Barbara Ehall, BSc.

Investigating the Role of Chemokine Receptors in Lymphomagenesis

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Priv.-Doz. Mag.rer.nat. Dr.scient.med. Alexander Deutsch

Medical University of Graz, Department of Hematology

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AFFIDAVIT

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Quote

”There is a single light of science, and to brighten it anywhere is to brighten it everywhere.”

Isaac Asimov

Danksagung

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Kurzfassung

Hintergrund: Chemokine und Chemokinrezeptoren, vor allem die CXCR4-CXCL12 Axe, sind für ihre signifikante Rolle in der Metastasierung und Verbreitung von Malignomen bekannt. Um diese Axe als neue Behandlungsoption für Lymphome zu erschließen, untersuchten wir die *in vitro* Effekte von drei CXCR4 Antagonisten namens AMD070, AMD3100 und WK1- letzterer ein von uns synthetisiertes Nicotinsäurederivat von AMD070- auf aggressive Lymphomzellen. Zusätzlich wurde CLL spezifische Chemokinrezeptorexpression mit mehreren erhobenen klinischen Parametern verglichen.

Methoden: Die Expression von CXCR4 und die Bindung von CXCL12 wurden mit $CXCL12^{AF647}$ Bindeassays und anschließender FACS Analyse untersucht. Mittels modifizierten MTS-Assays, Annexin V/ 7-AAD und Caspase-3 Färbung wurden die zellulären Effekte von drei CXCR4 Antagonisten analysiert. Das Chemokinrezeptor Expressionsmuster von CLL Proben wurde mittels RT-qPCR untersucht.

Resultate: AMD3100, AMD070 und WK1 waren in der Lage die CXCR4-CXCL12 Bindung zu blockieren, wohingegen ihr blockierendes Potential in Hinsicht CXCR7- CXCL12 vernachlässigbar war. Bemerkenswerterweise konnten wir demonstrieren, dass WK1 und AMD070 zytotoxische Effekte auf CXCR4+ CXCR7- Lymphomzellen besitzen, welche über Apoptose induziert werden. Die Chemokinrezeptor Expressionsanalyse in CLL Patienten ergab, dass CCR7 weniger stark exprimiert wird in Patienten welche Therapie benötigten.

Schlussfolgerung: Unsere Daten zeigen, dass AMD070 und sein Derivat WK1 pro-apoptotische Wirkungen auf aggressive Lymphomzellen besitzen und damit beide Moleküle für neue Lymphomtherapeutika in Frage kommen. In CLL könnte CCR7 als neuer Biomarker für die Risikoeinschätzung fungieren. Allerdings sind weitere Studien mit größerern Probensets unerlässlich, um diese Aussage zu validieren.

Abstract

Background: Chemokines and chemokine receptors, especially the CXCR4-CXCL12 axis, are well known to be implicated in cancer metastasis and progression. To investigate the CXCR4- CXCL12 axis as novel target for lymphoma therapy, we determined the *in vitro* effects of three CXCR4 antagonists, named AMD070, AMD3100 and WK1 - nicotinic acid derivative of AMD070 synthesised by us- on aggressive lymphoma cells. Additionally, CLL specific chemokine receptor expression patterns were compared to various collected clinical parameters.

Methods: CXCR4 expression and CXCL12 binding were analysed using $CXCL12^{AF647}$ binding assays followed by FACS analysis. By using modified MTS assays, Annexin V/ 7-AAD staining and caspase-3 staining we analysed the cellular effects of three CXCR4 antagonists. Chemokine receptor expression pattern analysis of CLL samples was performed with RT-qPCR.

Results: AMD3100, AMD070 and WK1 were able to block CXCR4- CXCL12 binding, whereas their blocking potentials on CXCR7- CXCL12 were negligible. Remarkably, we demonstrated that WK1 and AMD070 possessed cytotoxic effects on CXCR4+ CXCR7- lymphoma cells mediated by induction of apoptosis. In addition, our chemokine receptor expression analysis in CLL patients revealed that CCR7 was lower expressed in patients in who needed therapy.

Conclusion: Our data demonstrate that AMD070 and its derivative WK1 possess pro-apoptotic effects on aggressive lymphoma cells and thereby both represent molecules to develop novel lymphoma therapeutic agents. In CLL, CCR7 might serve as novel biomarker for risk stratification. However, further studies with larger sample sets are necessary to validate its impact.

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Abbreviations

7-AAD 7-amino-actinomycin D

ABC activated B-cell like lymphoma subtype

AID activation-induced cytidine deaminase

AML acute myeloid leukemia

BCL-2 B-cell lymphoma - 2

BCL-6 B-cell lymphoma - 6

BCR B-cell receptor

BL Burkitt lymphoma

cDNA complementary DNA

CLL B-cell chronic lymphocytic leukemia

CSR class switch recombination

CXCL12 chemoattractant a.k.a. SDF-1Îś (stromal cell-derived factor 1)

CXCR4 C-X-C chemokine receptor type 4

CXCR7 C-X-C chemokine receptor type 7

DLBCL diffuse large B-cell lymphoma

DMSO dimethyl sulfoxide

DNA deoxyribonucleic acid

EZH2 histone methyltransferase enhancer of zeste homologue 2

FBS fetal bovine serum

FDA Food and Drug Administration

FDC follicular dendritic cell

FL follicular lymphoma

GC germinal centre

GCB germinal centre B-cell lymphoma subtype

GEP gene expression profiling

GPCR G protein coupled receptor

HBSS Hank's balanced salt solution

HIV human immunodeficiency virus

HL Hodgkin lymphoma

HPRT1 hypoxanthine-guanine phosphoribosyltransferase 1

HSC hematopoietic stem cells

IRF4 interferon regulatory factor 4 a.k.a. MUM1

Ig immunoglobulin

MCL Mantle cell lymphoma

MEF2B myocyte enhancer factor 2

MYC v-myc avian myelocytomatosis viral oncogene homolog

NF-kB nuclear factor kappa-light-chain-enhancer of activated B-cells

NK Natural killer

NHL Non Hodgkin lymphoma

mRNA messenger ribonucleic acid

Pax5 paired box 5

PBS phosphate buffered saline

PCR polymerase chain reaction

PMBL primary mediastinal B-cell lymphoma

PRDM1 PR domain zinc finger protein 1 a.k.a. BLIMP-1

RAG recombination-activating gene

R-CHOP first-line treatment: rituximab with cyclophosphamide, doxorubicin, vincristine and prednisone

RNA ribonucleic acid

RPMI Roswell Park Memorial Institute

SHM somatic hyper mutation

WHO World Health Organisation

1 Introduction

1.1 Lymphoma

Malignant transformation of leukocytes, also known as white blood cells, leads to the development of lymphoma, leukaemia or myeloma depending on which type of immune cell the transformation occurs in. Lymphoma originate in lymph nodes, leukaemia in bone marrow and myeloma in plasma cells. [1]

Malignant immune cells start behaving abnormally and grow uncontrolled leading for instance to swelling in lymph nodes. Lymphoma can occur in different parts of the body such as thymus, lymph nodes, spleen, bone marrow and any other organs and tissues. [1]

The World Health Organisation (WHO) classified lymphomas in 2001 by cell type and molecular, phenotypic and cytogenic characteristics. These classifications have been updated in 2008 and 2017 and an overview is shown in table 1. [2] Not all classifications are relevant in terms of pathogenesis, but they have significant impact on treatment options. [3]

Table 1: WHO classification of hematopoietic and lymphoid malignancies [2]

WHO classification of lymphoid neoplasms
Mature B-Cell Neoplasms
Chronic lymphocytic leukaemia / small lymphocytic lymphoma and monoclonal B-cell lymphocytosis
Follicular lymphoma
Mantle cell lymphoma
Heavy chain disease
Diffuse large B-Cell lymphoma
High grade B-cell lymphomas
Burkitt Lymphoma
Mature T-cell and Natural Killer Cell Neoplasms
Hodgkin Lymphomas
Histocytic and Dendritic Cell Neoplasms
Post-Transplant Lymphoproliferative Disorders (PTLDs)

Lymphoma can be categorised broadly in Hodgkin (HL) and non-Hodgkin lymphoma (NHL), where around 90% of lymphoma NHL. [3] HL are characterized by the presence of Reed-Sternberg cells, which are giant tumour cells with atypical morphology, representing less than 1% of the cellular infiltrate. [4]

Lymphoma can originate from different immune cells namely T-cells, B-cells and natural killer (NK) cells. [2] Even though B- and T- cells are approximately equal in their prevalence in the human body, B-cell lymphoma account for around 95% of lymphoma in the western world. [3]

NHL encompass many lymphoma subtypes such as - in descending order of prevalence - diffuse large B-cell lymphoma (DLBCL; 30-40%), follicular lymphoma (FL; 20%), B-cell chronic lymphocytic leukaemia (CLL; 7%), MALT lymphoma (7%), mantle cell lymphoma (MCL; 5%), Burkitt's lymphoma (BL; 2%), nodal marginal-zone lymphoma (2%), primary mediastinal B-cell lymphoma (PMBL; 2%) and other even rarer subtypes. [3]

Haematological malignancies like the ones mentioned above represent a huge burden on the healthcare system and society as a whole. They are prone to relapses and require long-term and expensive treatments due to them quickly developing resistance to each round of treatment, thus requiring continuous research into new drugs to treat these malignancies. [1]

Comparable to TNM staging in solid tumours, lymphoid malignancies are staged using the An Arbour Staging System as seen in table 2. Staging is used to determine the malignancy and extent of lymphoid neoplasms, which is crucial in deciding an appropriate treatment and prognosis. [4, 5]

Table 2: Ann Arbor Staging System [6]

Stage	Features
I	Involvement of a single lymph node region or lymphoid structure
II	Involvement of two or more lymph node regions, or localized involvement of one extra-nodal site and one or lymph node regions, all on the same side of the diaphragm
III	Involvement of lymph node regions or structures on both sides of the diaphragm
IV	Diffuse or disseminated involvement of one or more extra-lymphatic organs, OR isolated extra-lymphatic organ involvement without adjacent regional lymph node involvement, but with disease in distant site(s), OR any involvement of the liver, bone marrow, pleura or CSF

1.2 DLBCL

DLBCL are aggressive lymphoma, which are mostly curable. Around one third of patients do not manage to go into remission and eventually relapse. [7, 8]

Currently, the R-CHOP treatment regimen (consisting of rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) is the first-line therapy and has curative effects on a significant number of patients. [9]

Diffuse large B-cell lymphoma have been subdivided thanks to gene expression profiling (GEP) into three astonishingly heterogeneous molecular subtypes after their cell of origin and the differentiation stage of B-cells. [8]

Germinal centre B-cell-like lymphoma (GCB) originate from centroblasts, activated B-cell-like lymphoma (ABC) are derived from plasma-blasts and primary mediastinal B-cell lymphoma (PMBL) derive from thymic B-cells. [8, 10] For a long time they were regarded and treated as a homogeneous group, even though apparently they behave very differently and pose varying degrees of danger. [8, 10]

PMBL patients have the best outcomes of these three subtypes responding to treatment regimens very well and usually do not require radiation therapy. [7] When comparing GCB- to ABC- DLBCL, patients suffering from GCB- DL-

BCL have a more promising prognosis after being treated with R-CHOP and also dose-adjusted (DA) EPOCH treatment. [7] Studies have also revealed a differential sensitivity of GCB- and ABC- DLBCL to particular drugs suggesting the development of personalized therapy. [10] As ABC- DLBCL are the most difficult to treat, research into exploitation of ABC-specific pathways is currently being conducted. As ABC type- DLBCL are the most difficult to treat, investigation on the development of therapies into targeting ABC-specific pathways is currently being conducted. [10]

1.3 Chronic Lymphocytic Leukaemia

Chronic lymphocytic leukaemia (CLL) is the most common type of leukaemia in the western world. [11] It is classified by the WHO as part of indolent mature B-cell neoplasms. [2]

CLL affects mostly people older than 65, this is the age at which 70% of diagnosis are made. The gender predisposition is skewed towards males as they are around twice as likely to be diagnosed with CLL. [11]

Despite previous epidemiological studies no distinct external risk factors for developing CLL could be determined. Genetic predisposition on the other hand was ascertained as a contributing risk factor. [11, 12]

Morphologically CLL exhibits elevated levels of small lymphocytes with scanty cytoplasm and clumped chromatin. Additionally, fragile cell membranes are very characteristic for this disease. [11]

Usually immunophenotyping using peripheral blood is enough to diagnose CLL while lymph node or bone marrow biopsies are not necessary. To differentiate CLL to mantle cell lymphoma cell, antibodies against CD19, CD20, CD5 and CD23 are used. CLL cell express these markers on their surface. [11]

1.4 B-cell development

B-cells are part of the humoral immune system and respond by producing antibodies to antigens. [13, 14] Initial B-cell development starts in the foetal liver and is then transferred to the bone marrow during the following embryonic development where hematopoietic stem cells differentiate to lymphoid precursor cells. [13, 14]

During the early B-cell developmental stages, DNA gene sequences, namely the variable (V), diversity (D) and joining (J) gene regions, are recombined in the immunoglobulin gene loci (heavy and light gene) to generate a functional immunoglobulin. [14] These are combined in one specific but always different way to encode the heavy chain of the variable region of the immunoglobulin. This process is called VDJ recombination and depicted schematically in figure 1. It is responsible for the high variability and thus binding ability of immunoglobulins and in particular the B-cell receptor (BCR). [14]

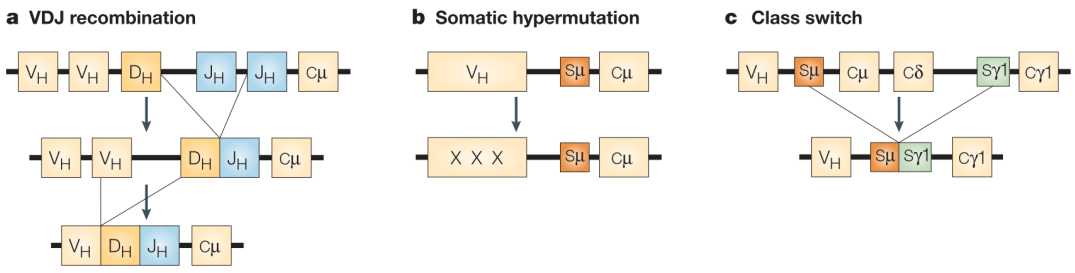

Figure 1: VDJ recombination, somatic hypermutation and class switch exemplified, adapted from Küppers et al. [3]

Recombination-activating gene (RAG) 1 and 2 are responsible for rearranging the heavy chain gene segments where first a D-segment (D_H) and a J-segment (J_H) are linked and then connection of V_H to D_HJ_H commences. If the new connection was made successfully, the cell has reached pre-B-cell status and presents the pre-B-cell receptor which consists of the rearranged heavy chain (VDJ-Cμ), substitute light chains (λ5 and VpreB) and Igα and Igβ subunits that transduce cellular signals. Expression of pre-B-cell receptors initiates cell cycle progres-

sion thus the cell divides a couple of times. If expression of the pre-B-cell receptor is malfunctioning even after the second allele of the heavy chain is rearranged the cell enters apoptosis. [13, 14]

Once the cell reaches pre-B-cell stage, rearrangement of the light chain V- and J-segments can commence. Now the light chain can pair with the heavy chain and a fully functional B-cell receptor is expressed. These mature B-cells leave the bone marrow at this stage and travel to the spleen of other lymph nodes where they interact with and can be activated by T-cells through recognition of an antigen that binds to the B-cell receptor (IgM). Depending on the affinity of the receptor to the antigen and whether it exhibits auto immune recognition, the cell either re-enters the recombination stage or begins apoptosis. [13, 14]

VDJ recombination, SHM and CSR are essential for a functioning immune system but these frequent genetic alterations can increase the probability of malignant B-cells developing. [14, 15]

How significant these mechanisms are in the formation of lymphoid neoplasms is best exemplified in the specific breakpoints of Ig loci. This is important due to the characteristic reciprocal chromosomal translocation of a proto oncogene under the regulatory control of an active Ig locus which in turn results in the constitutive expression of the oncogene. [3] For example the *BCL2-IgH* translocation that is associated with FL exhibits breakpoints directly adjacent to the Ig heavy chain J-region (J_H) or adjacent to where the Ig heavy chain D-region (D_H) is connected to the J-region ($D_H J_H$). These breakpoints often show that nucleotides have been lost at the ends and also that non-germline encoded nucleotides have been added. This strongly points to errors during VDJ recombination in the early stages of B-cell development as the reason for these translocations. [3] Other breakpoints occur in or adjacent to rearranged VDJ segments where the V-region is always somatically mutated. This observation strongly indicates that these translocations are the result of errors during the somatic hypermutation process. Breakpoints implicating errors during the class switch re-

combination occur in the *IgH* constant region switch regions. These regions experience DNA breaks during class switching implying that these translocations are linked to this process. [3] It has been shown that RAG, which is usually responsible for VDJ recombination, plays a crucial role in these translocations as it cleaves the major breakpoint region in the *BCL2-IgH* translocation. [16]

So far multiple proto oncogenes that are targeted by SMH have been identified in DLBCL and hence this could play a major role in B-cell pathogenesis. [17]

1.5 B-cell differentiation

Naive B-cells mature during their stay in the germinal centre (GC) and differentiate either into plasma cells of memory B-cells. The detailed process is depicted in figure 2.

In the dark zone of the germinal centre antigen-activated B-cells can undergo differentiation into centroblasts and start clonal expansion which is supported by interactions with T-cells. The variable regions of immunoglobulins experience somatic hyper mutation, a process involving the introduction of point mutations, deletions and other aberrations in the gene for the variable region, and afterwards the GC B-cells migrate to the light zone of the GC and develop into centrocytes. [14]

In the light zone, another selection of the mutated Ig occurs where cells expressing a malfunctioning Ig, but also cells expressing too sensitive or autoimmune Ig undergo apoptosis. Hence GC B-cells expressing Ig with improved antigen affinity are selected and experience immunoglobulin class switch recombination (CSR) in which the heavy chain of Ig is changed from IgM to IgG, IgA or IgE. This process is mediated by helper T-cells and follicular dendritic cells (FDC). This cycle of mutation and selection can be repeated several times. Finally GC B-cells differentiate into memory B-cells or antibody producing plasma cells depending on the kind of antigen that was bound and leave the GC. [14]

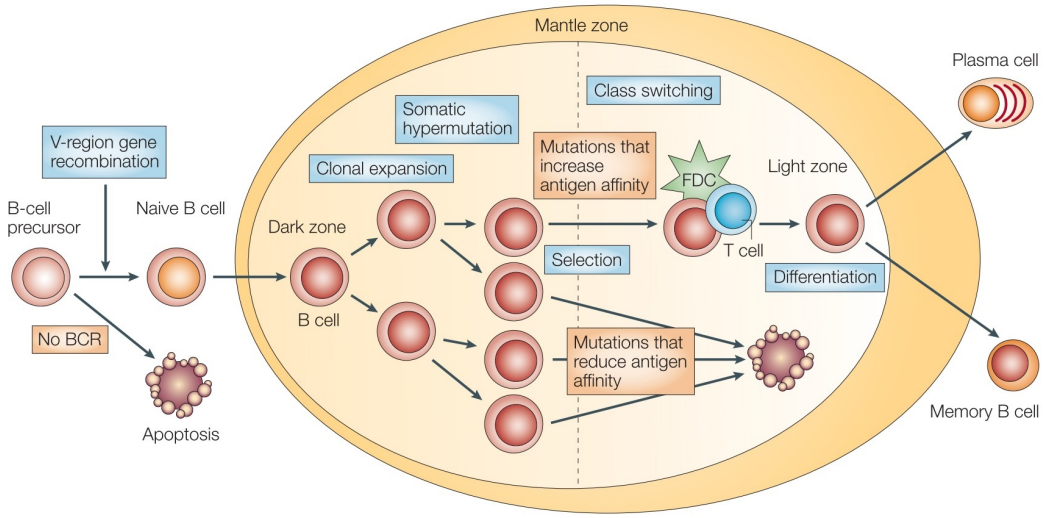

Figure 2: B-cell development and the germinal centre reaction adapted from Küppers et al, 2005. B-cell precursor cells that express a functioning VDJ combination in their BCR progress to naive B-cells. Naive B-cells then progress to the dark zone of the germinal centre where they undergo clonal expansion and somatic hypermutation. Cells that produce an high affinity BCR are selected to pass into the light zone GC while B-cells with suboptimal BCR undergo apoptosis. Here class switching happens and via interactions with follicular dendritic cells (FDC) or T-cells B-cells differentiate into either memory B-cells or Plasma cells. [3]

1.5.1 Cell of origin of GC derived lymphomas

Many B-cell lymphomas including BL, FL and DLBCL derive from GC B-cells. Their variable region of the Ig is somatically mutated. Using gene expression profiling these different cells could be attributed to different stages of the GC reaction. [15]

Aggressive B-cell lymphoma including BL, FL and a subset of DLBCL have been shown to possess a signature gene expression associated with GC B-cells hence supporting the hypothesis that these malignancies have their origin in the GC. In contrast, B- CLL show remarkable similarities to memory B-cells that have experienced somatic hypermutation. However, some cellular origins of DL-BCL - in particular ABC type lymphoma - could not be identified for sure. In ABC DLBCL it is thought that they develop from either a not well classified

subset of GC B-cells that underwent plasmacytoid differentiation or a post-GC immunoblast subset. [3]

Burkitt lymphomas apparently derive from dark zone B-cells based on their gene expression profile and DLBCL can be tracked back to B-cells that were stopped during their GC residence due to different transformations. Follicular lymphomas seem to derive from light zone B-cells just as GCB like lymphoma. On the other hand, ABC like lymphoma occur during differentiation and after the B-cells exit the GC (see figure 3). [15]

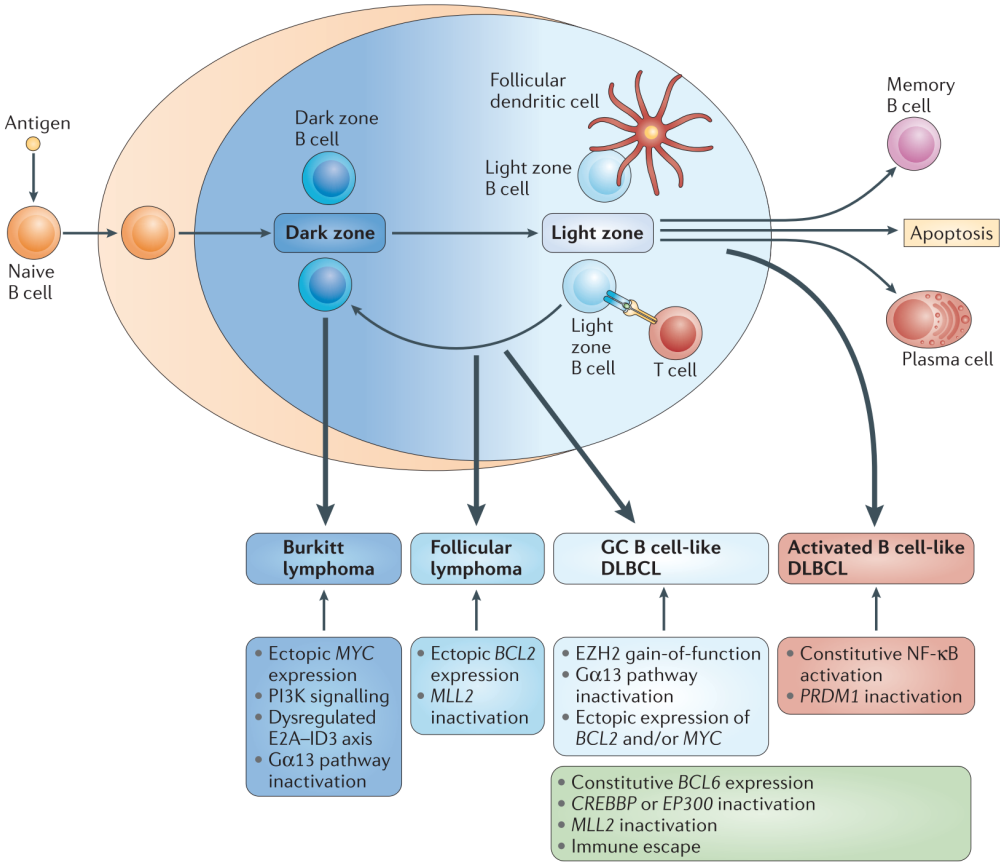

Figure 3: Cell of origin of lymphoid malignancies, adapted from Basso et al. Naive B-cells enter the GC and start the process of maturation in the dark zone of the GC before passing through the light zone to become memory B-cells or plasma cells. If apoptosis is not induced in abnormally matured B-cells, it can result in different types of lymphoma depending on the stage that the error occurred in. [15]

1.6 Molecular mechanisms of lymphomagenesis

During B-cell maturation and differentiation in the GC multiple molecular signalling pathways and transcription factors are being exploited by malignant B-cells. Major influencers on lymphomagenesis and their gene expression pattern over GC maturation can be seen in figure 4. [15]

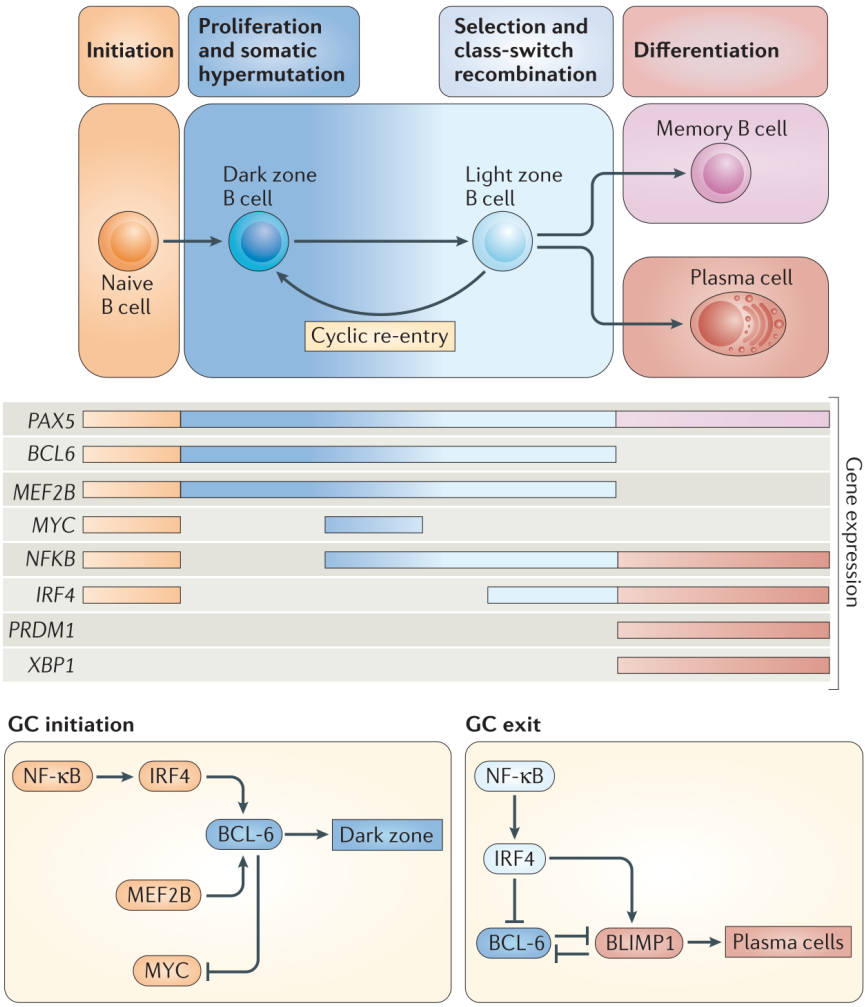

Figure 4: Mechanisms of lymphomagenesis and expression of genes involved with lymphomagenesis, adapted from Basso et al. [15]

Major factors in the GC initiation, expansion and exit are:

Nuclear factor kappa-light chain enhancer of activated B-cells (NF-kB) NF-kB is upregulated in early GC B-cells but is notably absent upon entry into the dark zone. If B-cells in the dark zone are triggered by their antigen, NF-kB is

induced once more as they enter the light zone of the GC. NF-kB is activated by the B-cell receptor (BCR), CD40 and TLR (Toll-like receptors) and it can then induce interferon regulatory factor 4 (IRF4), leading to transcriptional repression of BCL-6. [15]

MYC (v-myc avian myelocytomatosis viral oncogene homologue)

The first gene to be linked to lymphomagenesis in GC was MYC but the complete process by which it is involved in lymphomagenesis is still unclear. Characteristic is the transient expression which is then suppressed as soon as cells enter the dark zone of the GC. In some B-cells trying to re-enter into the light zone MYC is expressed. Interestingly, during the initial GC development MYC expression is essential but is silenced abruptly by BCL6. [15]

B-cell lymphoma 6 (BCL-6)

BCL-6 was characterised as an essential transcriptional repressor of multiple GC-involved mechanisms, such as promotion of GC reactions and the formation and maintenance of the GC. BCL-6 inhibits cell cycle arrest and B-cell activation. Its effects promote DNA rearrangement and prevent B-cells from departing from the GC prematurely. An abnormal expression of BCL-6 was shown to promote lymphomagenesis. BCL-6 is induced by IRF8, IRF4 and MEF2B. [15, 18]

Myocyte enhancer factor 2B (MEF2B)

MEF2B is expressed during early development of the GC. It has been shown to transactivate BCL6 and has been linked to lymphoma due to mutations that interfere with its activity. [15]

Interferon regulatory factor 4 (IRF4 =MUM1)

IRF4 enhances BCL6 expression during the early stages of GC B-cell development whereas it is inhibited in the dark zone just to be re-expressed by a specific subset of B-cells destined to develop into plasma cells. [15]

EZH2 (histone methyltransferase enhancer of zeste homologue 2)

As part of the polycomb repressive complex 2, EZH2 aids in the methylation of lysine 27 of histone 3 (H3K27), which is known as a marker of transcriptional

repression. It is also necessary for normal GC development and can be associated with lymphomagenesis. In B-cells it is only expressed during the stay in the GC. [15]

BLIMP1 (plasma cell master regulator B lymphocyte-induced maturation protein 1)

Upon repression of BCL-6 proteins like BLIMP1 can be expressed which is enhanced by NF-kB induced IRF4. BLIMP1, the product of PRDM1, in turn enforces the prolonged repression of BCL-6 and results in differentiation to plasma cells. [15]

PAX (Paired box 5)

PAX5 expression is continuous throughout the GC reactions where it enforces the B-cell transcriptional programme. Once a cell is destined to become a plasma cell, PAX5 is repressed resulting in initiation of a plasma cell specific programme that is mediated by IRF4, BLIMP1 and XBP1 (X-box binding protein). [15]

Multiple pathways involved with the GC reaction have been associated with distinct NHL subtypes and their particular phenotype. The transcription factors and regulators above therefore are often hijacked during lymphomagenesis. [15]

Burkitt lymphoma are characterised by their *MYC* translocations into Ig loci which are present in an astonishing 100% of cases. Also prevalent are mutations in transcription factor 3 (*TCF3*) which encodes E2A or mutations in inhibitor of DNA-binding 3 (*ID3*). These mutations result in dysregulation of E2A which in turn was shown to promote BCR signalling via the PI3K pathway. In contrast, in normal dark zone B-cells the PI3K signalling is undetectable. Additionally, around a third of BL cases exhibit inactivating mutations of various tumour suppressors. [15]

80% of follicular lymphoma exhibit the already mentioned *BCL2-IgH* translocation and also inactivation of the histone methyltransferase *MLL2* is as commonly observed. This expression of *BCL2* evades repression by BCL-6 and thus

starts an anti-apoptotic programme. The inactivation of *MLL2* affects the methylation status of H3K4, hence this mutation is thought to result in dysregulated transcription. Other factors that affect chromatin status and that are often mutated in FL and DLBCL are EZH2, CREB-binding protein and E1A-binding protein p300 (EP300). [15]

DLBCL subtypes GCB- and ABC- DLBCL exhibit alterations in BCL-6 regulation, chromatin remodelling mechanisms and immune recognition. Similarly to FL, EP300, CREB-binding protein and MLL2 are mutated in around a third of DLBCL. The main regulator in the GC reaction - BCL-6 - is often translocated to the *IgH* locus which puts this crucial regulator under control of a different promoter which in turn makes the downregulation during the later GC stages impossible. This is not the only way *BCL-6* can become dysregulated as for example interference in the auto-regulation of *BCL-6* - involving BCL-6 itself or IRF4 - is also commonly described. *BCL-6* dependent malfunctions occur in around 30% of DLBCL. Other still not clearly understood mechanism that is relevant in over 60% of DLBCL cases is the lack of the MHC class I complex. This lack of MHC I is quite specific for DLBCL and it results in immune evasion because natural killer cells and cytotoxic T-cells are unable to recognize and kill tumour cells. [15]

We currently do not know any GCB- DLBCL exclusive pathogenic mechanisms, but the already mentioned *MYC* and *BCL2* translocations are also observed in this type of B-cell lymphoma but to a lesser extent. MYC translocations are found in around 10% of GCB- DLBCL and BCL2 translocations in around 40%. If both MYC and BCL2 are dysregulated in the tumour, then the prognosis is particularly poor. Two malfunctioning pathways that are somewhat specific to GCB- DLBCL are epigenetic changes involving EZH2 and also B-cell migration away from the GC. [15]

ABC- like DLBCL exploit two distinct pathogenic pathways, one result is a constant activation of NF-kB signalling and the other inhibits differentiation to

plasma cells. The genetic changes that result in constitutive activation of NF-kB signalling conclude in the transcription of pro-survival genes. It was shown that ABC- DLBCL are highly dependant on the NF-kB pathway by *in vitro* experiments that exhibited cell death upon genetic or pharmacologic inhibition of NF-kB. ABC- DLBCL seem unable to terminally differentiate into plasma cells which appears to be due to negative regulation of BLIMP1 over two exclusive pathways. [15]

1.7 Chemokine Receptors and Chemokines

Chemokine receptors are essential for cellular functions such as migration and can be found on the surface of leukocytes and also other cells where they bind chemokines (chemotactic cytokines). [19] Chemokines are characterised by their function to directly induce cell movement based on the chemokine gradient mediated by binding to chemokine receptors on the surface of these cells. [20]

The typical structure of chemokine receptors, which are G-protein coupled receptors (GPCR) is depicted in figure 5A. These receptors are organized in 7 transmembrane domains with the N-terminus on the extracellular side and the C-terminus on the intracellular side on which a G-protein binds and binding of a chemokine to this receptors induces a signal transduction cascade, resulting most often in an intracellular flux of calcium ions (Ca^{2+}). The effect of this is mostly chemotactic movement toward the gradient of chemokines but cell growth, angiogenesis, anti- and pro- apoptotic effects are also known to be induced by chemokines. Best studied are the impacts of chemokines on migration of immune cells, for example leukocytes trafficking to damaged sites. [20, 19, 21]

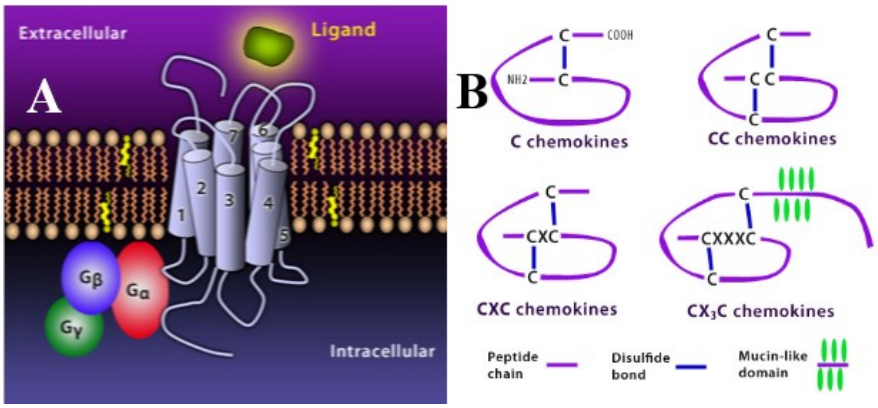

Figure 5: **A**: Structure of chemokine receptors. 7 transmembrane domains are embedded in the membrane with the N-terminus at the extracellular side and the C-terminus at the intracellular side. Ligands can bind at the extracellular loops and G-proteins are bound at the C-terminus. **B**: Chemokine subfamilies are classified according to their specific motives based on the position of the first two N-terminal cysteine residues. Image source: https://www.biolegend.com/chemokine_receptors

Chemokines are classified according to their specific motives based on the position of the first two N-terminal cysteine residues, namely CXC-motive, CC-motive, CX3C-motive or XC-motive (depicted in figure 5B) and bind to receptors that are classified into these same subfamilies depending on their ability to bind one class of chemokine. [20, 19].

Recently, chemokine receptors which now includes CXCR7 (ACR3), Duffy Antigen Receptor for Chemokines (DARC), D6 and CC-Chemokine Receptors like 1 and 2 (CCRL1 and CCRL2), were classified as so called atypical chemokine receptors. Although these receptors lack the ability to transduce signals directly they are capable of internalising a bound chemokine and thus are still able to sequester chemokines, hence affecting the pool of potential chemokine receptor ligands. [22]

Generally, most chemokine receptors and their ligands are promiscuous and redundant in their binding abilities meaning that one chemokine receptor can bind multiple chemokines and vice versa. This capacity of multiple binding partners adds enormous complexity to research concerned with chemokines and their receptors but on the other hand allows for incredible cellular fine tuning and compensation in response to various signals. Additionally, many chemokines

can have different activities depending on the circumstances and due to the redundancy of chemokines and their receptors, compensation mechanisms are to blame for low efficacy in anti-chemokine and anti-chemokine receptor experiments. Only 6 receptors, namely CXCR4, CXCR5, CXCR6, CCR6, CCR9 and CX3CR1 are thought to bind only one chemokine exclusively and therefore are most promising targets in the search for therapeutic targets for malignant neoplasms as well as markers for high-risk patients. [19, 23]

Chemokines are implicated in lymphomagenesis due to their involvement in regulating progenitor cells on their way to becoming hematopoietic cells.

Chemokines can recruit cells as well as keeping them at a site and also they induce further expression of cytokines. Additionally, chemokines support the formation and development of secondary lymphoid organs. [24]

The most studied chemokine- chemokine receptor pair are CXCL12 (also known as SDF-1) and CXCR4 due to the prevalent expression of CXCR4 in many different malignancies. [19]

CXCR4 has been shown to be expressed on normal B-cells and also malignant B-cell lymphoma cells. CXCR4 is therefore a chemokine receptor closely linked to circulation of malignant B-cells and metastasis formation. [25]

1.8 CXCR4-CXCL12 axis in cancer

CXCR4 and its ligand CXCL12 (CXC ligand 12 or stromal cell-derived factor 1; SDF 1) are one of the most promising targets for cancer therapy due to its simplicity as a non-promiscuous chemokine- chemokine receptor pair and additionally due to its profound involvement in lung, liver, bone marrow and brain metastasis formation. [26]

The importance and significant impact of CXCR4-CXCL12 was first observed in breast cancer metastasis and many following studies have elucidated some of the underlying mechanisms responsible for metastasis tissue preference

in other cancers too. [23, 26, 27]

Elevated CXCR4 and CXCL12 have been correlated with directed migration and dissemination of malignant B-cells in Burkitt lymphoma, DLBCL, follicular lymphoma and chronic lymphocytic leukaemia. Additionally, this axis is closely linked to decreased survival and increased tumour aggressiveness. [28, 29]

Recently, Chen et al. were able to link CXCR4 overexpression in DLBCL to the ABC like subtype in addition to Myc, BCL-2 or p53 overexpression and a couple of other characteristics. This CXCR4 overexpressing subset exhibited poorer progression free survival in GCB like DLBCL. ABC like DLBCL on the other hand were not correlated to poor survival, probably due to p53 activation intervening in CXCR4 signalling. [30]

A new study from 2016 showed that a ROS-induced CXCR4-CXCL12 signalling pathway may contribute to enhanced tumour cell survival in the bone marrow microenvironment via autophagy. [31]

Due to the undisputed role of CXCR4 and CXCL12 in cancer and in haematological malignancies in particular, there has been a lot of research into neutralization of this malevolent axis. Neutralization of CXCR4 with monoclonal antibodies [32] and various small molecules like AMD3100 [33, 34], AMD070 [35], BKT140 [29, 36] and pepducins [37] has resulted in reinvigorated effects of rituximab in addition to inhibition of cell migration and in some cases proliferation.

CXCR7

Although CXCL12 is thought to bind and activate only CXCR4 it has been shown that CXCL12 can also bind to the ACR CXCR7. As CXCR7 is incapable of transducing signals via the GPCR pathway it is still disputed how the interaction with CXCL12 might impact intracellular mechanisms. [38, 39, 22, 40, 41]

The most notable function of CXCR7 is its scavenger activity for CXCL12 and CXCL11. It has been shown to cycle between the plasma membrane and intracellular compartments and hence it is believed that it mediates internaliza-

tion and degradation of its ligands. CXCR7 also seems to be able to form heterodimers with CXCR4 as well as homodimers. This interaction with CXCR4 and CXCL12 makes it possible for CXCR7 to impact cell migration and it apparently inhibits down-regulation of CXCR4 expression. [38, 40, 42]

It was also proposed that CXCR7 can activate β-arrestin signalling pathways and hence affect receptor desensitization and receptor trafficking. Additionally, CXCR7 expression in hematopoietic cells is controversial, because some studies were able to provide evidence for its expression whereas others could not, CXCR7 expression in tumours however is upregulated. [43, 44]

1.9 CXCR4 antagonists

A lot of research has been done on the effects of neutralizing CXCR4 with different reagents. Monoclonal antibodies and small molecules like AMD3100, AMD070 and BKT140 all revitalised the chemotherapeutic effects of rituximab. [32, 33, 34, 35, 29, 36, 27]

One known CXCR4 antagonist that is currently in clinical use is Plerixafor (AMD3100). It has been approved by the U.S. Food and Drug Administration (FDA) for treatment of NHL to mobilize hematopoietic stem cells and it has been shown to enhance the effects of rituximab. [33, 45, 37, 29, 36] Structurally Plerixafor is a bicyclam molecule as seen in figure 6. [34] Plerixafor is able to reversibly bind to an extracellular binding site on CXCR4 resulting in CXCL12 being unable to bind and induce its effects. [33]

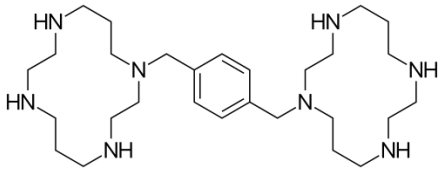

Figure 6: Chemical structure of Plerixafor/AMD3100 [34]

Another small molecule CXCR4 antagonist that was under research is AMD070.

Its clinical trials as a HIV treatment were discontinued but in vitro it had promising effects inhibiting CXCR4-CXCL12 mediated migration. [46, 47, 48]

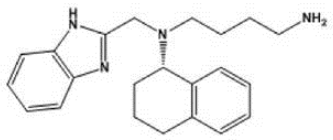

Figure 7: Chemical structure of AMD070. [46]

The newly synthesized CXCR4 antagonist WK1, a nicotinic acid derivative of AMD070, was investigated in regard to its potential properties as another treatment option for cancer, in particular B-cell lymphoma. Our previous studies indicated a higher effectiveness of WK1 than AMD070 in inhibiting cell growth in vitro. [49]

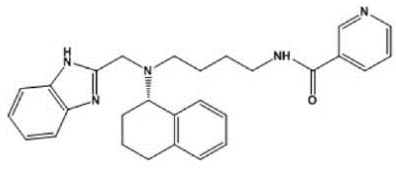

Figure 8: Chemical structure of niacin/nicotinic acid derivative of AMD070 [49]

1.10 Preliminary Results

In previous studies my group demonstrated that exclusively nodal lymphomas expressed CXCR4 when compared to extra-nodal lymphomas. Additionally, CXCR4 expression was associated with bone marrow infiltration. [50, 49]

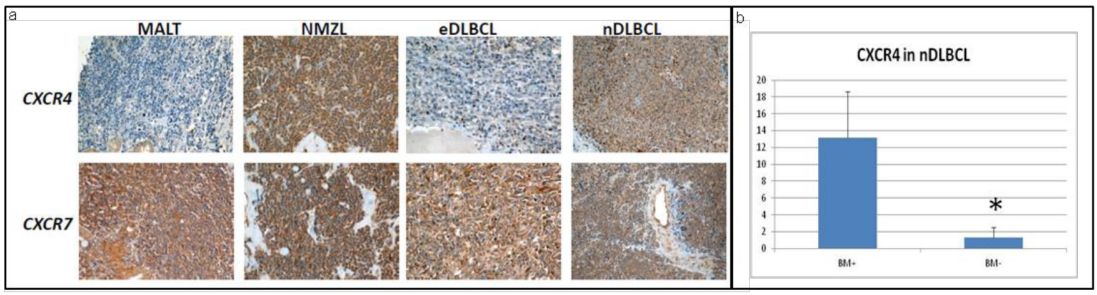

Figure 9: CXCR4 and CXCR7 expression in nodal and extra-nodal lymphoma. [50, 49]

They also showed that CXCR7 expression in extra-nodal gastric lymphoma is correlated with lymphoma progression. However, detailed functionality and effects of CXCR4 and CXCR7 expression are still mostly unknown in lymphatic malignancies. [49, 50]

1.11 Aims

Our primary aim was to investigate the molecular effects of CXCR4 antagonists, namely AMD3100 (aka Plerixafor), AMD070 and especially those of a novel side chain modified nicotinic acid derivative of AMD070, also known as WK1, on multiple lymphoma cell lines in comparison to niacin. In detail, we determined CXCR4 expression and performed CXCL12^{AF647} binding assay in combination with blocking antibodies by using FACS analysis to characterize CXCR4 and CXCR7 expression on lymphoma cell lines. CXCL12^{AF647} binding assays were also used to determine whether the three CXCR4 antagonists were able to block CXCL12-CXCR7 by using CXCR4 or CXCR7 positive cell lines in combination treatment. To study cell growth and cytotoxic effects of CXCR4 antagonists we exposed cells to varying concentrations of antagonists and afterwards analysed them with cell growth assays using tetrazolium compounds. Furthermore, we characterized the cytotoxic effects of all three CXCR4 antagonists by FACS analysis after Annexin V/ 7-AAD staining and additionally cleaved caspase-3 staining to investigate whether apoptosis is induced.

2 Materials and Methods

2.1 Cell lines and cell culture

To conduct our *in vitro* experiments we chose in total 6 cell lines representing 3 different lymphoma subtypes (see table 3). Representing BL we used BL2 and Raji cells, Karpas422 and SUDHL4 belong to the GCB subtype and RI1 and U2932 are classified as ABC subtype.

Cells were cultured in RPMI 1640 (Gibco®/Life Technology) supplemented with HyClone™Fetal Bovine Serum (FBS) (Thermo Fisher Scientific) and 1% antibiotic-antimycotic as described in table 3.

Table 3: B-cell lymphoma cell lines and subtypes that were used in experiments

Cell line	**DSMZ number**	**Cell type**	**Medium**
BL2	ACC625	Burkitt lymphoma (BL)	RPMI 20% FBS
Raji	ACC319	Burkitt lymphoma (BL)	RPMI 10% FBS
Karpas422	ECACC 06101702	DLBCL (GCB)	RPMI 20% FBS
SUDHL4	ACC495	DLBCL (GCB)	RPMI 10% FBS
U2932	ACC633	DLBCL (ABC)	RPMI 10% FBS
RI1	ACC585	DLBCL (ABC)	RPMI 10% FBS

The cells were kept and split as recommended by Leibnitz Institute DSMZ-German Collection of Microorganism and Cell Cultures and cultured at 37°C under 5% CO2 conditions.

Cell concentrations were determined with CASY or TC20 Biorad cell counter. Cell lines BL2, Karpas422, Raji and SUDHL4 were diluted to $5*10^5$ cells per ml and U2932 and RI1 cells were diluted to $1*10^6$ cells per ml. All cell lines were checked for mycoplasma regularly and were examined as negative.

2.2 EZ4U cell proliferation and cytotoxicity assay

This assay and was performed according to the manufacturer's protocol.

After diluting cells to the recommended cell concentration (5*10^5 or 1*10^6), cells were seeded into wells of a 96 well plate and treated with antagonists to final concentrations between 90 μM-1 μM. Experiments were always performed in triplicates. Untreated cells and pure medium acted as controls and blanks.

After treatment cells were incubated for 72 hours at 37°C and 5% CO2. To measure cell proliferation and cytotoxicity 20 μl EZ4U reagent was added to each well and incubated for another 4 hours at 37°C. We measured the samples at a wavelength of 492 nm with an additional reference measurement at 620 nm by using SpectroStar Photometer (BMG LABTECH).

2.3 CXCL12 binding assay and blocking of chemokine receptors with antagonists and antibodies

To determine the specificity of CXCL12 on CXCR4 and CXCR7 expressing cells we centrifuged 1 million cells and resuspended them in 100 μl HBSS before adding the CXCR4 antagonists AMD3100, AMD070 or WK1 to a final concentration between 20-0,01 μM. Cells were incubated for 45 minutes at 37°C before 0,5 μl of the Alexa Fluor®labelled CXCL12^{AF647} (BD) were added. After another 3 hours incubation time at 37°C, treated cells were washed by centrifuging them for 1 minute to create a pellet which was resuspended in 100 μl PBS after removing the supernatant. Measurement was performed on FACS BD^{TM} LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

To analyse the expression of chemokine receptors additional binding assays were performed using antibodies to block CXCR4 and/or CXCR7 before treatment with any chemokine receptor antagonists. Blocking antibodies (anti-CXCR4, anti-CXCR7) and isotype controls (IgG1 and IgG2A) were added to a final concentration of 10 μg/ml.

2.4 Cell viability and apoptosis staining using Annexin V and 7-AAD

The Annexin V Apoptosis Detection Kit APC (Affymetrix eBioscience) with Annexin V- APC and 7-AAD (7-amino-actinomycin D) was used to stain cells and hence determine the proportion of viable cells. For details about this Kit please refer to the manufacturer's instructions.

After diluting cell lines to $5*10^5$ or $1*10^6$ respectively, cells were seeded to wells of a 24-well plate and antagonists were then added to final concentrations of 40 μM, 20 μM, 10 μM, 5 μM and 1 μM in duplicates. Untreated cells were also included to normalise the effects of antagonist treatment. After 24, 48 and 72 hours 200 μl were removed from each preparation and centrifuged at 3500 xg for 5 minutes and supernatant was removed. Cells were resuspended in 100 μl Annexin V staining buffer and shortly before measurement on FACS LSRII 2,5 μl Annexin V and 2,5 μl 7-AAD were added to stain apoptotic and necrotic cells.

2.5 Caspase-3 staining

To investigate the apoptotic effects of CXCR4 antagonists WK1 and AMD070 in respect to them possibly inducing apoptosis, we conducted caspase-3 staining using the Caspase-Glo 3/7 assay (Promega). We chose BL2, Karpas422 and SUDHL4 cell lines to conduct caspase-3-staining as these cells reacted significantly to the treatment with AMD070 and WK1 demonstrated by cell growth and cell viability assay.

Firstly, cells were treated with chosen concentrations of the antagonists. For WK1 we chose the concentrations 40 μM, 20 μM and 10 μM and for AMD070 cells were treated with just 40 μM. Secondly, we fixed cells with the fixative reagent after spinning them down and removing the supernatant. We incubated them for 15 minutes before adding the permeabilisation reagent and caspase-3 antibody. After another 30 minutes of incubation we added "reagent number 3".

After centrifugation and resuspension in PBS we measured cells using FACS.

2.6 Analysis of chemokine receptor expression in CLL patient samples by qPCR

Frozen samples of CLL patients were kindly provided by the hematologic biobank of myeloid and leukaemia working group of the Division of Hematology, Medical University of Graz.

RNA was extracted using the Maxwell 16 LEV simply RNA Cells Kit by Promega. To test different qPCR reaction master mixes RNA extraction was also performed on control MNC cells. RNA was eluted in 50 μl of nuclease free water and was stored at -80°C until measurement on NanoDrop and cDNA synthesis.

cDNA synthesis was performed according to the RevertAid First Strand cDNA Synthesis Kit by Thermo Scientific. We used between 55 and 3000 ng RNA for the reaction and diluted the product in 380 μl nuclease free water and stored it at -20°C .

We used the Taqman Probe qPCR Kit and using a Master mix that contained per processed sample 5 μl Taqman Master mix, 2,5 pmol forward primer, 2,5 pmol reverse primer, 0,025 μl Taqman probe and 2,925 μl nuclease free water.

3 Results

3.1 Expression of CXCR4 in cell lines

To determine CXCR4 expression on the different lymphoma cell lines - BL2 and Raji as BL subtype, Karpas422 and SUDHL4 as GCB subtype and U2932 and RI1 as ABC subtype - we used fluorescent labelled antibodies against CXCR4. As shown in figure 10, BL2 and SUDHL4 cells exhibited strong CXCR4 expression, Raji, U2932 and RI1 cells moderate expression whereas Karpas422 practically possessed no detectable CXCR4 expression.

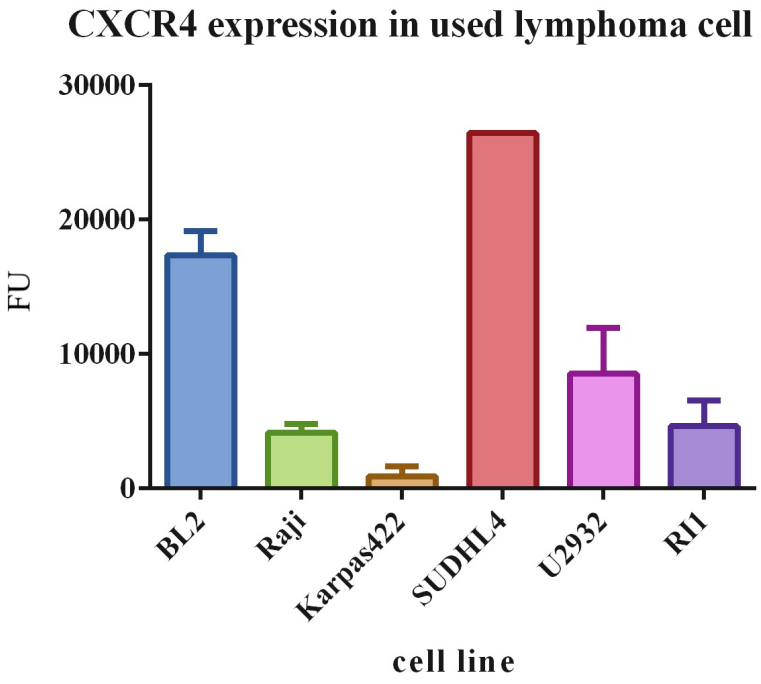

Figure 10: Expression of CXCR4 in aggressive lymphoma cell lines from BL, GCB and ABC subtypes. Using FACS an antibody binding specifically to CXCR4 was detected and the amount is plotted in fluorescent units (FU). Plotted are the median of two replicates with duplicates and error bars represent the standard deviation of the median.

3.2 CXCR4 and CXCR7 expression on lymphoma cells by using the CXCL12^{AF647} binding assays

To determine CXCR7 expression, we performed CXCL12^{AF647} binding assays in multiple replicates with chosen aggressive lymphoma cell lines. Since we blocked CXCR4 and CXCR7 with respective antibodies and compared the bound CXCL12^{AF647} binding of adequate isotype controls we calculated the relative amount of CXCL12^{AF647} that was able to bind to CXCR4 and/or CXCR7 as depicted in figure 11.

As seen in figure 11, we observed major differences in CXCL12^{AF647} binding. In BL2 we saw that CXCL12^{AF647} binds to CXCR4, whereas the other BL cell line Raji showed binding affinity to CXCR7. GCB subtype Karpas422 did not bind CXCL12^{AF647} as expected due to them lacking CXCR4 expression, as seen in figure 10. The other GCB-cell line SUDHL4 showed predominantly CXCR4 binding as well as ABC subtype cell line U2932. RI1 - belonging to the ABC subtype - bound CXCL12^{AF647} via CXCR4 and CXCR7.

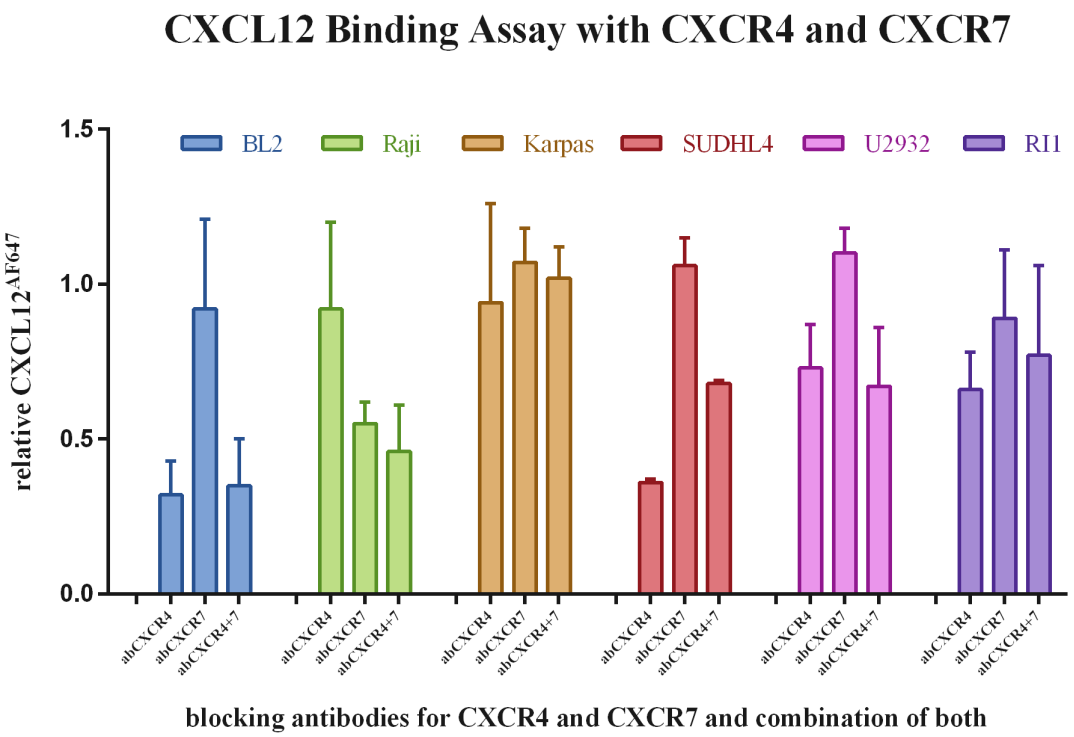

Figure 11: Relative expression of CXCR4 and CXCR7 was determined using blocking antibodies (abCXCR4/abCXCR7) for both receptors and detecting fluorescent CXCL12^{AF647} afterwards with FACS. abCXCR4 blocks CXCR4, therefore bars that are low indicate that less CXCL12 is able to bind. By comparison to bars of the combined blocking antibodies (abCXCR4+7) we can conclude which receptor is responsible for more CXCL12 binding. Bars represent the mean of 3 - 6 replicates which were always performed in duplicates. Error bars are the correlating standard deviation of the mean.

3.3 CXCL12 binding assay after treatment with CXCR4 antagonists

In order to analyse the potential of CXCR4 antagonists to block CXCL12 binding, we treated BL2 cells - a strongly CXCR4 expressing cell line - with WK1, AMD070 or AMD3100 with concentrations ranging from 0,1 -20 μM followed by CXCL12^{AF647} binding assays. Afterwards, we performed binding assays using CXCL12^{AF647} and FACS analysis.

As shown in figure 12 AM3100 and AMD070 reduced CXCL12 binding to 50% at rather low levels (0,025 μM for AMD3100 and 0,05 μM for AMD070).

In stark contrast, WK1 was able to reduce CXCL12 binding to 50% at a concentration of 2.5ÂţM. These data suggest that AMD3100 and AMD070 possess a greater potential to block CXCL12 than WK1.

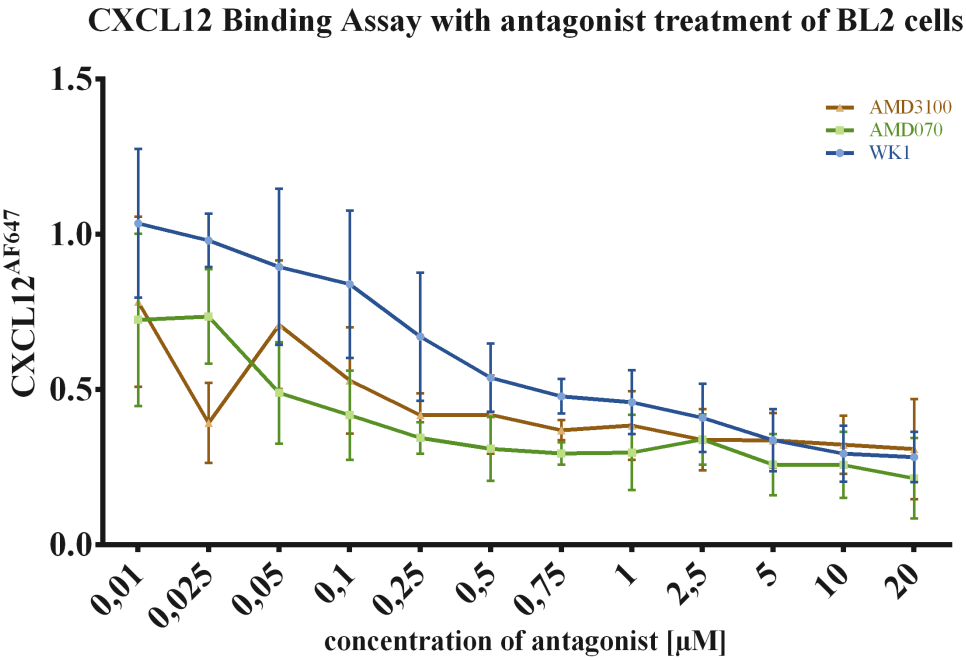

Figure 12: CXCL12^{AF647} binding assay after treatment of BL2 cells with CXCR4 antagonists AMD3100 (brown), AMD070 (green) and WK1 (blue). BL2 cells were treated with CXCR4 antagonists in increasing concentrations from 0,1 - 20 μM (x-axis). Following incubation, cells were exposed to excess CXCL12^{AF647}. Surplus was washed away and cells were then analysed using FACS during which the remaining CXCL12^{AF647} was detected and is plotted on the y-axis. Points in the graphs are mean values of 4 replicates (each in duplicates). Bars represent standard deviation of the mean that corresponds to each point.

To elucidate whether CXCR4 antagonists can block CXCR7-CXCL12, we conducted CXCL12^{AF647} binding assays with the CXCR7 expressing Raji cell line by treating these cells with WK1, AMD070 or AMD3100.

As shown in figure 13 CXCL12^{AF647} binding after AMD3100 treatment was reduced at a lower level than AMD070 and WK1 and this antagonist has hardly any effects. AMD070 and WK1 treated cells behave very similar in that CXCL12 binding decreases gradually over increasing antagonist concentrations, although AMD070 dependent CXCL12 binding decreases slightly compared to WK1 after

2,5 μM.

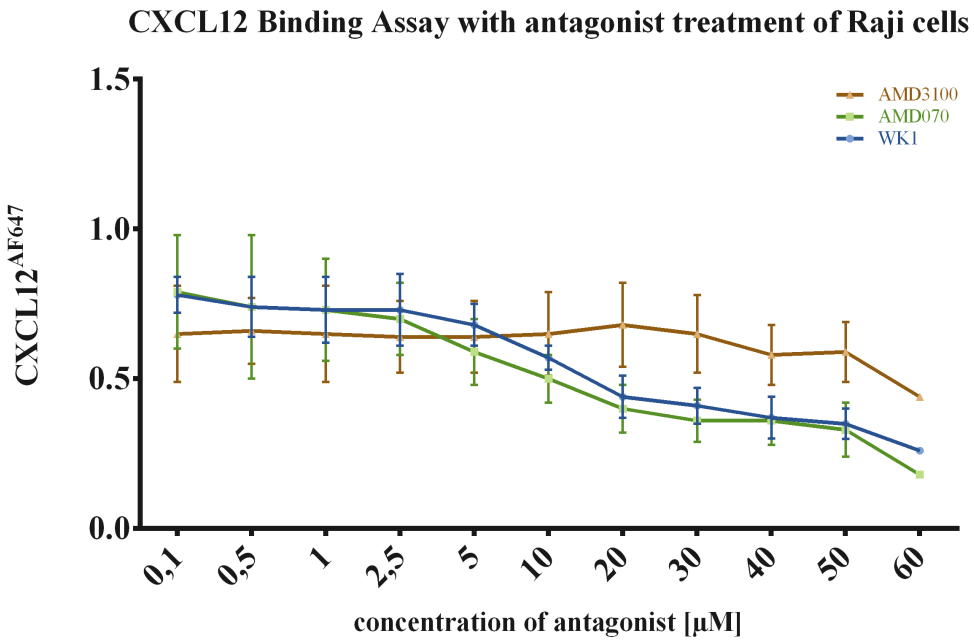

Figure 13: CXCL12^{AF647} binding assay after treatment of Raji cells with CXCR4 antagonists AMD3100 (brown), AMD070 (green) and WK1 (blue): After treatment of Raji cells with concentrations ranging from 0,1 to 60μM of CXCR4 antagonists (x-axis) we evaluated CXCL12 binding capabilities using a CXCL12^{AF647} binding assay. Following incubation of cells with antagonists, cells were exposed to excess CXCL12^{AF647}. Surplus was washed away and cells were then analysed using FACS during which the remaining CXCL12^{AF647} was detected and is plotted on the y-axis. Points in the graphs are mean values of 3 replicates (each in duplicates). Bars represent standard deviation of the mean that corresponds to each point.

3.4 EZ4U cell proliferation and cytotoxicity assay

To further characterize the three CXCR4 antagonists, whether they have any cytotoxic effects, we performed modified MTS assays (EZ4U assays) on WK1, AMD070, AM3100 and nicotinic acid treated cell lines. We treated our cell lines (BL2, Raji, Karpas422, SUDHL4, U2932 and RI1) with increasing concentrations ranging from 1 to 90 μM for 72h followed by the EZ4U in at least three technical replicates.

Our control set was treated with niacin, the residue that was added onto AMD070 to create WK1. As expected niacin had no effects on cell viability

and are shown in figure 17.

Displayed in figure 14 are the effects of WK1 on BL2, Raji, Karpas422, SUDHL4, U2932 and RI1 cell lines. Figures 14 A, C and D show significant cytotoxic effects of WK1. In figure 14 B, E and F a trend of impeded cell viability can be observed.

WK1 treatment resulted in a notable cell growth inhibition of BL2, Karpas422 and SUDHL4 as shown Figure 14A, C and D. In contrast, WK1 treatment resulted in no growth inhibitory effects on Raji, U2932 and RI1 cells as shown in Figure 14B, E and F.

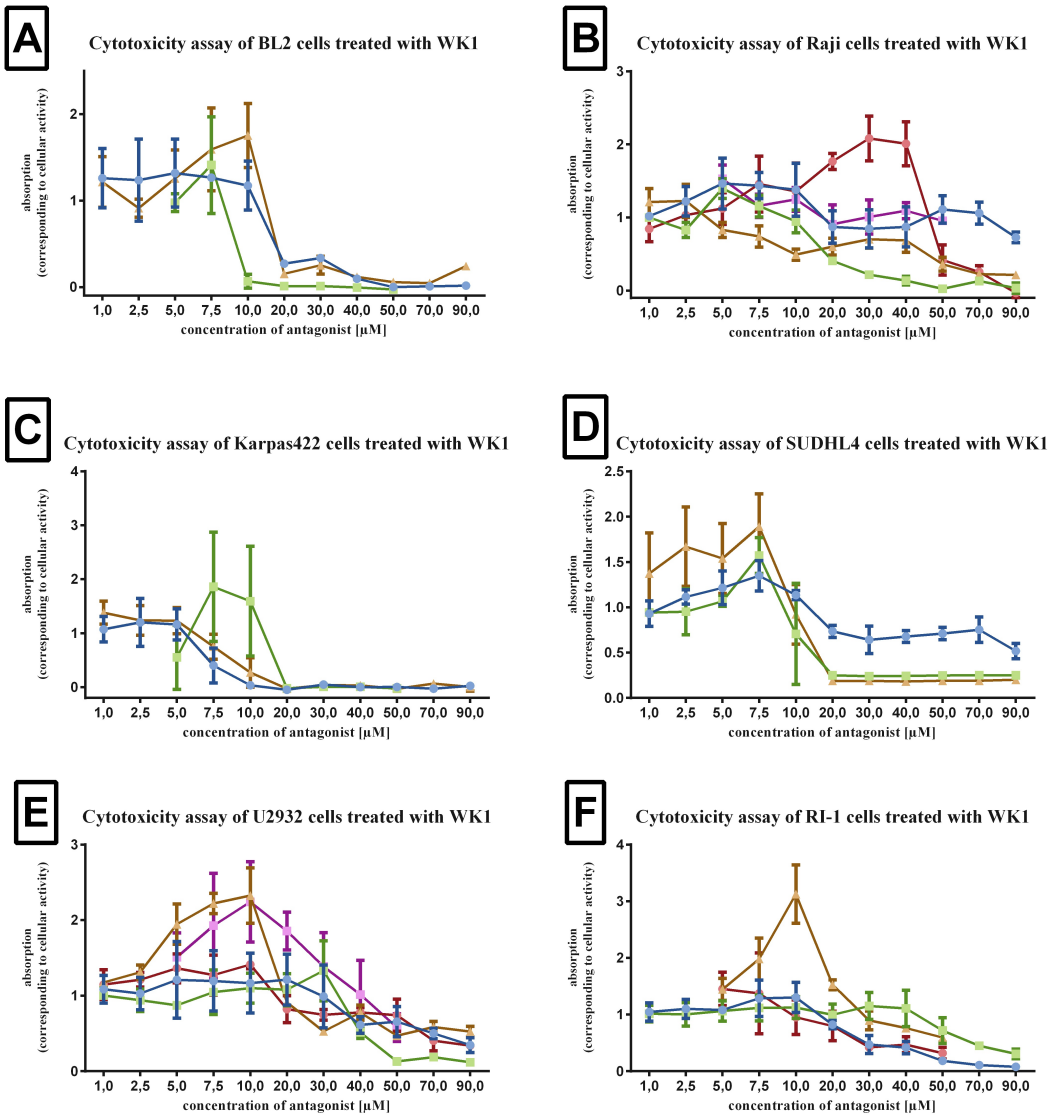

Figure 14: EZ4U assay of cell lines after WK1 treatment: Plotted in the graphs are the average absorption at 492 nm relative to the non-treated control (y-axis) to the respective WK1 concentration with which cells were treated (x-axis). Treatment concentrations ranged from 1- 90 μM. Colours indicate separate replicates which were always performed in triplicates. Bars represent the standard deviation. **A**: BL2 cells - belonging to the BL subtype - reacted to WK1 with decreased cell growth at 10 μM. **B**: Raji cells, also belonging to the BL subtype, was not affected. **C**: WK1 had considerable cytotoxic effects on Karpas422 cells (GCB subtype) as they started to be affected by WK1 at 5 μM. **D**: GCB cell line SUDHL4 also showed a significant decrease in cell growth in reaction to WK1 treatment starting at 7,5 μM. **E**: U2932 - a cell line belonging to the ABC- DLBCL subtype - showed a moderate trend of impeded cell viability in response to WK1. **F**: RI1 cells which are classified as ABC- DLBCL subtype, exhibit a gradual decreasing trend indicating moderate cytotoxicity of WK1 on these cells.

AMD070 treatment inhibited cell growth of BL2, Karpas422 and SUDHL4 in a notable manner as shown in Figure 14A and C, whereas for the other cell lines (Raji, SUDHL4, U2932 and RI1) no or just marginal effects were observed (Figure 14 B, D, E and F). Remarkable, the cell-growth inhibitory effects were just detected at higher AMD070 levels.

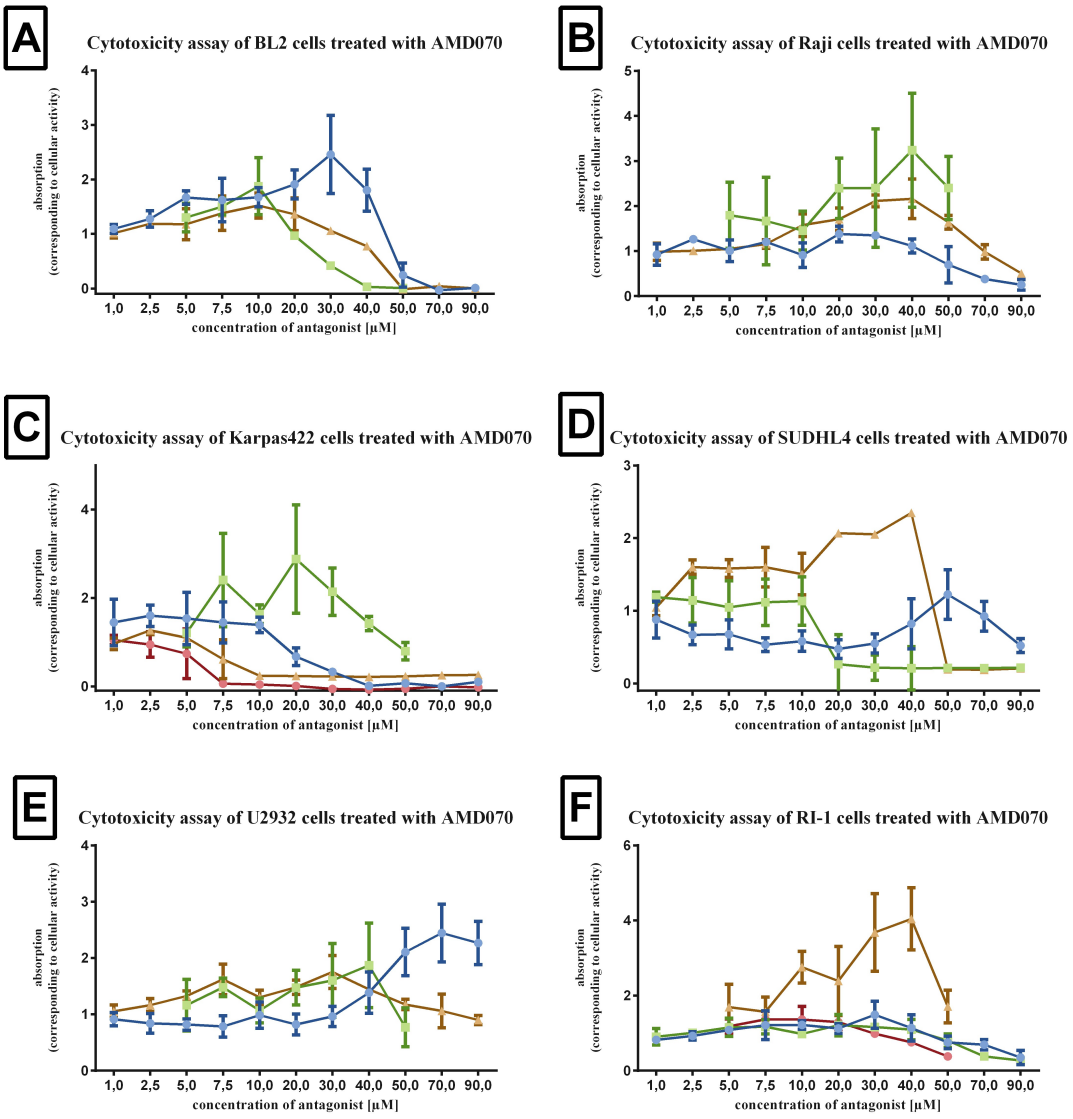

Figure 15: EZ4U assay of cell lines after AMD070 treatment: Plotted in the graphs are the average absorption at 492 nm relative to the non-treated control (y-axis) to the respective AMD070 concentration with which cells were treated (x-axis). Treatment concentrations ranged from 1- 90 μM. Colours indicate separate replicates which were always performed in triplicates. Bars represent the standard deviation. **A**: BL2 cells (BL subtype) responded to AMD070 with decreased cell growth around 20 - 40 μM. **B**: Raji cells, also belonging to the BL subtype, did not show significant decrease in cell death. **C**: AMD070 treatment showed mixed results in Karpas422 cells (GCB-DLBCL subtype) as some replicates indicate significant loss of cell growth at 7,5 μM, whereas in some replicates we could not reproduce these results at this concentration but rather observed it at higher concentrations. Generally though Karpas422 cells seem to be affected by AMD070. **D**: GCB- DLBCL cell line SUDHL4 also showed mixed cytotoxic effects in reaction to AMD070 treatment. **E**: U2932 cell line (ABC subtype) was not affected by AMD070 in regards to cell growth. **F**: RI1 cells (ABC-DLBCL subtype) exhibit a very slight decreasing trend indicating moderate cytotoxicity of AMD070 on these cells.

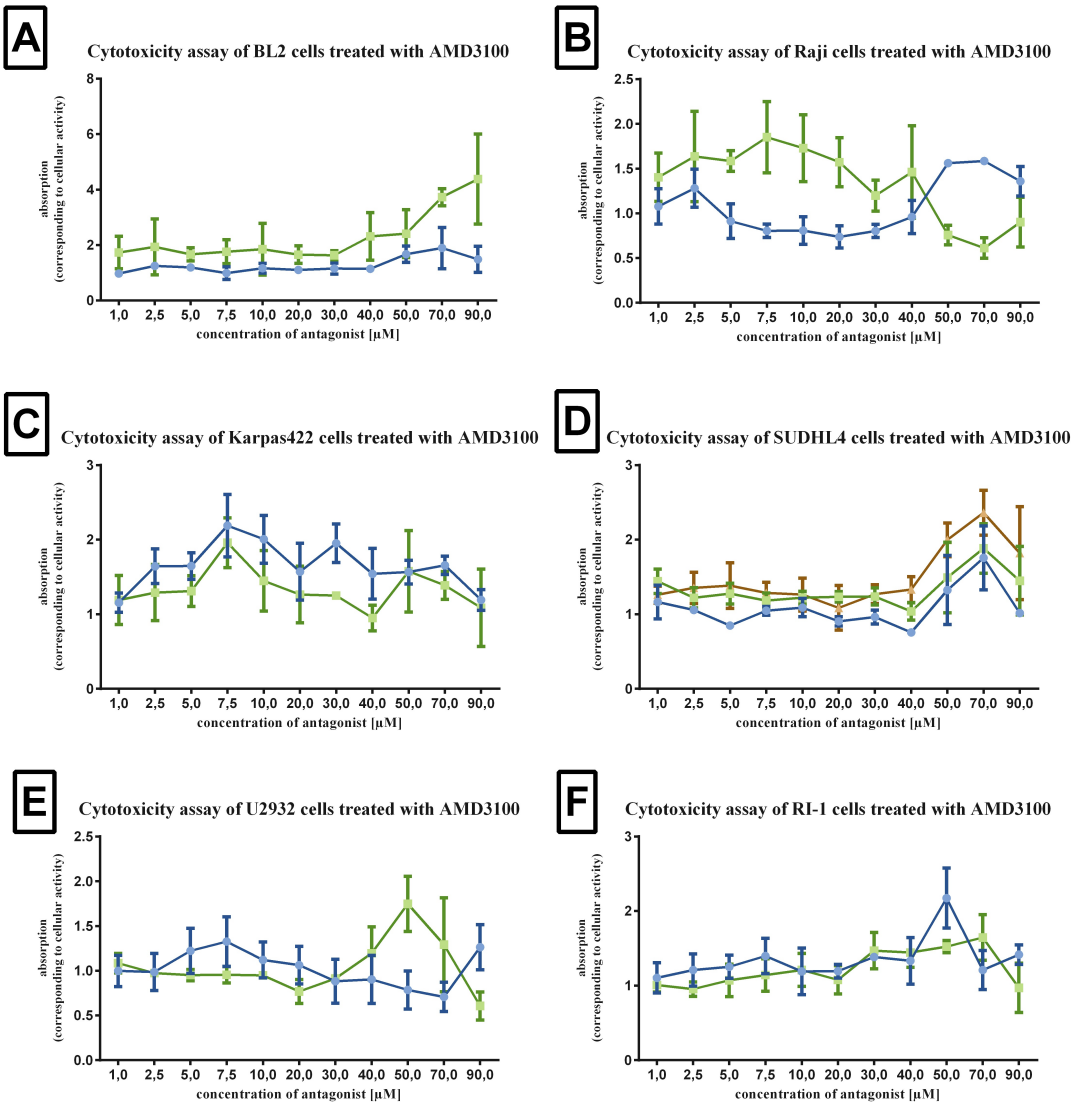

Figure 16: EZ4U assay of cell lines after AMD3100 treatment: Plotted in the graphs are the average absorption at 492 nm relative to the non-treated control (y-axis) to the respective AMD3100 concentration with which cells were treated (x-axis). Treatment concentrations ranged from 1- 90 μM. Colours indicate separate replicates which were always performed in triplicates. Bars represent the standard deviation. **A**: BL2 cells (BL subtype) were not inhibited in their growth by AMD3100. **B**: Raji cells, also belonging to the BL subtype, also did not react to AMD3100 in any significant manner. **C**: Karpas422 cells (GCB subtype) showed no notable change in cell growth. **D**: GCB cell line SUDHL4 had a reproducible peak at 70 μM but otherwise was also not affected by AMD3100. **E**: U2932 cells (ABC subtype) did not react to AMD3100 with decreased cell growth. **F**: RI1 cells (ABC subtype) were also not negatively affected by AMD3100.

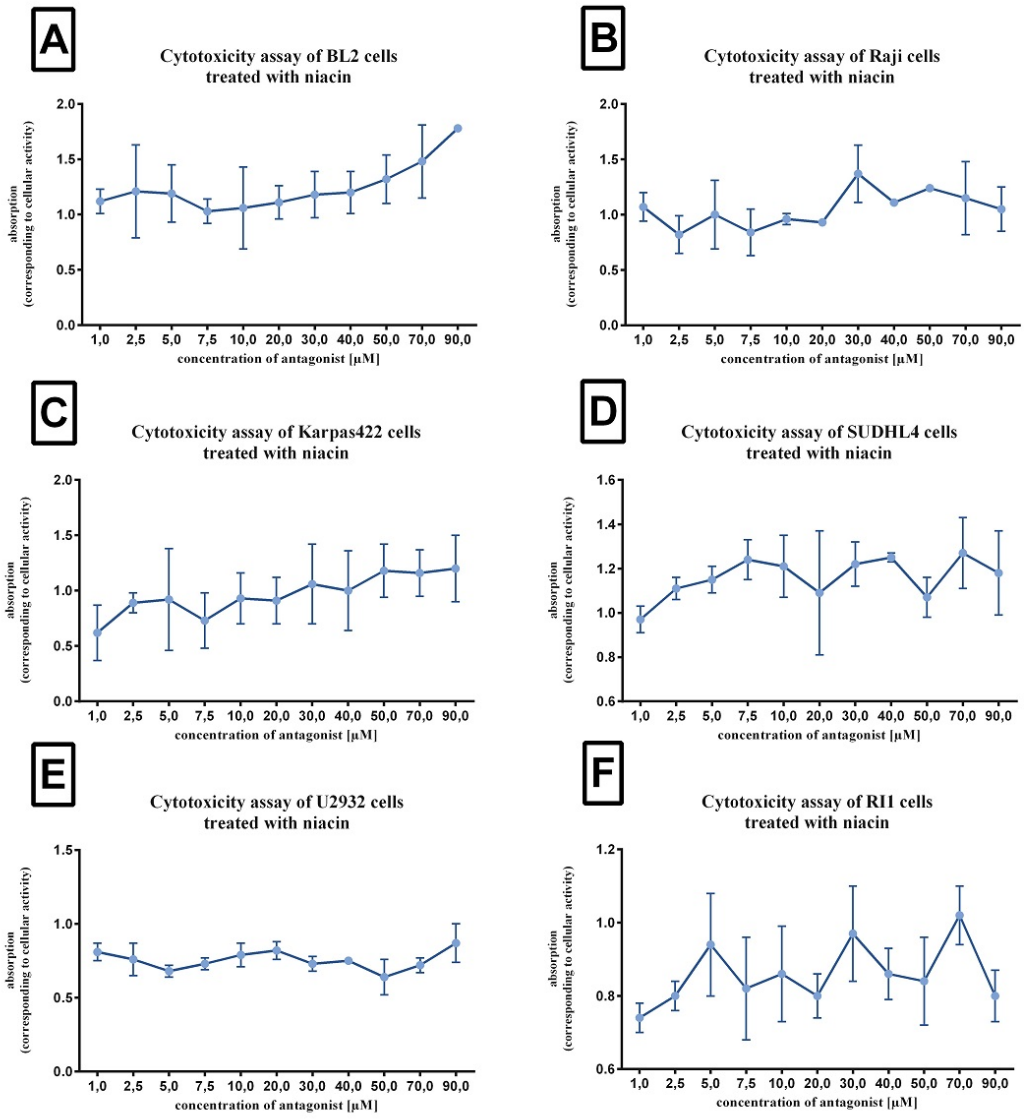

Figure 17: EZ4U assay of cell lines after niacin treatment: Plotted in the graphs are the average absorption at 492 nm relative to the non-treated control (y-axis) to the respective niacin concentration with which cells were treated (x-axis). Treatment concentrations ranged from 1- 90 μM. Each concentration was performed in triplicates. Bars represent the standard deviation. **A**: BL2 cells (BL subtype) were not inhibited in their growth by niacin. **B**: Raji cells, also belonging to the BL subtype, also did not react to niacin. **C**: Karpas422 cells (GCB subtype) showed no notable change in cell growth. **D**: GCB cell line SUDHL4 also exhibited no cell growth inhibition after niacin treatment. **E**: U2932 cells (ABC subtype) did not react negatively to niacin. **F**: RI1 cells (ABC subtype) were also not negatively affected.

3.5 Cell viability and apoptosis staining using Annexin V and 7-AAD after treatment with CXCR4 antagonists

To validate and further elucidate the cell growth inhibitory effects of WK1, AMD070 and AM3100, we performed Annexin V/ 7-AAD staining with FACS analysis after 24h, 48h and 72h of treatment with the three antagonists at a concentration of 0 μM, 1 μM, 5 μM, 10 μM, 20 μM and 40 μM. A concentration of 10 μM, 20 μM and 40 μM of WK1 reduced the cell viability of BL2, Karpas422 and SUDHL4 to remarkable extent (figure 18), whereas for other investigated cell lines (Raji, U2932 and RI1) no or just a slight reduction of the cell viability was observed (figure 18). Treatment of BL2 and Karpas422 with AMD070 also remarkably reduced the cell viability at a concentration of 20 μM and 40 μM. AMD070 treatment did not influence the viability of the other treated lymphoma cell lines (Raji, Karpas422, U2932 and SUDHL4, figure 19). In stark contrast, AMD3100 treatment did not affect the viability of any tested lymphoma cell line (figure 20). These data suggest that the cell growth inhibitory effects of WK1 and AMD070 are mediated by reduction of the cell viability and that the effects of WK1 are more potent than those of AMD070.

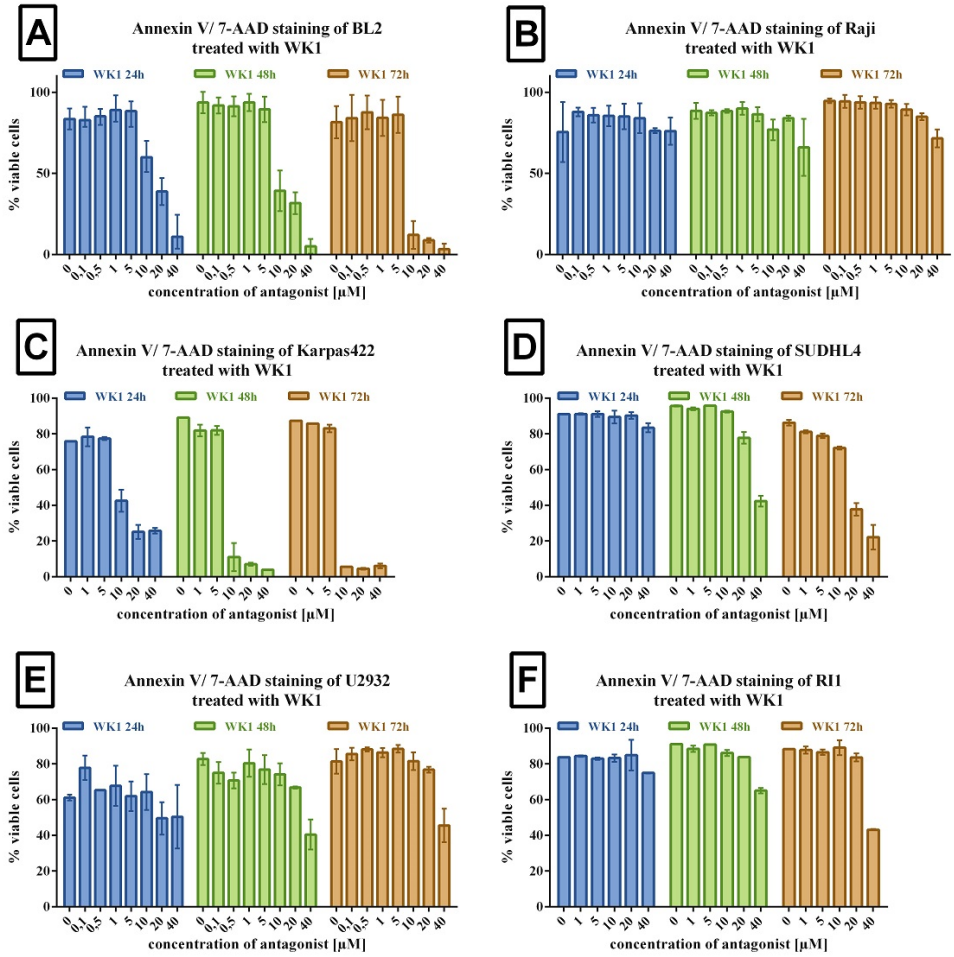

Figure 18: Annexin V staining of cell lines after treatment with WK1: Plotted on the y-axis is the mean percentage of still living cells - identifiable due to them being Annexin V / 7-AAD negative - after treatment with different concentrations of the CXCR4 antagonist WK1 which are plotted on the x-axis thrice. In colours blue, green and brown are incubation times 24h, 48h and 72h respectively. Error bars represent the standard deviation of the mean. Depending on concentrations and cell line these results stem from 2 - 8 replicates. BL2, Raji and U2932 were repeated most often and with additional concentrations (0,1 and 0,5 μM). **A**: BL2 cells (BL subtype) reacted with induction of cell death 24h after WK1 treatment at a concentration of 10 μM and up. **B**: Raji cells (BL subtype) did not show any significant inhibitory effects due to WK1 treatment, but a slight reduction was observable at 40 μM. **C**: Karpas422 cells (GCB subtype) showed pronounced inductions of cell death at WK1 concentrations over 10 μM at all 3 time points. **D**: SUDHL4 cells (GCB subtype) did not react in any significant manner to WK1 after 24h of incubation, but in the later measurements decreases in viable cells became obvious at 20 μM WK1. **E**: U2932 cells (ABC subtype) indicate a possible trend of inhibited cell growth at 40 μM WK1. **F**: RI1 cells (ABC subtype) did react to WK1 treatment at 40 μM. Generally, longer incubation times exacerbated the antagonist's effects.

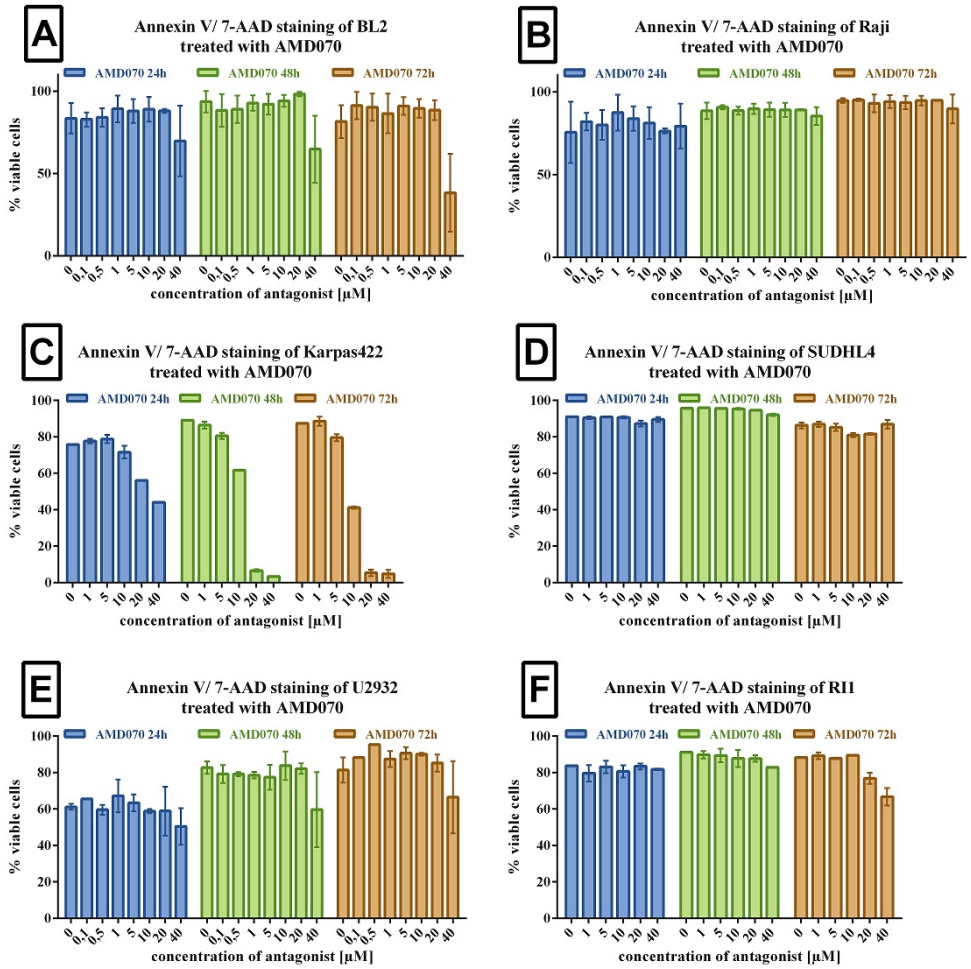

Figure 19: Annexin V staining of cell lines after treatment with AMD070: Plotted on the y-axis is the mean percentage of still living cells - identifiable due to them being Annexin V / 7-AAD negative - after treatment with different concentrations of the CXCR4 antagonist AMD070 which are plotted on the x-axis thrice. In colours blue, green and brown are incubation times 24h, 48h and 72h respectively. Error bars represent the standard deviation of the mean. Depending on concentrations and cell line these results stem from 2 - 8 replicates. BL2, Raji and U2932 were repeated most often and with additional concentrations (0,1 and 0,5 μM). **A**: BL2 cells (BL subtype) were inhibited by AMD070 at 40 μM. **B**: Raji cells (BL subtype) were not affected by AMD070. **C**: Karpas422 cells (GCB subtype) were inhibited after AMD070 treatment concentrations 10 μM and above. **D**: SUDHL4 cells (GCB subtype) were not affected by AMD070. **E**: U2932 cells (ABC subtype) appear to not be affected by AMD070 although after 48h and 72h the mean percentage of viable cells is decreased slightly. **F**: RI1 cells (ABC subtype) were not affected by AMD070 after 24h and 48h of incubation, however a slight decrease was apparent at 72h. Generally, longer incubation times exacerbated the antagonist's effects.

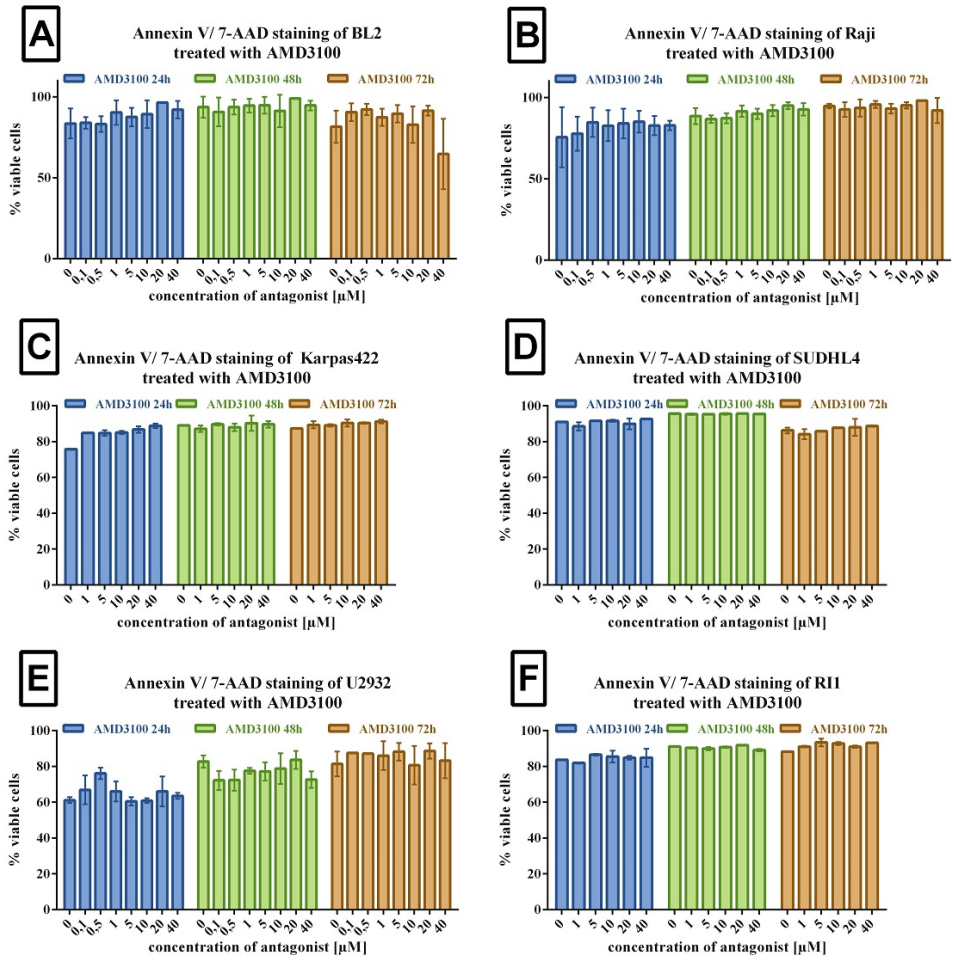

Figure 20: Annexin V staining of cell lines after treatment with AMD3100: Plotted on the y-axis is the mean percentage of still living cells - identifiable due to them being Annexin V / 7-AAD negative - after treatment with different concentrations of the CXCR4 antagonist AMD3100 which are plotted on the x-axis thrice. In colours blue, green and brown are incubation times 24h, 48h and 72h respectively. Error bars represent the standard deviation of the mean. Depending on concentrations and cell line these results stem from 2 - 8 replicates. BL2, Raji and U2932 were repeated most often and with additional concentrations (0,1 and 0,5 μM). **A** BL2 cells (BL subtype) , **B** Raji cells (BL subtype) , **C** Karpas422 cells (GCB subtype) , **D** SUDHL4 cells (GCB subtype), **E** U2932 cells (ABC subtype) and **F** RI1 cells (ABC subtype) treated with AMD3100 were not affected regarding cell viability.

3.5.1 Caspase-3 staining

To investigate whether the observed reduction of cell viability of our experiments with WK1 and AMD070 was mediated by induction of apoptosis we conducted caspase-3 staining on BL2, Karpas422 and SUDHL4 cells treated with 10 μM, 20 μM and 40 μM WK1 and 40 μM AMD070.

Treatment of all three lymphoma cell lines with WK1 caused cleavage of caspase-3 in a higher percentage of cells (Figure 21) indicating on the one hand that the cytotoxic effects of WK1 and AMD070 are mediated by induction of apoptosis and on the other that WK1 possesses a higher anti-lymphoma activity.

Cell lines were stained for caspase-3 after treatment with WK1 and AMD070. The results seen in figure 21 and show that especially WK1 induced apoptosis in BL2 cells as seen in the blue histogram. In green and brown we could also show that up to 20% of Karpas422 and SUDHL4 cells react to WK1 with apoptosis. AMD070 on the other hand did not initiate apoptosis as clearly as WK1.

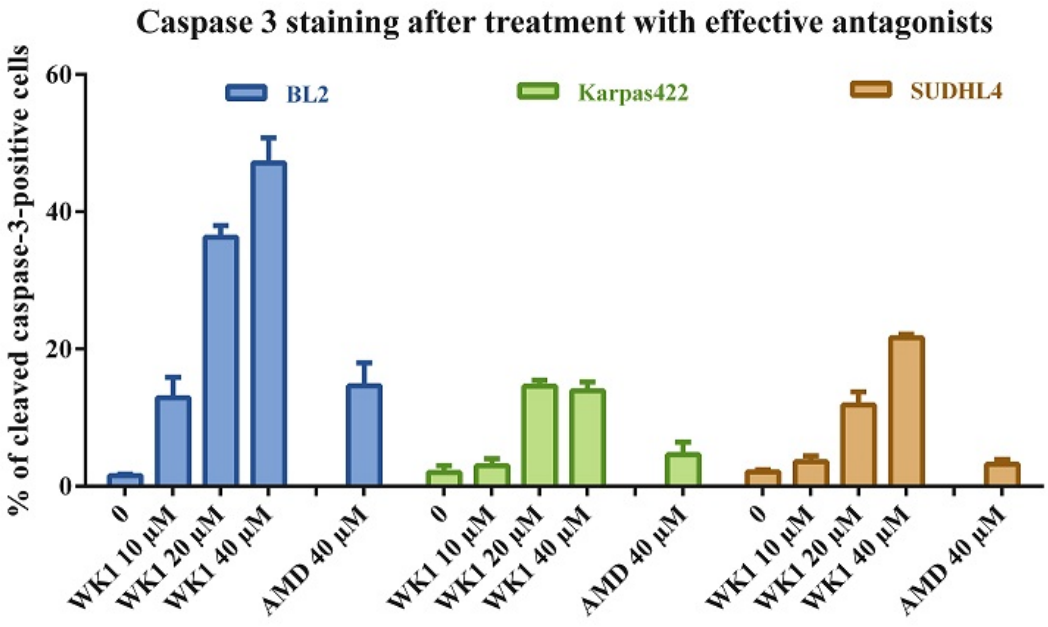

Figure 21: Caspase-3 staining of BL2 (blue), Karpas422 (green), SUDHL4 (brown) with different antagonists treatment concentrations plotted on the x-axis. Depicted are the means of 2 replicates with error bars representing the standard deviation.

3.6 Chemokine receptor expression analysis of CLL patient samples

To investigate the function of chemokine receptor (CR) expression in CLL, we determined expression levels of 17 well characterized CR - namely CCR1-10, CXCR1-5, CX3CR1 and XCR1- via RT-qPCR in analysed 20 clinically well documented patient samples. The expression of CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, XCR1 and CX3CR1 was determined via RT-qPCR and we compared CR expression levels to diagnosis, therapy start, progression, second neoplasia and also if a Δ17 mutation was diagnosed to be a cytogenetic risk parameter (deletion of 17q).

We found a trend for a lower expression level of CCR7 (n-fold, p=0.095) in patients who needed therapy in comparison to those who needed no therapy as depicted in figure 22. We did not find any further association due to the small patients cohort.

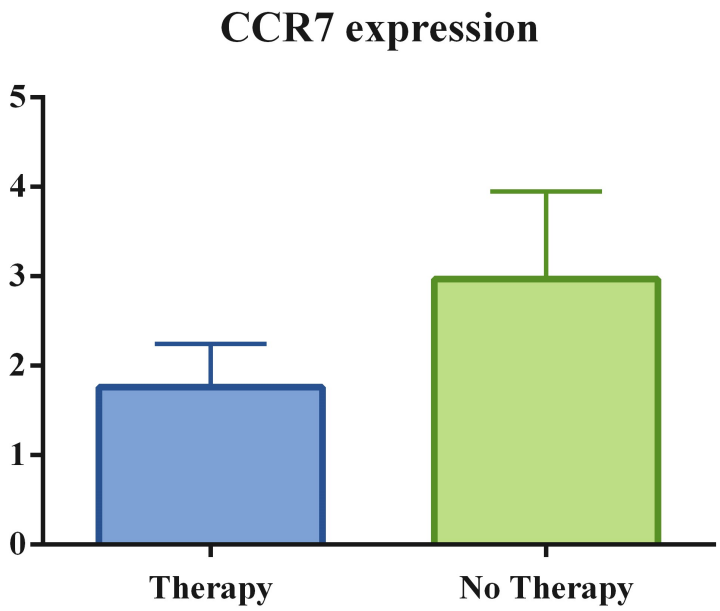

Figure 22: Comparison of the CCR7 expression between patients who needed therapy and who did not need therapy in patients who received therapy in comparison to patients without therapy.

4 Discussion

The impact of the CXCR4/CXCL12 axis is well described in many malignancies and also in haematologic malignancies in particular, but options and effects for antagonising the chemokine receptor in hopes for new and better treatments are sparse. Therefore, we aimed to evaluate the effectiveness and potential of the new CXCR4 antagonist WK1.

CXCR4/CXCL12 have important roles in the complex process required for metastasis and this pair have been linked to enhanced tumour cell survival, proliferation, angiogenesis and also to preferential metastasis to tissues with high expression of CXCL12 i.e. the bone marrow. [51]

First, we aimed to analyse the CXCR4 expression profile in our cell lines which resulted in a cell line specific CXCR4 expression pattern.

It is well known that immune cells and many cancers strongly express CXCR4 [52, 27] and we could confirm high CXCR4 expression levels in lymphatic malignant cell lines BL2 and SUDHL4 by using a CXCR4-specific FACS analysis. U2932, RI1 and Raji cells do express CXCR4 but to a lesser extent, while expression of CXCR4 is negligible in Karpas422 cells.

Second, we wanted to investigate CXCR7, known to also interacting with CXCL12. CXCR7 expression in hematopoietic cells is controversial as different studies showed inconsistent results. However, in tumour cells CXCR7 appears to be upregulated. [43, 44] By combining the CXCL12 binding assay with blocking antibodies, we were able to demonstrate that ABC- DLCBL cell lines (RI1 and U2932) and Raji cell lines expressed CXCR7 on their surface.

By combing the CXCL12 binding assay with AMD3100, AMD070 and WK1 treatment on CXCR4+ and CXCR7+ lymphoma cell lines, we were able to demonstrate that all three antagonists were able to block CXCL12-CXCR4 binding. Based on the fact that AMD3100 and AMD070 block CXCL12-CXCR4 binding with lower concentration compared to WK1 and that WK1 is the nicotinic acid derivative of AMD070, it might be speculated that the reduced blocking ability

might be cause by the large size of WK1 molecule. In contrast, the ability of AMD3100, AMD070 and WK1 to block CXCL12-CXCR7 binding was negligible for all of them.

Our cell growth-, viability- and apoptosis assays clearly demonstrated that WK1 and also to a reduced extent AMD070 possess the ability to induce apoptosis in lymphoma cells. Previous studies have shown that blockage of CXCR4 with either monoclonal antibodies or small molecules like AMD3100, AMD070 and BKT140 enhanced the chemotherapeutic effects of rituximab in tissues by blocking the interaction of lymphoma cells with protective stromal cells. [32, 33, 34, 35, 29, 36, 27] Additionally, direct cytotoxic effects of CXCR4 antagonizing agents were also found for BKT140 [29], NefM1 [55, 56], LY2624587 [57], MDX-1338 [58] and cyclic pentapeptide d-Arg3FC131 [59], indicating that the pro-apoptotic effects of CXCR4 antagonists might be a common feature. However, combining CXCR4 and CXCR7 expression pattern with our *in vitro* results, it was obvious that apoptosis was induced in cell lines exhibiting CXCR4 and no or low CXCR7 expression, indicating that CXCR7 might attenuate the apoptotic effects of CXCR4 antagonists. The Karpas422 cell line seems to be the only exception to that observation. Based on the fact that CXCR4 was rather weakly expressed, that the cytotoxic effects of WK1 and AMD070 were faster induced, and that the cleavage of caspase 3 was lower compared to the other cell lines, we speculate that both antagonists mediate their cytotoxic effects in CXCR4- and caspase-3 independent manner.

In the second part of my thesis, we demonstrated that CCR7 was lower expressed in patients who needed therapy of our cohort, indicating that low CCR7 expression may lead to symptoms that make therapy obligatory. It was shown that CCR7 expression levels in CLL were significantly elevated in patients with clinical lymphadenopathy compared to those without lymph node disease. Additionally, blocking of CCR7 resulted in inhibited transendothelial migration of CLL cells. [60] For other lymphoma entities, it was shown that CCR7 caused the

homing of lymphoma to lymph nodes and interaction of malignant B-cells and T-cell zone stromal cells. [61]

Taking altogether, we speculate that low CCR7 expression may cause extra-nodal dissemination of the CLL. However, due to the low numbers of samples further investigations are obligatory to elucidate this observation in CLL.

In conclusion, we demonstrated that the newly developed CXCR4 antagonist WK1 possesses mainly CXCR4-mediated apoptotic function, and thereby represents a novel molecule to develop novel treatments for aggressive lymphomas. Concerning CCR7 expression in CLL, we propose that low CCR7 might serve as novel biomarker for risk stratification.

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