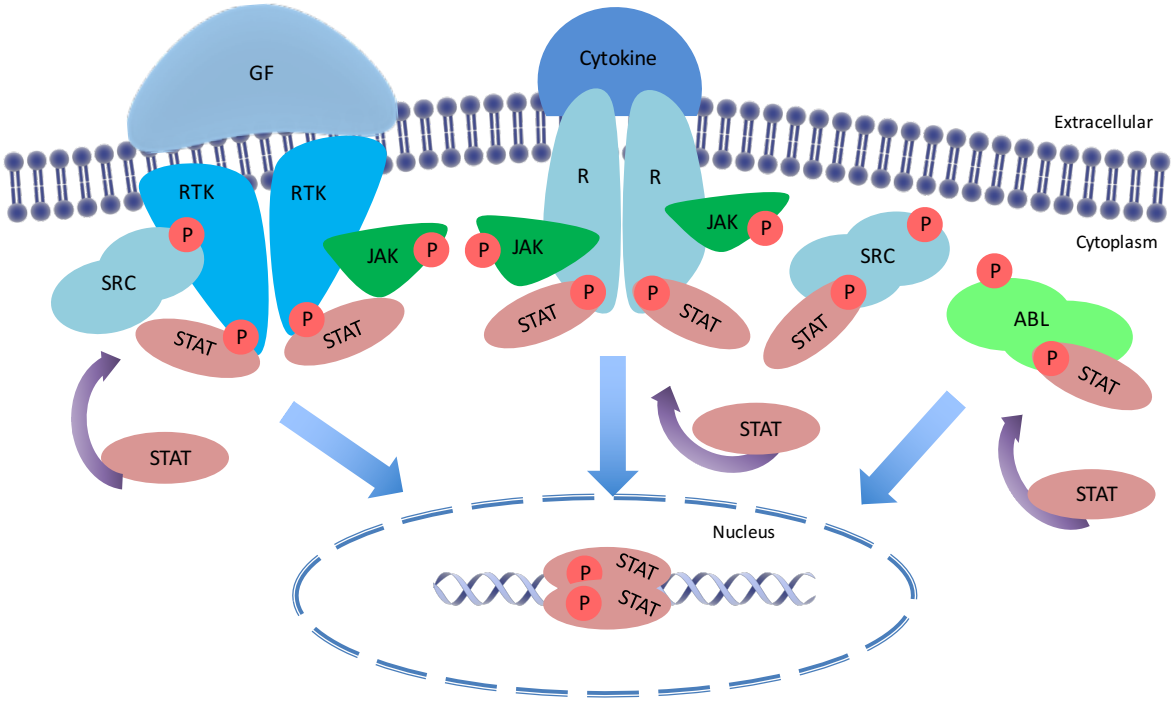


Figure 2. Schematic depiction of pathways that lead to the activation of STAT proteins.

STAT3 conveys signals from multiple ligands, therefore, it can be partially accountable for the resistance to targeted therapies when more than one pro-survival pathway is involved. Apart from that, it has been shown in the example of Erlotinib, that cells activate STAT3 as a feedback mechanism to protect themselves from the cell death upon prolonged treatment.[222] STAT3 activation occurs through the secretion of many STAT3-activating cytokines (e.g., IL6, IL10) upon the exposure of the cells to the drug. In a similar manner, the increased expression of STAT3 protects HER2+ breast cancer cells treated with Trastuzumab during a prolonged period.[223] Overall, acquired resistance to several of the targeted therapeutics is often mediated through a feedback STAT3 activation.[224]

The role of STAT3 in metastases was also convincingly demonstrated.[225] Recently, STAT3 hyperactivity in the immune cells has attracted much attention. It was shown that tumour-associated immune cells can prepare a new site for tumour dissemination by locally producing immunosuppressive cytokines and inducing immune tolerance towards cancer cells.[226]

Lastly, STAT3 was found to be involved in the maintenance of cancer stem cells. Similar to its role in maintaining the ESC, LIF and IL6 mediate activation of STAT3 and suppress differentiation. CSCs are thought to account for the failure of the many cancer treatments, as they can tolerate the majority of the drugs and re-populate the tumour site hypothetically from a single cell.[227]More details about CSCs can be found in the introductory chapter.

To summarise, STAT3 is an important regulator of many cellular events. Its activation is tightly controlled by different mechanisms to provide timely and adequate response. Once a dysregulation occurs, STAT3 can become a central knot of the tumour cell signalling. Therefore, targeting STAT3 holds promise for the improved therapy response and longer remissions in patients.

### 1.4.4 STAT4

This STAT family member is primarily expressed in T-cells and plays an important role in their differentiation and proliferation in response to IL12. Its activation occurs through IL12 binding to IL12R$\beta$1 and IL12$R\beta$2 and a classical cascade of phosphorylations with the involvement of JAK2 and Tyk2. Without stimulation, Th1 and Th2 cells express low levels of the transcriptionally inactive STAT4. In response to IL12, the STAT4 dimer regulates the expression of the IL12-responsive genes through a GAS element.[228] STAT4 activation happens mainly in the Th1 cells, causing them to secrete IFN$\gamma$.[229] Mutations and polymorphisms in STAT4 lead to autoimmune diseases (e.g., systemic lupus erythematosus, RA)[230] and are associated with the spontaneous clearing of the viral infections (e.g., HBV infection).[230] According to the reports in cancer, the IL12/STAT4 axis loss led to poor outcome in the hepatocellular carcinoma.[231] On the other hand, metformin treatment led to the downregulation of IL22/STAT4 signalling in a mouse model of hepatocellular carcinoma. This treatment was associated with the cell death and tumour shrinkage.[232] Similarly, chemotherapy led to the STAT4 protein degradation in lymphoma patients, but the outcome of the STAT4-loss is not clear.[233] Overall, the role of STAT4 was studied mostly in the autoimmune diseases; its role in cancer is not fully established. Clearly, the STAT4 gene has multiple polymorphisms that may differentially affect its functionality and lead to different phenotypes.

### 1.4.5 STAT5a and STAT5b

STAT5a and STAT5b (STAT5) are coded by different genes in chromosome 17 and are located in the same locus as STAT3. The difference between STAT5a and STAT5b lies mainly within their C-termini that have 20 unique amino acids for STAT5a and 8 for STAT5b.[234,235] STAT5a and STAT5b have both redundant and non-redundant functions. Both of them regulate gene transcription through the GAS-site binding. STAT5a has most transcriptional activity when it is in a tetrameric form, whereas STAT5b shows high affinity to DNA as a dimer.

Historically, STAT5a was discovered as a prolactin-responsive transcription factor in the mammary epithelium. Later, it was shown that numerous cytokines (such as IL3, IL5, IL2, IL7) and growth factors (e.g., GH, PRL, Epo) can activate STAT5a.[236] On the other hand, STAT5b was found in the muscle and liver tissues and seems to have a distinct function in the body growth.[237] Consistent with the tissue-specific expression, the knockdown of STAT5a and STAT5b in mice led to different phenotypes. STAT5a k/o led to a lack of mammary gland development and differentiation during pregnancy;[238] STAT5b$^{-/-}$ mice showed body growth abnormalities and liver dysfunction.[239]

STAT5 is necessary for the lineage differentiation in the mammary gland and the hematopoietic system.[238,240,241] Consequently, an abnormal activity of STAT5 can be most often seen in breast and blood malignancies. In the mammary gland, STAT5 is necessary for the differentiation and survival of a small number of cells that produce milk during lactation.[242] These secretory epithelial cells are located at the very end of the ductal tree and are postulated to be the primary site of the malignant transformation. Using transgenic mice, it was shown that STAT5a loss leads to delayed breast cancer development in several breast cancer models.[243,244] On the other hand, overexpression of STAT5a led to sporadic tumour development in older mice.[245] Some studies conclude that STAT5b is important for proliferation of breast cancer cells, as its deletion leads to apoptosis induction while other groups report a very mild effect of STAT5b overexpression on cancer development. [246,247]

Nuclear localisation and the activity of STAT5 are frequently detected in tumour tissues from breast cancer patients. Intriguingly, activated STAT5 correlates with an increased survival and generally a good prognosis in breast cancer.[245,248] Tumours with activated STAT5 are better differentiated and are more responsive to the endocrine treatment.[249,250] This points out that at the later stages of tumour development STAT5 seems to play an anti-tumourigenic role. One of the proposed mechanisms is inhibition of the invasive phenotype by the repression of MMP-2 and BCL6 expression.[251,252] Clearly, the functions of STAT5 in breast malignant transformation and tumour progression are different. However, the mechanism behind the role of STAT5 in the good prognosis of breast cancer remains to be fully investigated.

STAT5 has been shown to be constitutively activated in various tumours of the hematopoietic system (e.g., AML, CML, myeloproliferative disorders).[253,254] The persistent activity is usually driven by genetic aberrations in the upstream kinases, for instance, JAK2V617F hyperactivating mutation (in polycythemia vera), BCR-ABL fusion (in CML) or FLT3 kinase mutations (in AML).[255-257] Consistent with the physiological function of STAT5 to maintain the survival of pro-B-cells during lymphopoiesis (for example, through Mcl1 expression), abnormally activated STAT5 promotes survival, proliferation, and resistance to apoptosis of the malignant cells as well.[258,259] Additionally, it enhances the levels of such cancer-related genes as cyclin D1, BCL-xL, c-myc, Pim1 and represses the pro-apoptotic miRNAs miR15/16.[260-262]

Also, STAT5 may play an additional role in tumourigenesis through regulation of the tumour milieu. STAT5 regulates FOXP3 that induces differentiation of the Th17 cells providing immune tolerance.[263] On the other hand, STAT5 is critical for the maintenance and survival of the cytotoxic NK cells.[264] In conclusion, STAT5 plays a complex role in the immune regulation and immune surveillance that complicates the role of STAT5-specific inhibitors in blood cancers.

### 1.4.6 STAT6

The function of STAT6 has been primarily associated with the activation and proliferation of the immune cells. It is activated by IL4 and IL13 which signal through the IL4- and IL13-receptors, respectively (which share the IL4R$\alpha$ subunit). The receptor chains heterodimerise upon the ligand binding, causing JAK1 and JAK3 to cross-phosphorylate each other and the receptor subunits. Eventually, STAT6 is activated by phosphorylation on a single tyrosine residue, similar to other STATs. In the nucleus, a functional STAT6 dimer binds to GAS, although with low affinity. Instead, STAT6 has a unique binding site which contains a 4-base linker between the palindromes. Other STATs are not able to bind to this sequence, thus providing specificity for the IL4/STAT6 response.[265]

An additional characteristic feature of STAT6 as a transcription factor is its dependence on the complex formation with other transcription factors. Thus, a STAT6 target gene promoter cannot be activated by IL4 in vitro outside the promoter context, although a STAT6-dimer recognises its binding site. If C/EBP or NF-$\kappa$B sites are positioned in close proximity to a STAT6 site, the promoter gets fully activated.[266] The requirement of other proteins for the STAT6 transcriptional activity might explain the fact that the transactivation domain of STAT6 is drastically different from the TAD of other STAT proteins.[130,267]

The main transcriptional result of the STAT6 activity is the regulation of the Th2 cells activity and immunoglobulin switch. There is a report that shows the overexpression of STAT6 in the dedifferentiated liposarcomas and mesenchymal tumours.[268] Overexpression of STAT6 in breast

cancer cell lines leads to inhibition of cell proliferation.[269] The precise role and the mechanisms of STAT6 activity outside the immune cells are not clearly defined.[270]

## 1.5 THE DUAL ROLE OF STAT1 IN CANCER

STAT1 was discovered as a mediator of the IFN signalling. It was the first ever described example of the extracellular signal transduction to the nucleus without a direct contact.[271] The tumour suppressive activity of IFN has been attributed to STAT1 (or to STAT2 by different groups).[272,273]

STAT1 functions through the well-described transcriptional complexes, ISGF3 and STAT1/STAT1 homodimers, both of which require prior phosphorylation. Alternative complexes—ISGF3 without IRF9, for example—have also been described,[274] but the complete ISGF3 is a much more potent transcription factor. However, as it was demonstrated using a STAT1-null cell line U3A and its subline reconstituted with a STAT1 mutant that is unable to form dimers, unphosphorylated STAT1 also possesses transcriptional activity.[275]

Soon after the discovery of STAT1, it became evident that its function is also very context- and cell-type-dependent. This resulted in contradictory reports describing STAT1 both as a tumour suppressor and as an oncogene. However, one should distinguish between the protein levels of total STAT1, pSTAT1, and the mRNA expression level of STAT1, as they might not correlate with each other. It is not always obvious whether it is localisation or expression of STAT1 that reflects its activity in each cancer type. Also, the non-transcriptional functions of STAT1 cannot be excluded to play a role in tumourigenesis.[276-278] Furthermore, a recent ChIP-seq study that investigated the whole-genome binding sites of STAT1 concluded that a GAS-site is not a pre-requisite for STAT1 binding. GAS - sdjacent sites may provide a more complex regulatory mechanism of STAT1-driven transcriptional regulation.

The tumour-suppressive function of STAT1 is conveyed mainly by regulation of transcription of the target genes. It activates the expression of cell cycle regulators (e.g., p21 and p27), pro-apoptotic proteins (e.g., BAD, Bax, Bak, caspase 1, 2, and 3),[279,280] death receptor and its ligands FAS and FASL, DR5, and TRAIL.[281,282] STAT1 is also a potent transcriptional repressor as it inhibits expression of the genes such as anti-apoptotic Bcl-2 family members[283] and pro-angiogenic factors as VEGF and MMP2.[284] Some evidence suggests that STAT1 can complex with p53 thus releasing the repressive complex with MDM2 and facilitating the transcriptional activity of p53 and the consequent cell cycle arrest.[285]

The tumour promoting role of STAT1 was recognised soon after its discovery and continues to be reported in different systems.[286-288] In breast cancer, the oncogenic role of STAT1 was attributed to ISG15.[289] In other studies, it was shown that STAT1 can complex with MUC1, thus providing its own constitutive activation and expression of target genes including MUC1 and MUC4 that are considered the hallmarks of the epithelial transformation.[289-291] Some other mechanisms of the STAT1-induced cancer survival include the upregulation of anti-apoptotic proteins,[292] suppression of tumour cells immune recognition by the upregulation of PD-L1[291] and, possibly, many more to be discovered. In some cases, STAT1 acts as an unphosphorylated dimer, pointing out that an overexpressed protein can gain (un)expected functions even in the absence of activation.

To coclude, a former tumour suppressor STAT1 can have an opposite function when the context is changed. It appears that STAT1 is more than just a transducer of the signal from IFNs to the nucleus.

## 1.6 IRDS IN CANCER

A special case of the pro-tumourigenic effect of STAT1 is the induction of drug resistance. In an initial experiment by Khodarev et al. radiosensitive breast cancer xenografts were subjected to repetitive cycles of radiation. Eventually, the radio-resistant tumours were selected. When the gene expression profiles of the primary tumours and the radio-resistant tumours were compared, a gene signature later termed IRDS (interferon-related DNA damage signature) was discovered.[76] This gene set contained 31 known ISGs. Later it was established that IRDS is upregulated in tumours in response to the fractionated treatment with ionising radiation and chemotherapy.[293,294] Furthermore, these results were reproduced in the clinical samples of therapy-sensitive and therapy-resistant tumours of different origin, including breast cancer.[295,296]

Further studies by the same group identified STAT1 as the main driver of resistance. Overexpression experiments demonstrated that STAT1 conferred the resistance to breast cancer cells while stable downregulation of STAT1 by RNAi led to an increase in therapy sensitivity. Moreover, the docetaxel-resistant prostate cancer cells selected by a long-term exposure to low doses of the drug showed an increase in the expression of STAT1 compared to the parental cells. The cells could be re-sensitised to docetaxel when STAT1 was knocked-down.[297] Other groups obtained similar results in both haematological and solid cancers.[298-300] Notably, STAT1 and a subset of ISGs were found upregulated both in the primary and in the acquired resistance.

IFNs type I and II were proposed to be the triggers of STAT1 activity.[301] Chronic exposure to IFNs leads to constitutively elevated levels of ISGs long after treatment cessation. According to the suggested model, cells are selectively pressured for the survival during the early cancer development. Since the immune system tries to eliminate or growth-restrain the malignant cells, it will lead to the selection of the clone that is the most efficient in withstanding the growth-suppressive effects of the microenvironment. The resistance to cytotoxic drugs would be a secondary effect of this adaptation.[302,303]

Regarding the mechanisms underlying therapy resistance, not much is yet known. There is a report on the involvement of MUC-proteins into STAT1-mediated therapy resistance as described above,[290] however, the exact mechanisms and the contribution of the individual downstream ISGs into cell survival are not elucidated. Upon chronic exposure of the cells to the low-dose IFNs unphosphorylated STAT1 will accumulate. U-STATs (including U-STAT1) are transcriptionally active, and their target genes are not fully redundant with the classical IFN-induced genes but closely resemble IRDS.[304] Clarifying this issue would contribute to our understanding of the resistance mechanism and would greatly aid in the identification of novel biomarkers of therapy response.

A gene signature similar to IRDS was also associated with the reduced risk of bone metastases in breast cancer.[305] Parker et al. identified an IRF7-regulated gene signature that was absent in the bone metastases but present in the primary tumours. Moreover, they provided experimental evidence that IFN treatment and, hence, upregulation of ISGs leads to the reversion of the metastatic phenotype and prolongs overall survival. However, the IRF7-signature correlated inversely only with the development of the bone metastasis and did not correlate with the lung metastasis. In their recent work, the same group showed that the endogenous IFN-signalling is

necessary for the activity of the NK-cells.[306] This result might indicate that the IRDS downregulation (or the downregulation of a similar signature) can be beneficial for tumour cells to disseminate and establish themselves in a new site where an immunosuppressive environment has not yet been created.

The discrepancy between different studies on the role of ISGs in the cancer progression and therapy response remains yet to be explained. The tissue specificity may play a decisive role, although both studies described above (by Khodarev et al. and by Parker et al.) were done on breast cancer models. Additional investigations are necessary to determine whether these different reports contradict each other, or they are just two sides of the same coin.

## 1.7 HSP90 INHIBITORS AS AN ANTI-CANCER TREATMENT

Heat shock proteins are a special group of proteins involved in cancer development. There have been at least five main families of heat shock proteins described: Hsp100, Hsp90, Hsp70, Hsp60, and Hsp27. They are either constitutively expressed in distinct cell compartments or are induced by specific stimuli. Tumour cells are particularly dependent on Hsp90, much more than normal cells.[307]

Heat shock protein 90 (Hsp90) is a ubiquitously expressed molecular chaperone that maintains cell protein homoeostasis under normal and, especially, stress conditions. There are several proteins in the Hsp90 family: cytoplasmic Hsp90$\alpha$ (constitutive) and Hsp90$\beta$ (inducible), mitochondrial TRAP, and endoplasmic Grp94. The cytoplasmic forms are particularly important for cell survival. In normal cells, Hsp90 is involved in folding, modifications, and functionality of the key proteins necessary for cell survival and proliferation.[308] Also, Hsp90 recognises altered and terminally damaged proteins and mediates their incorporation into lysosomes and autophagic degradation[309] or degradation by a ubiquitin-proteasome system. It can also trigger an unfolded protein response upon the accumulation of damaged or non-folded proteins leading to the translation inhibition and the enhancement of heat shock proteins activity.

There are three major domains in Hsp90 proteins: an N-terminal domain with ATPase activity, a C-terminal domain with an interaction site between Hsp90 proteins, and a middle domain where a client protein binds. Under basal conditions, Hsp90 is kept in an open conformation (ADP-bound) and interacts with another Hsp90 protein through the C-terminal parts. The current model of the Hsp90 function implies a two-step activation process. First, Hsp70 together with its co-chaperones bind a client protein and bring it to Hsp90. In an open confirmation, Hsp90 interacts with a co-chaperone HOP, which binds the complex of the Hsp70/client protein and presents it to Hsp90. Binding of the client protein to the middle domain of the Hsp90 complex triggers the ADP to ATP exchange followed by the conformational switch from the open to the shut form where Hsp90 monomers interact through the N-terminal domains, middle domains through the client protein, and the C-terminal domain through ATP. At this stage, the dissociation of the Hsp70 complex takes place. Hsp90 co-chaperones bind to the shut Hsp90 complex and provide the ultimately functional protein state.[310]

Since many of the Hsp90 clients are critical for the tumour cell survival, they need to be maintained functional under endogenous and exogenous stress caused by hypoxia, acidosis, nutrients deprivation, chemotherapeutics, etc. Therefore, cancer cells actively utilise Hsp90 in the repair of damaged proteins and, in this way, Hsp90 contributes to sustaining survival and proliferation. Hence, overexpression of Hsp90 is often detected in cancer and is correlated with

poor outcome.[311-313] The clients of Hsp90 include growth factor receptors (e.g., EGFR), tyrosine kinases (e.g., MEK, AKT), transcription factors (e.g., p53, androgen receptors) and structural proteins (e.g., tubulins), many of which are upregulated in cancer. Hsp90 interacts with proteins in their mature form (compared to, for example, Hsp70 clients). It maintains the optimal conformation of the client proteins ready for the prompt response to a stimulus (e.g., it keeps phosphorylation sites and points of interactions with other proteins exposed, etc.). Despite a large number of clients, it has not been possible yet to identify any common motifs which Hsp90 could recognise.[314] A recent study demonstrates that there is a certain principle of recognition and that the number of clients is definite.[315] It is clear, however, that there is a selectivity comparing to Hsp70s which can bind virtually any protein.

Since Hsp90 clients include many known oncogenes or other proteins involved in tumour survival and progression, one could speculate that Hsp90 is involved in maintaining all main hallmarks of cancer. Experience with the proteasome inhibitor bortezomib has shown that cancer cells do not tolerate the accumulation of misfolded proteins and undergo apoptosis upon the treatment with this drug. Targeting Hsp90 also leads to the accumulation of unfolded proteins; therefore, Hsp90 inhibition was proposed to be an efficient strategy either by itself or in combination with other drugs.

Indeed, numerous studies report the induction of the cell death upon treatment with Hsp90 inhibitors and beneficial effects of combining them with the conventional treatments. To date, several generations of Hsp90 inhibitors have been developed.

The first class of the inhibitors included geldanamycin and radicicol. Although structurally unrelated to each other, these compounds functioned through outcompeting ATP binding to the N-terminal domain of Hsp90 proteins. The drugs also increased the recruitment of ubiquitin ligases to the Hsp90 complex, leading to the degradation of Hsp90 client proteins. The use of these inhibitors in the clinic was not pursued due to their intolerable toxicity.

The second generation of the Hsp90 inhibitors included two derivatives of geldanamycin, 17-AAG and 17-DMAG. 17-AAG was demonstrated to have ≈100-fold increased affinity to Hsp90 in cancer cells than in normal cells, thus pointing out for the first time that Hsp90 inhibitors are more specific to tumour cells.[316] Several clinical trials have been conducted using this drug either as a single agent or in a combination with docetaxel, bortezomib, trastuzumab, and rituximab.[317] Since HER2 is a proven target of Hsp90, many trials focused specifically on breast cancer. Good clinical response was observed in the metastatic HER2+ cancers in the combination with trastuzumab for the tumours that progressed on the monotherapy with trastuzumab.[318] Introducing 17-AAG into the clinic was hampered, though, by poor pharmacokinetics. As a derivative of 17-AAG with improved solubility, 17-DMAG was also tested in clinical trials and showed promising activity,[319] but its toxicity led to the termination of all trials as well. One of the proposed toxicity mechanism is gluthathione depletion.[320]

The third generation of the Hsp90 inhibitors includes synthetic compounds based on the structures of 17-AAG and radicicol. One of the most promising drugs tested in clinical trials is STA-9090 (ganetespib).[321-324]

Currently, there are 15 active clinical trials with Hsp90 inhibitors and about 50 trials are completed. The major problem, apart from the toxicity, is that there are very few proposed biomarkers for the response to Hsp90 inhibitors. Usually, the levels/activity of the likely important client protein is assessed. Also, peripheral levels of Hsp70 are controlled, its increased expression being a sign of the response to Hsp90 inhibition. However, these tests only show the pharmacodynamic response ( i.e., that the drug does affect Hsp90) and do not predict the clinical

response to treatment.[325] Another problem is that in cancer, Hsp70 can partially substitute the Hsp90 function, rendering cells resistant to the treatment. Double inhibitors of Hsp90 and Hsp70 have been reported to be efficient and are under currently in the preclinical development.[326,327]

Also, it became evident that not all Hsp90 clients are equally sensitive to the chaperone inhibition. HER2 in breast cancer and ALK in lung cancer, for example, are very sensitive, while AR, although also an Hsp90 client, does not seem to be affected by Hsp90 inhibitors.[328,329] Therefore, a careful selection of the responsive cancers should be carried out; currently, there is no available marker predictive of the response.

Multiple myeloma (MM) was proposed to be a cancer type that is highly dependent on the protein handling pathways. This partially explains the success of bortezomib in the treatment of this malignancy and suggests that the use of Hsp90 inhibitors would be beneficial as well. Also, it appears that several pro-survival pathways in MM cells are the clients of Hsp90 (e.g., IL6/STAT3, PI3K/AKT, MAPK). Indeed, pre-clinical studies showed that MM cell lines are relatively sensitive to the Hsp90 inhibitors, but there is a fraction of the cell lines and patient-derived cells which are resistant.[330,331] Several clinical trials have been conducted evaluating the safety and efficiency of the Hsp90 inhibitors in this type of cancer, either alone or in combination with bortezomib.[332,333] These studies also confirmed that there is only a subgroup of patients that shows a clinical response to the Hsp90 inhibitors.

In conclusion, targeting Hsp90 is a feasible strategy in anti-cancer treatment. To maximise the benefit of theHsp90 inhibition, treatment response biomarkers and rational combinational protocols should be developed.

## 1.8 STRATEGIES FOR TARGETING STAT3 IN TUMOURS

The first successful attempt to intervene with the STAT3 activity in cancer took place more than 15 years ago by H. Yu and R. Jove.[334] Using a dominant-negative mutant of STAT3 in a xenograft melanoma model, they demonstrated for the first time that inhibiting STAT3 activity prevents tumour growth. From these and later experiments using RNAi, dominant negative mutants, and conditional knock-outs, it was concluded that STAT3 inhibitors would be beneficial for treating cancer.[335]

The first proof-of-principle inhibitor was developed by Turkson et al. in 2001. It was a peptide binding the SH2 domain, thus preventing the phosphorylation and dimer formation.[336] Since then, inhibitors intended for the therapeutic use are constantly being developed. They all provide the evidence that it is possible to target STAT3 by non-peptide inhibitors, but their incorporation into the oncological treatment has not been successful yet.

Below, I outline the main groups of inhibitors developed so far and some of the approved drugs which indirectly influence STAT3 activity.

Peptidomimetics

The Tyr-SH2-domain binding peptide was effective in high concentrations in vitro. Non-peptide drugs that mimic its effect (ISS-610 and S3I-2001) were developed later and were shown to be effective in different malignant cell lines and xenograft models.[337,338] Phosphopeptides derived from LIF, gp130, EGFR and IL10 were also found to bind the SH2 domain of STAT3.[339] These studies resulted in the development of a peptidomimetic inhibitor of STAT3 with an IC50=150 nM.[340] The peptide from the SH2 domain of STAT3 was also modified and shown to bind STAT3,

albeit weakly, and inhibit cancer cell growth in vivo.[341,342] More attempts to improve phosphopeptides resulted in the development of the relatively potent SH2 domain binders in vitro, which failed, however, to inhibit important STAT3-regulated genes.[343] Currently, it is believed that the problems with stability and permeability of phosphopeptides and mimetics outweigh their efficiency in vivo.

Decoy oligonucleotides

An elegant approach for the STAT3 activity inhibition is sponging the activated dimers by short decoy oligodeoxynucleotides (ODN). Initially, STAT3 ODN decoys were designed using the nucleotide sequence of the STAT3 binding site from the FOS promoter that was slightly modified to increase the efficiency. When injected intratumourously, ODN decoy promoted tumour regression in glioblastoma[344] and was able to resensitise bladder cancer and head and neck squamous carcinoma cells to Cetuximab and Erlotinib.[345] Moreover, initial safety trials showed a lack of toxicity from decoy ODN and evident inhibition of the STAT3-regulated genes in the tumours. There are studies currently under way to evaluate the possibility of STAT3 ODN modification to provide stability sufficient for systemic administration. [346-348]

Natural product derivatives

Traditional medicines and plant extracts are a rich source of biologically active substances. During the most recent decade, attempts have been undertaken to study their effects in experimental systems with the aim of developing novel drugs. Although tumour-suppressive and pro-apoptotic effects of natural compounds are often observed, it is usually problematic to identify the mechanisms of their action and primary targets of such treatments. Inhibition of the STAT3 phosphorylation correlated with the induction of apoptosis has been one of the effects pointing at the potential of some of these natural products.

Curcumin, a product from Curcuma longa, showed effectiveness in lung and gastric cancer xenograft models.[349,350] The inhibition of IL6 secretion and the abrogation of STAT3 and NF-κB activities have been attributed to the activity of this compound. The promising results prompted the development of more potent and bioavailable curcumin derivatives (FLLL32, HO-3867, LLL12, etc.) with reported activity in different cancer types.[351-354] As far as the mechanism is concerned, there is a report that some of the derivatives can prevent STAT3 dimerisation through binding to the SH2 domains.[353,354] For the majority of curcumin-like derivatives, the mechanism and primary targets are not clear. Due to their potency, curcumin-like inhibitors are now being tested in clinical trials for different cancer types and the inflammatory diseases. Interestingly, curcumin has been named as one of PAIN compounds 'worst offenders' and it is claimed to be a covalent modifier and membrane disruptor.[355] It is likely that it blocks multiple signalling pathways coming from membrane receptors and, therefore, can be a potent but highly unspecific STAT3 inhibitor.

Some of the other clinically relevant natural product derivatives with activity against STAT3 are 3,3'-diindolylmethane,[356] oleanolic acid[357] and resveratrol.[358] The derivatives of these substances are currently undergoing evaluation in clinical trials (most at phase I-II) for cancer, multiple sclerosis, RA, and even Alzheimer's disease. Pre-clinical experiments demonstrated that treatment with these compounds leads to the downregulation of important STAT3 target genes. The mechanisms of action are not well understood, and the inhibition of STAT3 activity might be a secondary effect.

Apart from those named above, there are a plethora of other natural product derivatives with anti-cancer activity that affect STAT3. As was mentioned earlier, STAT3 gets inputs from

multiple signalling molecules. Therefore, it is not surprising that the tumour - suppressive effect of many natural products is associated with the inhibition of STAT3 phosphorylation.

Tyrosine kinase inhibitors

Receptors with tyrosine kinase activity are frequently mutated or amplified in cancer. This leads to the self-sufficiency of cancer cells, makes them independent of the external stimuli and provides constitutive pro-survival signalling.[9] Many relatively specific tyrosine kinase inhibitors were developed during the last 15 years, both antibodies and small molecules. Although good clinical response is observed upon the initial treatment, resistance develops in almost 100% of the cases. The mechanisms of resistance to targeted therapies are the subject of extensive study. In some cases, STAT3 activation upon drug treatment can be one of the mechanisms that provide a survival advantage for cancer cells, especially in tumours that originally did not harbour a constitutively activated STAT3.

On the other hand, tumours with STAT3-addiction can regress upon treatment with tyrosine kinase inhibitors. Indeed, several drugs have a documented inhibitory effect on STAT3. For example, Imatinib, Erlotinib, and Sorafenib were designed to target specific tyrosine kinases (BCR-ABL, EGFR, and Ras respectively), and they all inhibit STAT3 as well.[359-361] Thus, one of the mechanisms of their action might be the STAT3 inhibition.[362-364]

A special case of tyrosine kinase inhibitors affecting STAT3 phosphorylation is JAK inhibitors. JAK-activating mutations have been seen in several cancer types; therefore, there is a good basis for the use of JAK inhibitors (selective or pan) in oncological treatment. However, it was in the autoimmune diseases (e.g., RA) that JAK-inhibitors were first used. Currently, there are several FDA-approved JAK inhibitors in use (e.g., Tofacitinib, Ruxolitinib) and in trials for cancers, and many more are under development and in early stages of trials.[365-370]

Platinum-based and microtubule-targeting drugs

Traditional chemotherapy remains the main treatment for cancer. Therefore, a lot of studies have focused on the mechanism of action and resistance to these drugs. Although multiple pathways are affected by chemotherapeutics, STAT3 inhibition was proposed as one of the contributing factors to the cell death in response to the treatment.[371]

At the same time, resistance to these chemotherapeutics has been attributed to STAT3 activation in some cancers.[372-374] For example, ovarian carcinoma cells gradually upregulate STAT3 in response to cisplatin treatment and eventually become resistant to the treatment. However, they can be treated by oxaliplatin, other platinum-based compound, that downregulates STAT3 phosphorylation on tyrosine, but induces phosphorylation on serine and the increases the expression of STAT3β.[375]

TLR-conjugated siSTAT3.

Tumour microenvironment has been shown to be important for tumour growth support. Abnormal activity of STAT3 has been detected not only in cancer cells but also in the immune cells of the tumour niche. This results in the reciprocal activation circle between cancer cells and residing immune cells, providing a constant flow of growth factors and cytokines. On the other hand, myeloid cells with constitutively expressed STAT3 secrete immunosuppressive cytokines, thus preventing the immune-mediated elimination of the tumour. In an attempt to exploit this phenomenon, Yu et al. and Kortylewski et al. have developed a system of intracellular siSTAT3 delivery where they conjugated a TLR9 derivative with siSTAT3.[376] Through TLR-part, this

complex is recognised and engulfed by the residing Th1 lymphocytes, which results in the ablation of their activity. Under these conditions, adoptively transferred T-lymphocytes can efficiently target tumour cells.[348] Also, TLR9 signalling was found to be an important contributing factor for tumour development and recurrence, also through the regulation of IL6 production. Therefore, a TLR9-siSTAT3 conjugate might potentially kill two birds with one stone.[377] The modified system with CpG-conjugated STAT3 decoy oligonucleotides was also effective in targeting STAT3 in acute lymphoblastic leukaemia.[378]

Direct STAT-specific small molecule inhibitors

The last five to 10 years have been prolific regarding the development of STAT3 inhibitors. A multitude of small molecules with different potency and specificity are reported to inhibit STAT3 activity in different experimental systems. I would like to briefly outline the milestones of the STAT3 small molecule inhibitors development (more from the the point of view of a cancer biologist than a medicinal chemist).

STA-21 was one of the first inhibitors with STAT3 inhibitory activity that was identified by the virtual screening of the National Cancer Institute (NCI) library collection combined with the drug libraries from Sigma-Aldrich and Ryan.[379] The 3D structures of the compounds were generated and docked against the SH2 domain of STAT3. One hundred compounds from the NCI library were then validated in breast cancer cell lines with constitutively active STAT3. A STAT-responsive luciferase reporter was stably transfected, and the clones with the highest luciferase expression were subjected to the 48h treatment with 20μM of the compounds. STA-21 was selected after this screening and was shown to inhibit the STAT3 DNA-binding activity in the cells. A derivative of STA-21 with improved pharmacological characteristics showed the activity against glioblastoma in a mouse model.[380] LLL compound series based on the functional centres of STA-21 was patented in an attempt to optimise SAR of STA-21.

STATTIC was identified by screening the collection of ≈18,000 compounds in a fluorescence-based polarisation assay.[381] Due to its small size and, possibly, promiscuity[382] it has not become a drug, but an important tool for studying STAT3 activity. Also, the paper reporting the discovery of STATTIC set a standard and the minimal requirements for the validation procedure of STAT3 inhibitors: in vitro binding assay, cellular essay (e.g. a reporter), EMSA for DNA binding, immunofluorescent staining for the nuclear translocation, Western blotting for the induced phosphorylation of STAT3 and STAT1, effect on other proteins in the pathway (e.g., JAKs, JNK for IL6-induced STAT3) and the differential effect in the STAT3-dependent and -independent cell lines.

Turkson et al.[383] identified S3I-201. Virtual screening of diverse compounds identified a molecular probe structurally different from previously described STA-21 and STATTIC. The potency of this compound was rather low (up to 100 μM for 24h in the reporter assay in a SRC-transformed cell line). However, this report was the first to show not only the selectivity of the inhibitor towards the cells harbouring activated STAT3, but also inhibition of a STAT3-dependent gene transcription (Cyclin D1, Bcl-xL, and survivin). Also, S3I-201 was tested in vivo and showed a tumour suppressive effect in a breast cancer xenograft model.

BP-1-102 is a derivative of S3I-201.[384] It was designed using a computer-aided lead optimisation program. It is much more potent than S3I-201, but, most importantly, it is the first

orally bioavailable STAT3 inhibitor. Recently, though, a concern was raised regarding its specificity and suitability as a drug.[385]

OPB-31121 is the only small molecule inhibitor of STAT3 that has reached the clinical trials stage and demonstrated some clinical efficacy.[386] It is described in the initial report as a 'STAT inhibitor' and is shown to inhibit both STAT3 and STAT5. The structure was recently described, and it seems to target the SH2 domain of STAT3, however, at a non-conventional site.[387] The modified version of the compound (OPB-51162) is currently being tested in clinical trials, also with variable response.[388] It is clear now that not all tumours with activated STAT3 respond equally well,[389-391] and it remains to be seen whether this compound will make it into a routine anti-cancer treatment.

## 1.9 A DISEASE MODEL USED IN THE THESIS

### 1.9.1 Multiple myeloma

Clinical view

Multiple myeloma is a malignancy characterised by the clonal expansion of plasma cells in the bone marrow. It accounts for about 1% of all diagnosed cancers and 13% of haematological malignancies. The median age at diagnosis is 65-70 years; the incidence is higher in men; African-American background is an additional risk factor. The disease-associated deaths are tightly connected to the age: In the group diagnosed before 65 years, the five-year overall survival is ≈50% while the mortality rates rise steadily and sharply with each additional five years.

The progression of myeloma, as with any other cancer, is a multi-step process of accumulation of genetic and epigenetic mutations causing the survival advantage of a particular clone of plasma cells. A non-malignant, asymptomatic condition called monoclonal gammopathy of undetermined significance (MGUS) precedes the development of multiple myeloma in most of the patients. MGUS progresses to myeloma at the rate of about 1% per year.[392] The next step in MM progression is smouldering (indolent) myeloma (SMM), a more aggressive, but still a non-malignant and asymptomatic condition with a 10% rate of progression to clinical MM per year.[393]

The current staging system of MM is based on serum concentrations of β-macroglobulin and albumin (the higher the concentration, the higher the is a disease stage and the worse prognosis patient has). Also, cytogenetic FISH assessment of infiltrating bone marrow cells can be used: t(4;14), t(4;16) and 17p deletion are associated with poor prognosis.

The induction therapy usually includes bortezomib and dexamethasone, with or without the addition of the third drug, such as thalidomide, doxorubicin, or cyclophosphamide.[394] In the event of a good response, the same therapy is prescribed for three to four cycles before the cell transplantation.[395,396] The patients who do not qualify for the stem cell transplantation (the elderly or the cachectic) receive a treatment with melphalan and prednisolone, often with thalidomide, bortezomib, or cyclophosphamide.[397,398]

Despite the grading system and some prognostic value of cytogenetic data, the patients are currently not stratified based on these criteria. Therefore, even patients with a relatively good prognosis receive the full treatment due to a general high mortality risk in this disease.

Biological view

MM arises from the transformation of the plasma cells that are terminally differentiated B-cells arrested in the $G_0/G_1$ cell cycle phase. The primary event leading to MM development is believed to be the acquisition of the proliferation ability through activation of D-type cyclins by plasma cells. One proposed mechanism for this event is the chromosomal translocation t(11;14)(q13;q32) that leads to the expression of Cyclin D1 under the control of the heavy chain enhancer. Alternative mechanisms include deregulations in histone methyltransferase MMSET through the t(4;14)(p16;q32) translocation that leads to the increase of H3K36me2 across the genome, that in turn de-represses the transcription of a Cyclin D gene. Upon other chromosomal re-arrangements, Maf transcription factors, which also control the expression of Cyclin D2, can be activated. Apart from that, hyperdiploidy (especially trisomy 11) can also lead to the increased expression of cyclins.

Since the number of plasma cells in the bone marrow increases during the progression from MGUS to SMM and MM, the cells are believed to get additional proliferation advantages. This may happen through the upregulation of Ras and Myc.[399,400] Additionally, chromosome 13 might be deleted leading to insufficiency in the production of a tumour suppressive Rb-protein.[401]

The terminal stage of MM is characterised by the stroma-independent growth that leads to extramedullar diseases and plasma cell leukaemia.

Pro-survival pathways in multiple myeloma

Although it has been shown that plasma cells can survive and even slowly proliferate outside the bone marrow,[402] stroma of the bone marrow (BMS) is essential for their survival and progression. Since the bone marrow is normally a place of haematopoiesis, stroma cells produce a variety of cytokines and growth factors meant to support rapid proliferation and survival of the blood cells (e.g., IL6, IL10, IL-1β, VEGF, bFGF, etc.). Tumour cells hijack the bone marrow microenvironment to maximise their own growth, proliferation, and survival. To avoid the immune system-mediated control, proliferating tumour cells suppress the local immune system and cause irreversible lytic destruction of the bone marrow matrix.

Accumulated genetic abnormalities lead to the abnormal expression of the cell surface adhesion molecules, thus retaining the cells within the bone marrow microenvironment. Interactions of the plasma cells with the stromal cells lead to the upregulation and alteration of cytokine and growth factor secretion reciprocally by the stromal cells and by the plasma cells. Concurrently, an interaction with the proteins of the extracellular matrix (e.g., collagen, fibronectin, etc.) together with a changed cytokine repertoire lead to apoptosis inhibition and cell cycle progression.[403]

IL6 is considered to be the main growth factor for myeloma cells.[404,405] At earlier stages of myeloma development, it is produced by stromal cells; however, continuous exposure to IL6 leads to the autocrine secretion of this cytokine by MM cells. IL6 activates at least three signalling pathways: JAK/STAT3, RAS/MAPK, and PI3K/Akt that were shown to provide the proliferation and survival advantage of the malignant plasma cells.

IGF-1 is also an important factor that stimulates the proliferation of both IL6-dependent and IL6-independent cell lines through the regulation of PI3K pathway, and also of the MAPK cascade.[406,407]

VEGF is yet another essential growth factor for MM cells, especially for the refractory MM. It has been shown that VEGF increases the expression of anti-apoptotic proteins such as Mcl-1 and survivin, and also induces cell proliferation by activating a Ras/MEK/Erk pathway. Pre-clinical studies show that anti-VEGF antibodies and small molecule inhibitors of VEGF slow down

proliferation and induce cell death in MM cell lines.[408] Also, there is experimental evidence that a combination of bortezomib with an anti-VEGF drug has a synergistic effect.[409]

Activated Wnt signalling has also been detected in MM. It leads to nuclear accumulation of β-catenin and promotes the proliferation of plasma cells. Also, Wnt signalling increases the expression of the Wnt inhibitor Dkk1 required for the survival of osteoblasts and prevention of bone resorption under normal conditions.

To summarise, MM remains an incurable disease where it has been difficult to achieve long remissions. It also appears that 'multiple myeloma' is a term that encompasses several distinct diseases with the common feature of clonal plasma cells expansion in the bone marrow and antibody production. Several signalling pathways collaborate in plasma cells to promote their survival. Moreover, populations of myeloma cells with a different phenotype can be identified in a single patient. The use of proteasome inhibitors revolutionised the treatment; however, acquired resistance is a serious problem. Taken together, the drug combinations for the treatment of multiple myeloma should be tailored for each patient. Therefore, it is paramount to investigate different cellular subtypes and their signalling pathways.

## 2 THE AIMS OF THE THESIS

The overall aim of this thesis was to investigate the mechanisms leading to therapy resistance in human cancers and to identify the markers for the treatment response in different systems. I attempted to contribute to the understanding of factors leading to treatment success or failure, as this knowledge might lead to the identification of novel drug targets and, eventually, the development of new therapeutics.

More specifically, we aimed:

1. to investigate the relative contribution of different signalling pathways leading to apoptosis in multiple myeloma cells upon their treatment with IFNα **(Paper I);**

2. to explore the connection between the expression of IFN-stimulated genes and therapy resistance in colon carcinoma **(Paper II)**;

3. to study the involvement of a JAK/STAT3 pathway into apoptosis induction by Hsp90 inhibitors in multiple myeloma **(Paper III)**;

4. to develop novel compounds targeting an IL6/STAT3 signalling pathway and to investigate their mechanisms of action **(Paper IV)**.

# 3 RESULTS AND DISCUSSION

## 3.1 PAPER I

'Activation of STAT1 is required for interferon-mediated cell death.'

### 3.1.1 Background and Rationale

IFN is a cytokine produced by cells to trigger the transcription of genes helping in the clearance of the viral infections. Recombinant IFN has been used in the treatment of severe viral infections (e.g., Hepatitis B), but also in the oncological practice as a therapy for some cancers (e.g., malignant melanoma, multiple myeloma, renal cell carcinoma). Although the anti-viral function of Type I IFN is studied rather thoroughly, the mechanisms of its pro-apoptotic activity are not clearly understood.[410] This is one of the limiting factors of the clinical IFNs usage, probably leading to the underuse of this pluripotent tool in cancer management.

IFN binding to its receptor triggers the signalling through several pathways (e.g., JAK/STAT, PI3K/Akt, mTOR, etc. also illustrated in figure 3).[61] Our group has previously shown that although JAK/STAT is a major pathway induced by IFNα, pharmacological inhibition of PI3K or mTOR leads to a partial protection of the U266 multiple myeloma and the Rhek-1 keratinocyte cells from the IFN-induced cell death.[411] Therefore, we set out to investigate if JAK/STAT signalling also plays a role in the IFN-mediated apoptosis of MM cells.

### 3.1.2 Main findings

The protection of the myeloma cells from the IFN-induced cell death by PI3K or mTOR inhibitors was partial. It points out that an additional pathway(s) is involved in the IFN-mediated apoptosis. We came across the observation that deguelin, a natural compound of the rotenoid family, inhibited the IFN-induced phosphorylation of STAT1 in the U266 cell line. Inhibition of the STAT1 phosphorylation by deguelin correlated with protection from the IFN-induced apoptosis, as demonstrated by the reduction of AnnexinV/PI double-positive cells. The protective effect of deguelin was also partial, similar to the effects of PI3K and mTOR inhibitors. When deguelin was combined with either inhibitor, the cell death induced by IFN was completely blocked. Thus, our experiments have suggested that several pathways need to be involved for the full apoptotic effect of IFN. Also, it appears that the phosphorylation of STAT1 is essential for this effect of IFN.

To further investigate the role of the phosphorylated STAT1 in the apoptosis induction, we transfected the mutant forms of STAT1 (STAT1-Y701A and K410/413A) into the IFN-sensitive keratinocyte cell line Rhek-1. The STAT1-Y701A mutant is not phosphorylated on a crucial Tyr701 residue and the STAT1-K410/413A mutant is not able to move to the nucleus. Upon treatment with IFNα, apoptosis was induced in the mock-transfected cells, as shown by FLICA staining for active caspases. However, in the cells transfected with either of the two mutants apoptosis induction was impaired. This data provides additional evidence that the STAT1

phosphorylation and transportation to the nucleus (hence, most probably, the transcriptional activity) are necessary for the full pro-apoptotic effect of IFNα.

Since both chemical and genetic inhibition of the STAT1 phosphorylation protected from the IFN-induced cell death, we can conclude that the JAK/STAT pathway activity contributes to the induction of the cell death by IFNα. Also, a cooperation between the JAK/STAT pathway with other pathways is required for the maximal pro-apoptotic effect of IFN. This data indicates that in order to reach the clinical benefit by the IFNα treatment, the functionality of these pathways should be preserved.

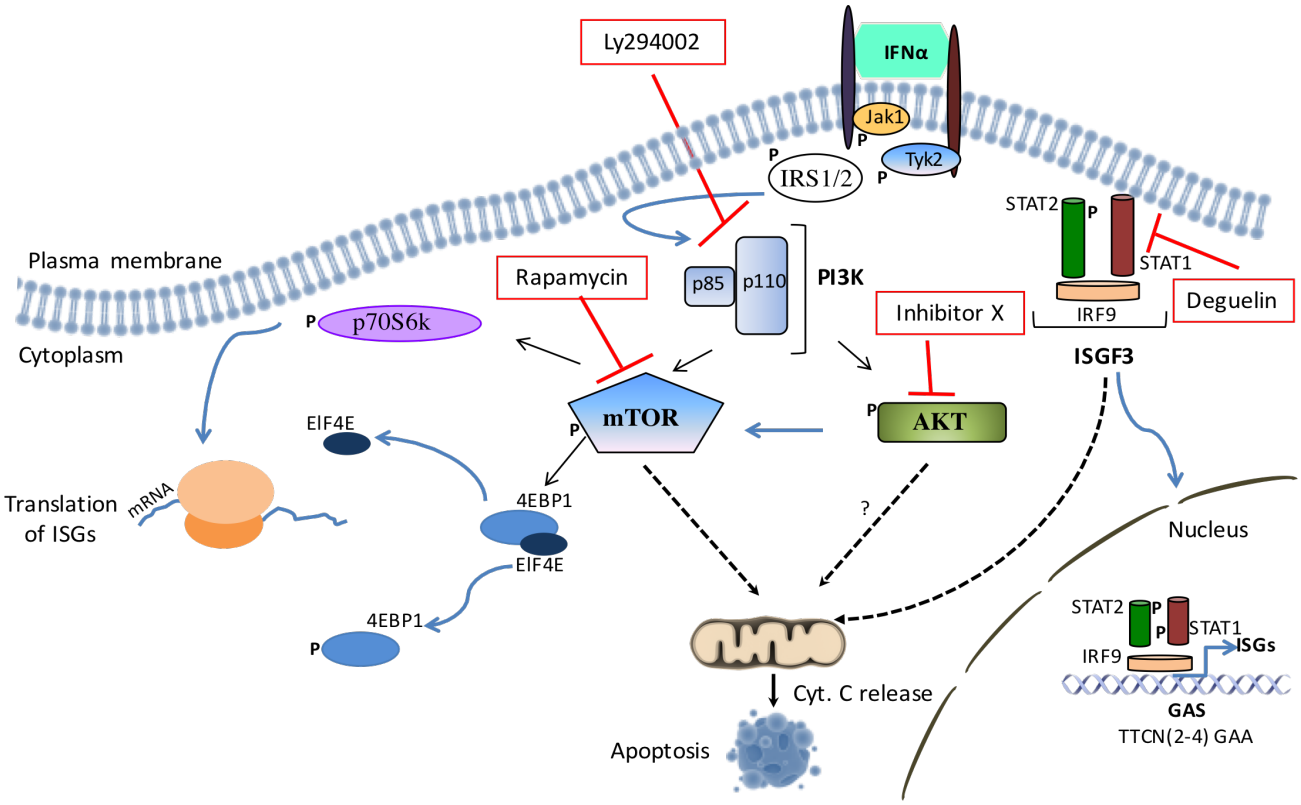


Figure 3. Schematic illustration of the IFN**α**-induced pro-apoptotic pathways in a U266 cell line used in **Paper I**. Chemical inhibitors relevant for this study are shown in red rectangles.

## 3.2 PAPER II

'Cell crowding induces interferon regulatory factor 9, which confers resistance to chemotherapeutic drugs.'

### 3.2.1 Background and Rationale

Since the discovery of Gleevec, many drugs selectively affecting cancer cells have been approved. Being designed as "targeted" therapeutics and aimed at specific patient groups, they revolutionized the treatment of some types of cancer. However, acquired resistance to therapy is inevitably developed, thus preventing cancer cure.[412,413] Similarly, chemo- and radiotherapies,

although effective during the initial treatment, lose their efficiency with each tumour recurrence.[414,415] Besides, there is a phenomenon of the intrinsic resistance in aggressive cancer types that are able to withstand any treatment.[416,417] Therefore, further understanding of the mechanisms is needed to shed light on the molecular events steering cancer cell survival under the treatment.

Multicellular spheroids (MCS) were entailed as a model in cancer drug development since they resemble in vivo tumours in higher degree than conventional 2D culture.[418,419] The monolayers (2D) provide all the cells with the uniform access to the abundant nutrients and drug(s), and it eliminates heterogeneity and a metabolic stress that are involved in the development of treatment resistance.[420,421]

We hypothesized that since MCS are more resistant to drugs than 2D cultures,[417] this might be reflected in their gene expression profile. Therefore, we set out to investigate which genes determine drug resistance of the MCS.

### 3.2.2 Main findings

We performed the microarray comparing the gene signatures of the HCT116 cells (colon adenocarcinoma) grown as a monolayer to the same cell line grown as MCS. The upregulated genes can be grouped into several gene signatures, such as those reflecting the metabolic changes caused by the compromised environment in the 3D culture (e.g., solute carriers *SLC2A3*, *SLC9A7*, stress-regulated members of MAPK-signalling cascade) or the genes described to be involved into non-specific multidrug resistance (e.g., *ABC1* transporters).[422] However, the most prominently upregulated genes were so called interferon-stimulated genes (ISG) that included the members of the ISGF3 transcriptional complex.

We investigated whether this gene signature is restricted to the MCSs of the HCT116 cell line. We cultured cell lines of different origin (ovarian, breast, colon carcinomas, transformed fibroblasts) in 3D and in 2D and measured the expression levels of the ISGF3 complex members (*STAT1*, *STAT2*, *IRF9)* and three ISGs upregulated in the array (*IFITM1*, *IFI27* and *OAS1)*. We concluded that the increase in the expression of these genes is rather a general phenomenon. The differences in the amplitude of ISGs regulation are cell line specific and, probably, depend, among others factors, on the morphology of the spheroids.

When we used monolayer cultures of HCT116 cells to investigate possible stimuli leading to the upregulation of ISGs, we observed that culturing cells over prolonged time leads to a gradual accumulation of *IRF9, STAT1, STAT2* (on protein as well as on mRNA levels), and to a successive increase of *IFITM1, IFI27, OAS1* mRNAs. By comparing cells seeded in different amounts (sparse to confluent) cultured for 24h, we reasoned that the upregulation of the genes was dependent on the density of the cells and, probably, on the cell-to-cell contact, coined by us as “crowding”.

Since the expression of ISGs is transcriptionally regulated by the ISGF3 complex,[423] we investigated whether knocking down STAT1, STAT2 and IRF9 in 2D would lead to the downregulation of ISGs *IFITM1, IFI27, OAS1* upon crowding. The RNAi-mediated STAT1 targeting did affect neither the expression of ISGs nor IRF9 and STAT2. Knocking down STAT2 led to the ISGs partial downregulation. On the other hand, reducing the expression of IRF9 most prominently affected the genes, including *STAT1* and *STAT2*. Additional experiments with the use of a STAT1-negative cell line U3A[424] and its parental line 2f-TGH demonstrated that STAT1 was not necessary for the ISG density-dependent upregulation, but its presence led to much higher gene

expression in this model. Hence, the STAT2/IRF9 complex can effectively induce ISG expression, but the presence of STAT1 might promote even higher expression of ISGs in other systems.

It was described in the literature that a set of genes highly similar to that in the under study in this paper (termed "IRDS") was associated with radio- and chemotherapy resistance in different cancer types.[295] Moreover, it was shown in mice models that tumours, primarily highly responsive to radiotherapy, became successively more resistant with each tumour recurrence.[76] The development of the resistance was associated with IRDS acquisition. We set to investigate if the increased expression of IRF9 changes drug sensitivity in our system. Indeed, moderate overexpression of IRF9 alone led to the upregulation of ISGs, and also rendered the cells resistant to chemotherapeutics.

To summarize, we have demonstrated that the 3D culture growth is accompanied by the upregulation of ISGs in different cancer types. We have also shown that crowding of cells over time and by increasing their density leads to the upregulation of the investigated set of ISGs. The regulation is STAT1-independent, but IRF9- and STAT2-dependent in the HCT116 cells, however, in other systems STAT1 might be involved to promote even further upregulation of the ISG expression. Lastly, the elevated expression of IRF9 alone is sufficient to increase the ISGs expression and to promote resistance to the drugs. Establishing the connection between IRF9, the ISGs and therapy resistance could lead to the evolution of novel approaches in cancer treatment.

## 3.3 PAPER III

'An activated JAK/STAT3 pathway and CD45 expression are associated with sensitivity to HSP90 inhibitors in multiple myeloma'

### 3.3.1 Background and Rationale

Hsp90 is a molecular chaperone ubiquitously expressed in normal cells and elevated in cancer cells of different origin.[425] Presumably, the increased expression of the chaperoning complex in cancer occurs as a response to the proteotoxic stress caused by the overproduction of proteins needed for the survival of a cancer cell.[426] The inhibition of Hsp90 leads to cell death in several cancer types, and several generations of Hsp90 inhibitors have been developed and tested in clinical trials although with modest success so far.[427]

Multiple myeloma is a cancer type that particularly relies on the molecular machinery maintaining protein homeostasis. This dependency underlies the efficiency of the proteasome inhibitor bortezomib in the treatment of this malignancy.[428] It was also proposed that the inhibition of Hsp90 might provide an additional benefit in the clinical management of MM patients by inhibiting critical signalling proteins and by the induction of an unfolded protein response.[429] The experimental evidence confirmed the effectiveness of the Hsp90 inhibitors in a subset of MM cell lines and patient-derived cells.[331] Several generations of the Hsp90 inhibitors were tested in clinical trials in bortezomib-resistant patients, however, their clinical efficiency was insufficient for further implementation. The inclusion criteria into the clinical trials of Hsp90 inhibitors were not based of molecular profiling of the malignant cells that could, possibly, explain a modest overall clinical response.[430] We hypothesized that the efficiency of the Hsp90 inhibitors might correlate with the activity of a signalling pathway particularly dependent on Hsp90 and important for the survival of myeloma cells.

### 3.3.2 Main findings

A predominant activity of either IL6/STAT3 or PI3K/Akt pathway was proposed to be determined by the presence of an IL6-induced phosphatase CD45 which binds to the IGF-1R and dephosphorylates it thereby inhibiting the PI3K pathway activity[431,432]. We investigated whether the response of MM cells to the Hsp90 inhibitors was correlated with the activity of either of these pathways.

First, we characterized a panel of MM cell lines by assessing the basal levels of pSTAT3, pAkt, CD45, CD138 and evaluating their sensitivity to the JAK inhibitor Pyr6 and to the PI3K inhibitor Ly294002. To summarize, we found that the pSTAT3 positive cell lines expressed CD45 and responded to the Pyr6 treatment. Conversely, in line with the previous reports, $CD45^-$ cells had higher levels of pAkt and were sensitive to the inhibition of the PI3K .[433] Importantly, all the investigated cell lines expressed similar levels of the Hsp90 protein.

After that we studied the sensitivity of all the cell lines to the Hsp90 inhibitor 17DMAG. We could conclude that the $pSTAT3^+CD45^+$ cell lines were much more sensitive to the treatment than the $pSTAT3^-CD45^-$ cell lines. In addition, basal pSTAT3 levels of the sensitive cell lines were inhibited upon the treatment with 17DMAG.

When we cultured the $pSTAT3^-CD45^-$ cell line LP1 in the presence of IL6 (as described in[431]), we were able to force the phosphorylation of STAT3 and the expression of CD45 in a small fraction of the cells. This treatment sensitised the cells to 17DMAG, mostly at the expense of the $pSTAT3^+CD45^+$ cell population, thus providing evidence that the JAK/STAT3 pathway is vulnerable to the Hsp90 inhibition.

Further, we investigated the presence of distinct cell populations in the MM patient samples. Although each sample was a mixture of different subpopulations, it was possible to divide the limited number of patient samples we had at our disposal into $CD45^{high}$ - and $CD45^{low}$-expressors. In line with our observations of the cell lines used in this study, $CD45^{high}$ cells had a high number of $pSTAT3^+$ cells and a low share of $pAkt^-$ expressing cells and, vice versa, for $CD45^{low}$ - cells. These results suggest the existence of different patient groups among MM patients, with predominantly activated either JAK/STAT3 or PI3K/Akt pathway.

We proceeded to treat the patient-derived tumour cells with 17DMAG *ex vivo*. After 24h, the levels of caspase 3 were measured as an estimate of the drug -induced apoptosis. Notably, $CD45^{high}$ patient samples had a reduced number of $pSTAT3^+$ cells and the elevated number of caspase $3^+$ cells upon the treatment with 17DMAG. Thus, we could confirm that the $CD45^{high}$ $pSTAT3^+$ cells were more sensitive to the Hsp90 inhibitors than the $CD45^{low}$ - cells, which is in line with our observations of the cell lines. Besides, this experiment further confirmed our hypothesis that the cells with the activated JAK/STAT3 pathway (as measured by the presence of pSTAT3), are mostly responsible for the 17DMAG-induced apoptosis.

To further prove that the activated JAK/STAT3 axis confers sensitivity to the Hsp90 inhibition, we utilized a STAT3C-transfected subline of a MM cell line U266. When treated with 17DMAG, the STAT3C - subline was more resistant to apoptosis than the mock-transfected subline. Thus, STAT3C protected myeloma cells from the apoptosis induced by the Hsp90 inhibitor.

STAT3C is retained in the nucleus longer as compared to the endogenous STAT3.[434] Therefore, the STAT3C protective function might be executed at the level of the target genes.

Indeed, 17DMAG inhibited the expression of a STAT3-regulated gene Mcl1 stronger in the mock-transfected cells than in the STAT3C clone.[435] This observation provides some additional evidence that Hsp90 inhibitor 17DMAG induces cell death by inhibiting the phosphorylation of STAT3. It reduces the transcription of an Mcl1 anti-apoptotic protein, which is particularly important for the survival of MM cells[436,437]. Also, the basal levels of another anti-apoptotic protein Bcl2 increased when STAT3C was overexpressed in the myeloma cells. Although not affected by the 17DMAG treatment, this protein can provide additional protection against apoptosis induction.[438-440]

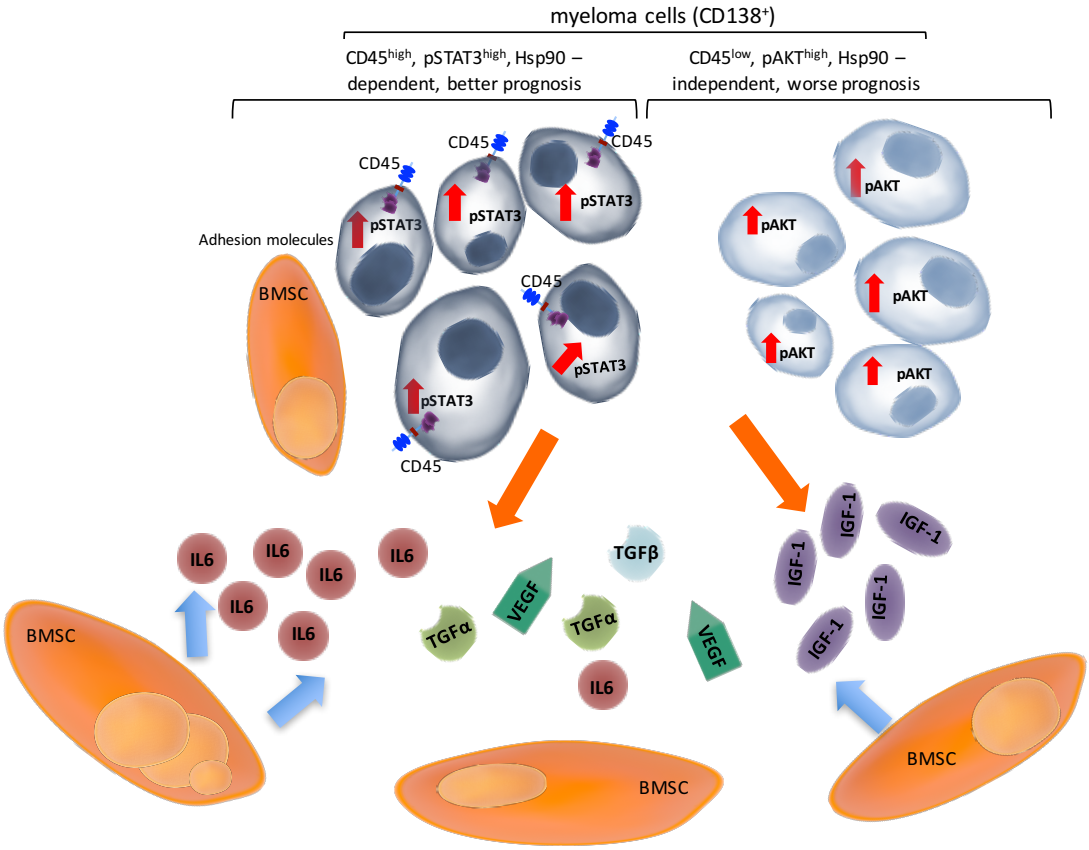


Figure 4. Schematic illustration of the signalling pathways in multiple myeloma cells according to the model described in **Paper III.**

In conclusion, our findings demonstrate that MM cell lines as well as patient samples differ in the CD45 expression and the activity of the IL6/STAT3 pathway. The constitutively activated IL6/STAT3 pathway makes myeloma cells vulnerable to the treatment with the Hsp90 inhibitors through the downregulation of the STAT3 phosphorylation and the expression of its target genes. On the contrary, MM cells relying on the PI3K/Akt signalling pathway are much more resistant to the treatment with 17DMAG (Figure 4). Thus, our data may suggest that the predominant activation of the JAK/STAT3 signalling pathway can be used as a predictive biomarker for the treatment response of myeloma tumours with Hsp90 inhibitors.

## 3.4 PAPER IV

'Development and characterisation of novel inhibitors of STAT-mediated transcription'

### 3.4.1 Background and Rationale

STAT3 is a transcription factor that acts as a convergence point downstream of cytokine and growth factor receptors as well as non-receptor tyrosine kinases. It is phosphorylated in the cytoplasm (e.g., by JAKs, SRC) and then transported to the nucleus where it binds to the gene promoters. Many of the STAT3 target genes are cell type and context specific. The general vector of STAT3-dependent transcription points towards oncogenesis by promoting cell survival, proliferation, inhibition of apoptosis, immune suppression, tumour-promoting inflammation and metastasising.[335] Although a lot of studies proved that STAT3 inhibition leads to cancer cell death,[441] only few inhibitors have reached clinical trials, and none has been approved for clinical use.

One of the reasons for the difficulties in the development of STAT3 inhibitors is the structure of the STAT3 complex. The transcription factor lacks easy-targetable domains, such as ATP-binding pockets frequently exploited during cancer-related drug development. Also, the STAT3 dimer is formed by large interacting surfaces and a lot of energy is required to break them. In spite of these difficulties, attempts to inhibit STAT3 are not abandoned and we undertook our own campaign to identify and develop low molecular weight compounds that affect the transcriptional activity of STAT3.

### 3.4.2 Main Findings

To construct a screening system we decided to use a pair of sublines derived from the colon adenocarcinoma line DLD1: A4 (where STAT3 was homozygously deleted) and A4wt (A4 reconstituted with the exogenously expressed wtSTAT3 at the levels similar to DLD1).[442] The A4wt cell line was stably transfected with the promoter reporter construct made containing 4x SIE upfront of the luciferase gene with a relatively short half-life. The reporter is activated is response to the IL6 stimulation and can be inhibited by a commercially available inhibitor of STAT3 STATTIC and a pan-JAK inhibitor Pyr6. To increase the specificity of our screening in favor of the inhibitors of the STAT3 transcriptional activity (thus excluding inhibition of the upstream kinases), we treated the cells with the library compounds 1h after the stimulation with IL6. Since IL6 induces STAT3 phosphorylation and nuclear translocation within minutes,[443] we reasoned that after 1h of stimulation the JAK kinases would become inactivated by the phosphatases.

After hit selection, we assessed the compounds for their chemical promiscuity and selected the compounds with a pharmacological potential. We carried out additional biological assays to filter out the compounds with a highly unspecific mechanism of action (e.g., the inducers of rapid cell death, general transcription inhibitors, compounds highly toxic for the non-transformed fibroblasts). Also, we utilized the screening system to determine the $IC_{50}$ of the inhibitory activity of the compounds. Simultaneously, we used a STAT3-null subline A4 transfected with the same reporter and stimulated with IFNγ, to study whether the compounds had a differential effect on the

IFNγ-induced luciferase activity (Figure 5 illustrates the complexes formed in response to IL6 and IFN$\gamma$ stimulations).

The compounds were docked against the SH2 domain of STAT3 to assess their potential to bind it. Although the SH2 domain is one of the most popular sites for STAT3 inhibitors binding, we did not restrict our selection to this particular site, as there are other loci described as crucial for the STAT3 transcriptional function. Therefore, based on the initial biological tests and also on the results of docking, we chose 4 compounds for further development and named them KI 1, KI 4, KI 12 and KI 16.

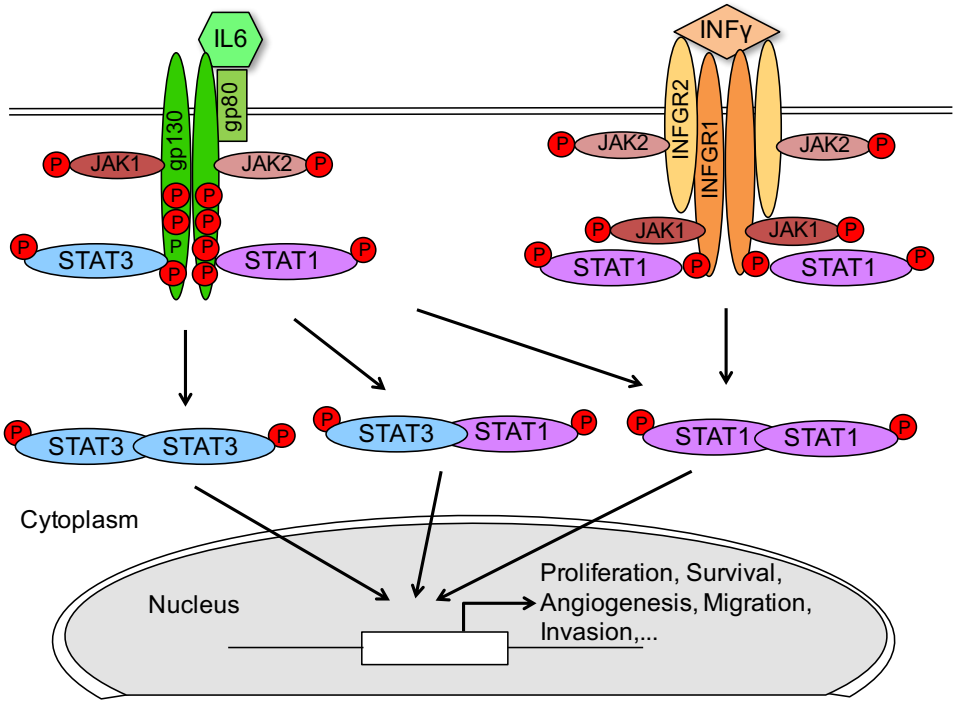


Figure 5. IL6- and IFN**γ**-induced signalling pathways (the courtesy of Y.Yu)

Next, the impact of the compounds on the viability of 4 cancer cell lines with or without constitutively activated STAT3 was assessed. The STAT3-dependent cell lines MDA-MB-468 and DU145 have documented autocrine production of cytokines and growth factors that leads to STAT3 constitutive phosphorylation (such as IL6[444,445] and EGFR[446,447]). These cell lines were more sensitive to our experimental compounds than the cells without the activated IL6/STAT3 pathway (MCF7 and PC3). Moreover, the sensitivity to the drugs was proportional to the basal levels of pTyrSTAT3 in whole cell lysates that gives an indication that the compounds induced the cell death through STAT3 inhibition.

Since STAT3 oncogenic function is exerted to a large extent through its transcriptional activity, we further tested whether the compounds can inhibit the genes induced in our screening system (A4wt cells, treated with IL6).[435] We chose a panel of 4 genes (*MUC1, JUNB, BCL3, TRIM15*) regulated by IL6 as determined by a microarray (J. Yang, unpublished). Cells were pre-treated with the compounds and then treated with IL6. We could see that the IL6-induced gene expression was abolished. These compounds affected the transcription of different genes with varying efficiency which can be explained not only by yet un-optimized chemical structures of the drugs, but also by a complex regulation of these genes promoters.

A different gene set was used to assess the effect of the compounds on gene transcription in the STAT3-dependent cell lines MDA-MB-468 and DU145. When treated with the compounds for

4h, we observed the downregulation of *BCL3*, *CCND1* and *MUC1* genes, although with different efficiency. Since these cell lines express pSTAT3 constitutively, they might require longer treatment to achieve prominent downregulation of the genes.

To further understand the mechanism of action of the compounds, we assessed how they affected the phosphorylated levels of STAT3 and its closely related protein STAT1. When using IL6 to induce the STAT3 phosphorylation on Tyr705, the compounds did not affect pSTAT3 (apart from the compound KI 16, which inhibited pTyr705STAT3 in a dose-dependent manner). When applied in the MDA-MB-468 and DU145 cell lines without external activation of STAT3, KI1 and KI16 demonstrated some activity against the phosphorylated STAT3.

Taken together, we identified several compounds that inhibited STAT3 transcriptional activity. KI1 and KI4 resemble the structure of Erlotinib, whereas KI12 and KI16 are unique structures for STAT3 inhibitors. Consistently with its alignment with the SH2 domain of STAT3 KI16 inhibited the IL6-induced phosphorylation of STAT3.

## 3.5 GENERAL DISCUSSION AND FUTURE DIRECTIONS

Targeted therapy was thought to revolutionize the treatment of cancer by selectively killing cancer cells and sparing normal cells. It would result in minimal side effects. The concept worked in experimental systems, but turned out less efficient in patients.[448] First, the blame was laid on the absence of the molecular diagnostic criteria that led to the overtreatment of a large group of patients when only a small fraction could benefit from a drug. This problem was addressed, and the patients receiving a drug were screened for a targeted mutation.[449] However, it did not lead to the expected success.[450] The same mutation in different cancer types may result in a differential response to the same therapy. Taken together, it points out that cancer is extremely intricate, and it is unlikely to be defeated with a single agent. The efficiency of the available therapeutics can be increased when a set of three biomarkers is used that assess the prognosis of the intrinsic resistance, prediction the most efficient therapy and the evaluation of the clinical response.[451]

Three papers of this thesis address the issue of biomarkers (**papers I-III**), while paper **IV** describes our attempt to hit cancer cells by knocking off one of the central nods in the signalling.

Empirical evidence showed that IFNs have a potent biological activity. Therefore, they were used to treat cancer and infectious diseases in the absence of better alternatives. IFN use declined as more effective drugs were introduced, and now it is used in several specific types of cancer only. This can be partially explained by its limited efficiency as a single treatment agent, but also by some side effects of the IFN treatment tilting the balance of risk vs benefit against its use.

It appears that although almost all cells are growth arrested upon treatment with IFN, the direct pro-apoptotic effect on tumour cells occurs in a limited number of cancers.[452] In our study (**paper I**) we use two cell line models: the multiple myeloma cell line U266 and the human keratinocyte cell line Rhek-1, that both undergo apoptosis upon the treatment with IFN. What determines whether the cells (even of the same origin, e.g. multiple myeloma) will undergo apoptosis is not yet clear. Using pharmacological inhibitors of different pathways, we have demonstrated that phosphorylation of STAT1 is essential for the IFN-induced apoptosis. We have confirmed this observation by overexpressing the STAT1 dominant-negative mutants. Both chemical and genetic inhibition of the STAT1 phosphorylation caused a partial protection from the IFN-induced apoptosis. The complete protection was achieved when, at least, two signalling pathways were inhibited (e.g., the JAK/STAT1 and the PI3K/Akt pathways).

After the discovery of the JAK/STAT pathway as the main cascade propagating the IFN-signal, it soon became obvious that IFN induced other pathways as well. Among them, for example, is a MAPK/p38 pathway that was shown to be essential in the IGSs transcription separately from the JAK/STAT pathway activity.[453,454] The PI3K/Akt pathway has also been extensively studied in relation to the antiviral effects of IFNs through the transcriptional regulation of ISGs. Akt deficiency impaired the translation of ISGs, but not the transcription, showing that JAK/STAT-induced transcription is an independent event.[455]

In our study, we show that PI3K is important for the IFN-induced apoptosis and that its pharmacological inhibitors do not affect the JAK/STAT pathway activity. In a similar vein, previous reports demonstrate that PI3K pathway signals downstream of JAKs, but independently of the JAK/STAT arm. Deletions of Akt (either of one or both alleles) had a dose-dependent inhibitory effect on the translation of ISGs and an antiviral function of IFNs, but no effect on the phosphorylation, nuclear translocations, or activity of STATs.[455]

Recent studies have shown that type I IFNs can induce autophagy in some cancer cells.[456,457] IFNβ-triggered apoptosis was induced independently of the PI3K-regulated autophagy in a glioma model. In this case, autophagy induction was a pro-survival mechanism that counteracted the pro-apoptotic effect of IFNβ.[458] Rapamycin, an mTOR inhibitor used in our study, is a known inducer of autophagy.[459] Akt inhibitor X (also known as 10-NCP) has been found to induce autophagy in an Akt- and PI3K-independent manner.[460] In other systems, Ly294002 (a PI3K inhibitor) was shown to block IFN-induced STAT1- and STAT2-dependent autophagy in a range of cell lines.[456] At the same time, this compound induced autophagy and cell death in other cell lines independently of the IFN treatment.[461] It is recognised now that autophagy in cancer cells can both protect from and induce cell death depending on the context, applied drug, the stage of tumour development, etc.[462] Autophagy in MM may also play both protective and pro-apoptotic roles.[463] We have not addressed the autophagy regulation upon the IFN treatment in our system; it is likely, however, that autophagy might protect multiple myeloma cells from the IFN-induced cell death.

Apelbaum et al. performed an siRNA screening upon treatment with type I interferon to determine which genes were important for the IFN-mediated cell death. They confirmed our observation that STAT1 (as well as JAKs, IFNARs, and IRFs) were of utmost importance, but, surprisingly, they did not find that RNAi-mediated inhibition of any of the PI3K downstream targets protected from the IFN-induced cell death.[464] Tthe same group earlier reported the discovery of an IFN mutant with much higher affinity to IFNAR1 than the wild type and with 100-fold decreased EC50 for the induction of apoptosis.[465] Antiviral properties of the mutant IFN were hardly affected, demonstrating again that the induction of apoptosis and of antiviral state are regulated separately from each other.

The use of IFNs as anti-cancer drugs was based largely on empirical evidence. Our study exemplifies the importance of delineating the exact mechanisms of the anti-tumour activity of drugs, including IFN. On one hand, this might aid in understanding the mechanisms of resistance, and, on the other hand, will provide the basis for appropriate drug combinations in individualised anti-cancer therapy. Since a tumour is currently viewed as a heterogeneous organ, where the dominance of a particular clone is governed by intricate mechanisms, it is thought that a combination of traditional radio- and chemotherapy with targeted drugs (simultaneous or sequential) is the future of clinical oncology.[466]

External immune system activation during immunotherapy either requires IFN for the immune cell activation ex vivo or the immune cells use IFN as an effector. Therefore, the use of IFNs as part of complex immunotherapy is not improbable. Considering our findings that the PI3K

and mTOR inhibitors inhibit the IFN-induced cell death, one should be careful when designing the combinations of targeted therapies with IFNs or with immunotherapy.[467,468]

From a wide perspective of tumour biology and treatment sensitivity, this study leads to two general conclusions. Firstly, a signal (such as IFN) generates a massive response in a cell, and cooperation between seemingly separate pathways determines the nature of the response. Secondly, a canonical pro-tumourigenic pathway can participate in orchestrating the pro-apoptotic effect when interacting with other signalling pathways. These findings are important for the rational use of IFN.

In the study described in **Paper II** we investigated the mechanisms of intrinsic drug resistance of the cells and demonstrated that it correlated with the expression of a set of interferon-stimulated genes. Several groups came across a similar observation using tumour samples of different origin (see section 'IRDS in cancer'). The analysis of the available expression data demonstrates that ≈30-40% of breast, head and neck, ovarian carcinomas, as well as a fraction of glioblastomas and hematological tumours express IRDS, a set of genes initially described by Khodarev et al., and considered to be a marker of drug resistance. About 50% of tumours bearing IRDS are more aggressive and have worse prognoses than tumours without IRDS (Khodarev et al., unpublished).

Several studies conclude that STAT1 is the main inducer of the IRDS.[469] Although also induced in our system (both in MCS and in crowded monolayers), we found that STAT1 is disposable for the expression of ISGs, at least in the HCT116 cell line. In the other system (U3A-2fTGH pair), STAT1 expression contributed to the induction of ISGs, but was not necessary for it, since a STAT1-null subline U3A still showed upregulation of the genes and protein levels of STAT2 and IRF9. According to our data, IRF9 upregulation is sufficient for both ISGs induction and for driving drug resistance. Since knockdown of IRF9 and STAT2, but not STAT1, led to the reduced expression of ISGs, we hypothesise that the IRF9/STAT2 complex is a transcriptional regulator of ISGs. Notably, we were not able to detect the phosphorylated forms of STAT proteins when cultured over time, suggesting that unphosphorylated ISGF3 (U-ISGF3) or U-STAT2/IRF9 drive the ISGs in this system.[151]

There is a view that tumours and cell cultures secrete low levels of IFNs in response to the exposure to DNA of neighbouring cells that undergo necrosis or in response to the DNA of oncogenic viruses.[470] In our system, though, no traces of IFNs were detected, neither on the mRNA level nor in the secreted form. As additional evidence, we demonstrate that cells without STAT1 are not able to adequately react to IFNs by inducing IRF9 levels, once again demonstrating that ISGs in our system are unlikely to be driven by IFN.

The question remains as to why the role of IRF9 is undermined in the analysis of the publicly available expression data.[470] The reason for this might be that IRF9 does not have a transactivation domain and, therefore, cannot solely drive the transcription of ISGF3-regulated genes while STAT1 homodimers are strong transcriptional enhancers. As shown in **paper II**, overexpression of IRF9 about two- or three-fold led to the induction of ISGs and drug resistance. This might indicate that moderately increased IRF9 levels stimulate complex formation with STAT1/STAT2 or solely with STAT2 to drive gene transcription.[143,144] As reported by Luker et al., overexpression of IRF9, but not STAT1 and STAT2, confers drug resistance. The same group also reported that IRF9 was overexpressed in 50% of breast carcinomas resistant to antimicrotubule agents.[471] This result is consistent with our own data, where siRNA-mediated downregulation of IRF9 had a more pronounced effect on the transcription of ISGs than the downregulation of STAT2.

Taken together, the role of IRF9 in mediating drug resistance might still be underestimated. Unveiling the mechanism of IRF9 induction and ISG-induced cell death will allow evaluating the potential of IRF9 as a resistance biomarker. Additional experimental and epidemiological studies are needed to reveal whether IRF9 promotes any other phenotype than treatment resistance.

In **paper III**, we show that a typical pro-oncogenic pathway predisposes to the drug sensitivity. Hsp90 inhibitors were developed as anti-cancer drugs based on the assumption that cancer cells require high chaperoning activity. Multiple proteins involved in oncogenesis have been shown to be clients of the Hsp90 complex, thus potentially rendering cancer cells vulnerable to the Hsp90 inhibition while sparing normal cells. In our study, we observed that MM cells have predominantly activated either the IL6/JAK1/STAT3 or the PI3K/Akt pathways, probably due to IL6-regulated expression of CD45 which shuts down the PI3K/Akt signalling. Both pathways being oncogenic, it appears that the IL6/STAT3 pathway is particularly dependent on the Hsp90 activity, whereas the PI3K/Akt pathway is less dependent. This finding was also applicable to primary cells where we could identify a subgroup with high CD45 and pSTAT3 expression and with low CD45 and high pAkt expression. The $CD45^{high}$ population was sensitive to the Hsp90 inhibition, showing signs of apoptosis and decreased pSTAT3 levels upon the treatment with 17DMAG. Moreover, the external induction of the JAK/pSTAT3 pathway was sufficient to sensitise the cells to the Hsp90 inhibitors, as was demonstrated by prolonged treatment of a resistant $CD45^{-}$ cell line LP1 with IL6. Taken together, we observed that an active IL6/STAT3 pathway might serve as a predictive factor for the sensitivity to Hsp90 inhibitors.

When the primary chemotherapy or even targeted therapy is applied, it is common that cells develop resistance through the induction of STAT3.[222,223,472-474] In this case, it might be reasonable to apply the inhibitors of STAT3 or Hsp90 inhibitors to reach further tumour regression. The use of Hsp90 inhibitors for the co-treatment with other conventional drugs has already been found beneficial.[475,476] Surely, the dependence of pSTAT3 signalling pathway on Hsp90 should be further investigated to understand whether this phenomenon applies to other cancer types. Although STAT3 was described as a client of Hsp90,[477,478] inhibition of STAT3 phosphorylation by Hsp90 inhibitors occurs most probably due to degradation of JAK1 and JAK2; therefore, the mode of activation of STAT3 in a particular cancer type should be taken into consideration.[479,480] Importantly, the redundancy in the chaperoning pathway should be considered when inhibiting Hsp90.[481,482]

In **Paper IV** we describe the identification and initial preclinical characterisation of novel inhibitors targeting STAT3 signalling in cancer. We identified the inhibitors by screening a compound library in a cellular system designed to monitor STAT3 transcriptional activity. Using this system allowed us to account for the drug penetration and solubility issues, but we had to address the issue of the primary drug target later. Therefore, we applied very stringent criteria to the lead selection from the primary and secondary screenings and the counter-screens, and also performed the compound structure analysis to identify the most promising and structurally novel compounds.

By assessing the expression of STAT3-target genes, we could see that all selected compounds inhibited the IL6-induced expression of *MUC1, BCL3, TRIM15*, and *JUNB* genes. Also, the compounds inhibited the transcription of the IFNγ-induced genes, which indicates that the compounds are likely to be more specific to STATs than to the upstream kinases.

When we performed the docking of the studied compounds to the SH2-domain of STAT3, we did not detect high glide scores for the compounds KI 1, KI 4, and KI 12. It is consistent with a Western blotting analysis that showed that these compounds (KI 1, KI 4, and KI 12) did not influence the IL6-induced phosphorylation of STAT3. On the other hand, KI 16, which had the highest glide score of the four compounds, reduced the levels of pSTAT3. Interestingly, none of the compounds (including KI 16) had high gliding scores when docked against the SH2 domain of STAT1 or against JAK1.

It should also be noted that the compounds tested in the biological assays were identical to the ones in the library and have not been subjected to any chemical modification yet. The average molecular weight of these compounds is ≈300 (for comparison, Gleevec is 493.6). Thus, there remains the opportunity for structure-activity relationship development in the functional group.

Our findings indicate that it is likely that the compounds we selected act through different mechanisms, which remains to be studied. Using the docking, we can assess the potential of the compounds to bind only to specific domains with available crystal structures (for example, the SH2-domains for STAT3 and STAT1), but not to the protein as a whole. A common method to verify the drug binding to its target is the assessment of protein stability upon subjecting it to increasing temperatures (thermal shift assay and CETSA).[483-485] On the other hand, to get a more comprehensive idea of the drug effect on the cells, a gene expression-based approach is widely utilised. After generating a drug-specific gene signature in a cell line(s) of interest, it is queried against disease-specific and drug-specific expression data in the Connectivity Map Project, allowing for all-embracing analysis of the compound effects.[486] Both physical binding of the candidate compounds to STAT3 protein and the functional analyses should be performed to elucidate the mechanism of action and to predict side effects of the compounds under study.

Finally, another question which often arises in conjunction with the development of STAT3 inhibitors is the feasibility, novelty, and the potential of the attempts. Considering the abundance of the published compounds claimed to inhibit STAT3 while none has been approved as a drug, it is reasonable to admit that STAT3targeting is a difficult task. On the other hand, STAT3 is way too attractive a target to be abandoned, and, regardless of how much effort it takes, there will always be dreamers who will keep trying. After all, everything is impossible until someone has done it, and I strongly believe that any big discovery is based on multiple small discoveries. Therefore, with every developed compound we learn a bit more about STAT3 and come a bit closer to the development of a drug.

# 4 CONCLUSIONS

1) IFN induces several signalling pathways in the cells, and a cooperation of at least two pathways (the JAK/STAT1 and the PI3K/Akt) is necessary to trigger apoptosis in a multiple myeloma cell line. Phosphorylation of STAT1 is required for the pro-apoptotic effect of IFN **(Paper I)**.

2) Multicellular spheroids express a set of genes that belong to the signature previously associated with therapy resistance. Crowding of cells of different origin also results in the upregulation of the IFN-stimulated genes without the production of IFN. The genes are regulated by IRF9 and STAT2 while STAT1 is dispensable. Overexpression of IRF9 alone leads to multidrug resistance in a colon carcinoma model **(Paper II)**.

3) The IL6/STAT3/CD45 and the PI3K/Akt pathways are mutually exclusive in multiple myeloma cell lines. Hsp90 inhibitors induce apoptosis in multiple myeloma cells through downregulation of STAT3 phosphorylation and and the expression of its target genes. The IL6/STAT3 pathway activity and the expression of CD45 phosphatase predict the sensitivity of multiple myeloma cell lines and *ex vivo* treated primary myeloma cells to Hsp90 inhibitors **(Paper III)**.

4) We report the development of novel inhibitors of STAT transcriptional activity in cancer cells. Four investigated compounds differently affect phosphorylation of STAT3 and are likely to have different mechanisms of action. Regardless of their effect on pSTAT3, the compounds inhibit the expression of STAT-regulated genes **(Paper IV)**.

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