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Targeting chronic lymphocytic leukemia cells using anti-ROR1 monoclonal antibodies and small molecule inhibitors

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"The only thing we'll ever agree on is that we'll never agree on anything"
Bacha Khan

"Compromise your approach but never compromise your goal"

To My Sisters

Abstract

Chronic lymphocytic leukemia (CLL) is characterized by accumulation of malignant B cells in blood, bone marrow, spleen and lymph nodes. It is the most common leukemia in the Western world. Major progress has been made in recent years to prolong the survival and improve the well-being of the patients. Despite advances, CLL is still a disease with no cure. New therapeutic strategies, based on new targets and novel drugs, are needed.

Receptor tyrosine kinase-like orphan receptor (ROR1) belongs to one of the twenty families of receptor tyrosine kinases (RTKs). RTKs are involved in important cellular processes including proliferation, differentiation, survival, signaling and migration. Many RTKs are deregulated in various types of cancer and may act as a therapeutic target. ROR1 is highly expressed in CLL and other malignancies but not in normal adult cells. ROR1 has been shown to be a survival factor for CLL cells. The main goal of the study was to develop novel ROR1 targeting therapeutics for CLL.

In the **first study**, five monoclonal antibodies (mAbs) directed against different domains of the extracellular part of ROR1 were produced and their apoptotic activity was analysed. All five mAbs recognized surface bound ROR1 and induced selective apoptosis of CLL cells but not of normal B cells. Antibodies alone against CRD and KNG domains of ROR1 were the most effective in inducing apoptosis of CLL cells without the need of complement or immune effector cells. KNG and CRD mAbs induced higher cytotoxicity than rituximab in vitro. Crosslinking of ROR1 mAbs with $F(ab)_2$ fragments significantly enhanced apoptosis. Two of the mAbs also induced complement dependent cytotoxicity (CDC) similar to rituximab and one KNG mAb (IgG1) induced antibody-dependent cellular cytotoxicity (ADCC).

In the **second study**, different ROR1 isoforms as well as the phosphorylation pattern in CLL cells were analysed. Two major ROR1 bands with the size of 105 and 130 kDa were identified which may correspond to unglycosylated (immature) and glycosylated (mature) ROR1 respectively. The 105 kDa band was significantly higher expressed in non-progressive than in progressive CLL patients. Two other ROR1 bands with the size of 260 kDa and 64 kDa were also noted which might represent dimerized ROR1 and truncated ROR1, respectively. The 64 kDa band was localized to the nucleus indicating a possible role as a transcription factor. ROR1 isoforms of 64, 105, 130 and 260 kDa were all phosphorylated at tyrosine and serine residues. The phosphorylation intensity for the 130 kDa ROR1 isoform was significantly higher in progressive than in non-progressive CLL patients. ROR1 mAbs against the CRD and KNG domains induced dephosphorylation of ROR1 and subsequent apoptosis of CLL cells.

In the **third study**, the effect of ROR1 mAbs on PI3K/AKT/mTOR signaling was analyzed. Anti-CRD ROR1 mAb induced apoptosis of CLL cells and reduced phosphorylation levels of ROR1 as well as of SRC, PI3K, AKT, mTOR, and CREB. An anti-ROR1 mAb against the Ig-like domain did not induce apoptosis.

In the **fourth study**, the expression of dishevelled (DVL) proteins was investigated. DVL proteins (DVL1, DVL2 and DVL3) were significantly upregulated in CLL cells compared to normal PBMC. DVL1 and DVL3 expression was higher in progressive than in non-progressive CLL patients whereas DVL2 was significantly higher expressed in non-progressive compared to progressive CLL. DVL1, DVL2 and DVL3 were phosphorylated at tyrosine and serine residues. The data indicate that DVLs are involved in the pathobiology of CLL probably as part of the Wnt signaling pathways.

In the **fifth study**, a small molecule that inhibits activated ROR1 (KAN0439834) was described for the first time. KAN0439834 induced apoptosis in CLL cells, about 60-fold higher compared to normal PBMC. KAN0439834 dephosphorylated ROR1 and seemed to dephosphorylate PI3K, AKT and CREB. KAN0439834 showed higher specific killing activity against CLL cells compared to other kinase inhibitors. Treatment of NOD-SCID mice xenografted with human CLL cells with KAN0439834 significantly reduced the number of CLL cells and dephosphorylated ROR1.

In **conclusion**, mAbs and a first-in-class tyrosine kinase inhibitor against ROR1 showed promising results with regard to specific cytotoxicity of CLL cells. Based on these data, ROR1 appears to be a suitable target for a novel therapeutic approach of CLL, as well as of other malignancies with a different mechanism of action than currently available drugs.

List of publications

I. Daneshmanesh AH, **Khan SA**, Hojjat-Farsangi M, Jeddi-Tehrani M, Akhondi MM, Bayat AA, Ghods R, Mahmoudi A-R, Hadavi R, Österborg A, Shokri F, Rabbani H, Mellstedt H: Monoclonal antibodies against Ror1 induce apoptosis of chronic lymphocytic leukemia (CLL) cells. *Leukemia, 26:1348-1355, 2012*.

II. Hojjat-Farsangi M, **Khan S**, Daneshmanesh A, Moshfegh A, Sandin Å, Mansouri L, Palma M, Lundin J, Österborg A, Mellstedt H. The tyrosine kinase receptor ROR1 is constitutively phosphorylated in chronic lymphocytic leukemia (CLL) cells. *PLoS One, 8: e78339, 2013*.

III. Daneshmanesh AH, Hojjat-Farsangi M, Moshfegh A, **Khan AS**, Mikaelsson E, Österborg A, Mellstedt H. The PI3K/AKT/mTOR pathway is involved in direct apoptosis of CLL cells induced by ROR1 monoclonal antibodies. *British Journal of Haematology, 169(3):455-458, 2015.*

IV. **Khan AS**, Hojjat-Farsangi M, Daneshmanesh A, Hansson L, Österborg A, Mellstedt H, Moshfegh A. Dishevelled proteins are significantly up regulated in chronic lymphocytic leukemia. *Experimental Hematology, accepted, pending revision 2015.*

V. **Khan AS**, Hojjat-Farsangi M, Daneshmanesh A, Vågberg J, Byström S, Olsson E, Löfberg C, Norström C, Schultz J, Norin M, Olin T, Österborg A, Mellstedt H, Moshfegh A. On-Target Effects of a ROR1 Tyrosine Kinase Small Molecule Inhibitor by Apoptosis Mediated Death in Chronic Lymphocytic Leukemia Cells in vitro and in vivo. *Manuscript.*

Related publication:

Hojjat-Farsangi M, Moshfegh A, Daneshmanesh AH, **Khan AS**, Mikaelsson E, Österborg A, Mellstedt H. The receptor tyrosine kinase ROR1 – an oncofetal antigen for targeted cancer therapy. *Seminars in Cancer Biology, 29:21-31, 2014.*

List of abbreviations

ADC	Antibody drug conjugates
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired immunodeficiency syndrome
AIHA	Autoimmune hemolytic anemia
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
ATM	Ataxia telangiectasia-mutated
Bcl-2	B-cell lymphoma 2
BCR	B-cell receptor
BIRC3	Baculoviral IAP repeat containing 3 gene
BMSC	Bone marrow stem cells
BR	Bendamustine-rituximab
BTK	Bruton's tyrosine kinase
CAR	Chimeric antigen receptor
CD40L	CD40 ligand
CDC	Complement-dependent cytotoxicity
CDK	Cyclin-dependent kinase
CK1	Casein kinase 1
CLL	Chronic lymphocytic leukemia
CLLU1	CLL up-regulated gene 1
CML	Chronic myelogenous leukemia
CRD	Cysteine-rich domain
CR	Complete remission
CREB	cAMP response element-binding protein
CTCL	Cutaneous T-cell lymphoma
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DVL	Dishevelled
DLBC	Diffuse large B-cell lymphoma
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
FC	Fludarabine-cyclophosphamide
FCR	Fludarabine-cyclophosphamide-rituximab
FDA	Food and drug administration
FGFR	Fibroblast growth factor receptor
FISH	Fluorescence in situ hybridization
FL	Follicular lymphoma
GTP	Guanosine triphosphate
HDAC	Histone deacetylase
HEK cells	Human embryonic kidney cells
HER	Human epidermal receptor
hROR1	Human ROR1

HSC	Hematopoietic stem cell
IC50	The half maximal inhibitory concentration
Ig	Immunoglobulin
IGHV	Immunoglobulin heavy chain variable genes
IL	Interleukin
IP	Immunoprecipitation
kDa	Kilodalton
KNG	Kringle
LDT	Lymphocyte doubling time
LPL	Lipoprotein lipase
LRP6	Low density lipoprotein receptor-related protein 6
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MBC	Monoclonal B cell lymphocytosis
MCL	Mantle cell lymphoma
Mcl-1	Myeloid leukemia cell differentiation protein 1
MDS	Myelodysplastic syndrome
miRNA	Micro RNA
MRD	Minimal residual disease
mRNA	Messenger RNA
mROR1	Mouse ROR1
MSC	Mesenchymal stromal cell
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYD88	Myeloid differentiation primary response gene 88
MZL	Marginal zone lymphoma
NFκB	Nuclear factor kappa-light chain enhancer of activated B cells
NGS	Next generation sequencing
NK cells	Natural killer cells
NLC	Monocyte-derived nurse like cells
NOD-SCID mouse	Non-obese diabetic-severe combined immunodeficiency mouse
NOTCH1	Notch homolog 1 translocated associated
NTRKR	Neurotrophic tyrosine kinase receptor related
NSCLC	Non-small cell lung carcinoma
ORR	Overall response rate
OS	Overall survival
pAb	Polyclonal antibody
PARP	Poly ADP ribose polymerase
PBMC	Peripheral blood mononuclear cells
PC	Proliferation centers
PCR	Pentostatin-cyclophosphamide-rituximab
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PFS	Progression free survival
PI	Propidium iodide
PI3K	Phosphoinositide-3 kinase

PKC	Protein kinase C
PNA	Purine nucleoside analog
PR	Partial remission
PRCA	Pure red cell aplasia
PRD	Proline-rich domain
RCC	Renal cell carcinoma
ROR	Receptor tyrosine kinase-like orphan receptor
RT-PCR	Real-time PCR
RTK	Receptor tyrosine kinase
S/TRD	Serine/threonine rich domain
Sβ2m	serum β2 microglobulin
sCD23	Soluble CD23
SF3B1	Splicing factor 3B subunit 1
SH2	Src homology 2
SHM	Somatic hypermutation
shRNA	Small hairpin RNA
sIg	Surface membrane Immunoglobulin
SLL	Small lymphocytic leukemia
SNAI 1	Snail family zinc finger 1
STAT	Signal transducer and activator of transcription
sTK	Serum thymidine kinase
SYK	Spleen tyrosine kinase
TCL	T-cell lymphoma
TCL1	T-cell leukemia/lymphoma protein 1
TITF-1	Thyroid transcription factor 1
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptors
TP53	Tumor protein p53
VEGFR	Vascular endothelial growth factor receptor
ZAP-70	Zeta-chain associated protein kinase 70
ZEB1	Zinc finger E-box-binding homeobox 1

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PAPERS I-V

1 CHRONIC LYMPHOCYTIC LEUKEMIA

1.1 Introduction

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in the Western world which mainly affects individuals with an age more than 50 years and is characterized by the progressive accumulation of CD5+ B cells in the peripheral blood, bone marrow and lymphoid organs such as lymph nodes and spleen [1-3]. Previously CLL was considered to be a homogenous disease with mature B cells accumulation due to defective apoptosis but now several reports have indicated that the high lymphocyte count is due to both prolonged survival and high proliferation [4-6]. B cells in CLL express CD5, CD19, CD23 and low levels of IgM, IgD and CD79b, which is a phenotype of mature and activated B cells [7-9] as depicted in Figure 1. CLL is now considered as a disease of high heterogeneity with regard to cell morphology, cytogenetics and immunophenotype and with a varying clinical behavior [10]. One-third of CLL patients do not require treatment and survive for more than twenty years [11] while some may progress quickly from time of the diagnosis, accompanied by complications such as autoimmune phenomena, infections and deterioration.

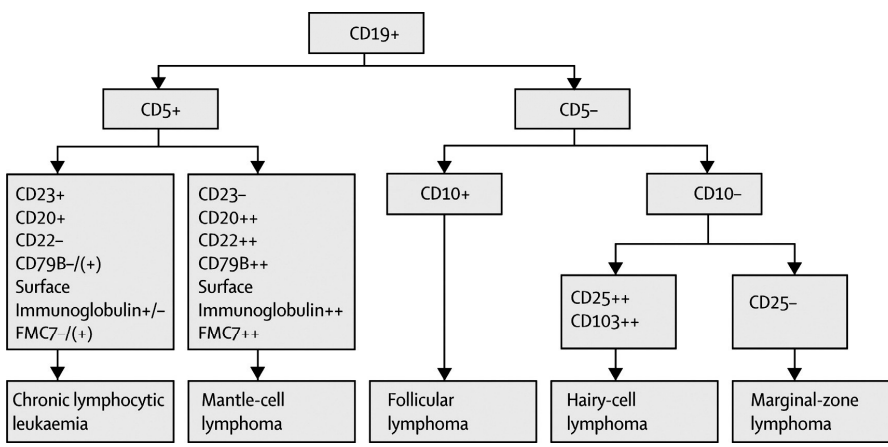

Figure 1. Differential diagnosis of CD19+ lymphocytosis.

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1.2 Epidemiology

The incidence of CLL varies with age and sex in a given population. CLL is more common in men than women with ratio of 1.5-2:1. The number of new CLL cases in Sweden is around 500 per year with a median age of 71 years at diagnosis [12]. However, one third of new CLL cases are diagnosed under the age of 55 [13]. The prevalence of CLL is around 30-50/100,000 which is the number of all diagnosed cases at a given moment in time [14]. CLL is very rare in Chinese, Japanese and Filipino populations for unknown reasons [15]. The incidence rate among them is 5 times lower even if they have fully adopted an American lifestyle compared to White Americans [16].

1.3 Risk factors

There is no evidence that the occurrence of CLL is due to environmental factors such as radiations and chemical compounds. No correlation with immunodeficiency problems has been reported [2]. The occurrence of CLL is very rare in China and Korea and almost non-existent in Japan and this extremely low incidence frequency is maintained in Japanese emigrants and in their following subsequent generations excluding the environmental modifiers of genetic susceptibility [16, 17]. There is also indication that 5-10% of cases might be due to familial susceptibility to CLL and other lymphoid malignancies resulting in two or more members in the same family with disease [18-20]. The first relatives of CLL patients have 2 to 7 times higher risk to develop the disease than the general population [21]. The familial predisposition is also related to the earlier onset of a more aggressive course of disease in the progeny of CLL patients [20, 22-24].

1.4 Biology and pathogenesis

The development of cytogenetic and molecular biology during the last two decades has made a considerable progress in the field of CLL. It is currently suggested that inhibition of apoptosis is of major importance for the development of CLL, where B cells show a high anti-apoptotic activity with a strong Bcl-2 expression. There exists imbalance between the rate of cell birth and death in CLL cells [25, 26]. The accumulation of transformed B cells due to defective apoptosis machinery may result in CLL as there exists a very small proliferating population of B cells which is one of the hallmark of CLL [27].

Monoclonal B cell lymphocytosis (MBL) is the pre-leukemic stage of CLL with $< 5x10^9$/L of circulating B cells with the absence of lymphadenopathy and disease symptoms [28]. Majority of patients go through MBL state for 6 months to 7 years prior to development of CLL [29]. Most MBL clones show the same genotype as CLL and recently some driver mutations within subclones for development of CLL have been suggested [30].

CLL is a dynamic disease with clonal architecture that gets changed over time and is also influenced by a selection process. The first oncogenic transformation from MBL to CLL is not known but the presence of oligoclonal B cell clones in MBL and CLL indicate the probability of generating B cell clones may have been acquired as back as self renewing hematopoietic stem (HSC) cell with germ line IGH genes [31]. Next generation sequencing (NGS) has made it possible to detect mutations in smaller subclones. The presence of mutations in subclones may lead to heterogeneity and different response to therapies in CLL [32, 33]. However, more studies are needed to understand the exact mechanism of formation of mutated subclones.

The BCR (B-cell receptor) is present on the surfaces of both normal and malignant B cells and composed of antigen-specific surface membrane immunoglobulin M (sIgM) and the CD79a and CD79b heterodimers. Somatically mutated immunoglobulin (Ig) variable genes are present in CLL cells. The number of somatic hypermutations (SHM) in the heavy chain variable region of immunoglobulin genes (IGHV) may determine two CLL subgroups as mutated and unmutated CLL. In unmutated CLL, there is 98% or more sequence similarity of the IGHV gene with corresponding germline sequence while less than 98% for mutated CLL [34, 35]. About half of the CLL patients belong to each group with different prognosis associated with IGHV mutational status [34-36].

CLL cells may originate from antigen-experienced B cells as there are similarities between normal memory B cell and the CLL cell as well as mutated and unmutated CLL cells [37]. This observation may suggest the involvement of antigen-driven process in the pathogenesis of CLL. The presence or absence of somatic mutations in the stereotyped receptors may suggest the contribution of common set of antigens to CLL pathogenesis [38-40]. The stereotyped receptors are more common in unmutated than mutated CLL patients and is associated to affect prognosis as demonstrated for IGHV3-21 subgroup [38, 39, 41]. Many exogenous as well as autoantigens have been suggested to be involved in the malignant transformation process.

The BCR signaling in CLL may be induced independent of antigens. About 50% of CLL patients have self-recognizing epitopes that result in the autonomous and constitutive activation of BCRs [42]. Basal signaling has been shown to be higher in CLL cells as compared to normal B cells and it was particularly elevated in IGHV mutated CLL cells [43]. The absence of activating mutations in BCR genes in CLL may suggest the involvement of both autonomous and external antigen in the stimulation of receptors [44].

1.5 Diagnosis of CLL

The diagnosis of CLL is carried out by blood count, analyzing blood smears and immune phenotype from blood samples. The presence of $5x10^9$/L or more B-lymphocytes in the peripheral blood is the prerequisite of CLL [45]. CLL cells are characterized by weak sIg levels and express CD5, B-cell antigen CD19, CD20 and CD23. The levels of CD20 and CD79b in CLL cells are very low as compared to normal B cells. A bone marrow aspirate is recommended in cases with cytopenia to differentiate between bone marrow infiltration or autoimmune cause. Analysis of bone marrow is also recommended before treatment initiation [45].

Most patients have no symptoms at the time of diagnosis. The most common symptoms are night sweat, fatigue, weight loss, lymph node swelling, hepatosplenomegaly and other symptoms due to bone marrow failure. Patients with CLL may be accompanied by autoimmune complications and recurrent infections [1, 45-47].

1.5.1 Staging systems

CLL is categorized into different prognostic groups by a system based on clinical staging introduced by Rai et al [46, 48] and Binet et al [47]. Clinical stages of CLL are defined by the presence of anemia/thrombocytopenia, number of regions with enlarged lymph nodes, hepatomegaly and splenomegaly. The platelet count to define Binet stage C and Rai stage IV is the same at 100,000 platelets/μl [45] but defining anemia based on hemoglobin level is different for the two systems with cut-off of 100 g/L for a Binet stage C and 110 g/L for Rai stage III.

Both staging systems estimate the prognosis of patient; the median survival for Binet stage A or Rai stage 0 (low risk) is more than 10 years, 5-7 years for Binet stage B or Rai stage I-II (intermediate risk) and 2-3.5 years for Binet stage C or Rai stage III-IV (high risk), the patients in high risk group are accompanied by anemia and thrombocytopenia [46-

[48]. A limitation of the Rai and Binet staging systems is that they are unable to predict disease progression at an early stage (Binet A or Rai 0).

1.5.2 Morphology of CLL cells

The staging systems are based on the morphology of CLL cells in the blood and bone marrow, mostly of mature appearance. Atypical morphology of CLL cells in a blood smear and a high number of pro-lymphocytes or diffuse infiltration in the bone marrow are linked to a poor prognosis [46, 47, 49, 50]. On the other hand, high number of smudge cells may indicate a good prognosis [51].

1.5.3 Lymphocyte doubling time

Lymphocyte doubling time (LDT) is the time period during which the absolute lymphocytes count gets double indicating the activity of the disease. LDT longer than 12 months is related to increased progression free and overall survival [52, 53]. On the other hand LDT of less than six months indicates active disease and should be recommended for treatment but only when lymphocyte count is above 30,000 lymphocytes/μl [45]. The shortcoming of using LTD is that once high lymphocytes counts have been reached and some patients with stable absolute lymphocyte counts have still high CLL cell birth rates than 1% of the entire leukemia clone per day [5]. However, using LTD for disease assessment is simple and reliable.

1.5.4 Serum markers

There are three serum markers which act as important prognostic factors for CLL patients; serum β2-microglobulin (sβ2m), soluble CD23 (sCD23) and serum thymidine kinase (sTK). The advantage of serum markers is the simple way of blood sampling and reliability of assessments, but still they are not widely applied in Europe for CLL patient management.

High level of sβ2m in CLL patients relates to poor prognosis such as advanced disease stage, bone marrow infiltration and high tumor burden [54, 55]. The sβ2m levels are also correlated with the expression of other adverse prognostic factors as CD38 and zeta-associated protein (ZAP70) [56].

The cellular enzyme sTK is usually found in dividing cells and absent in non-dividing cells which serves as a useful marker for proliferation and is associated with disease progression and advanced stage [57, 58]. Elevated level of sTK has been shown to correlate with other prognostic factors such as LDT, ZAP70, cytogenetic abnormalities and CD38 in Binet stage A patients [59].

High levels of sCD23 correlated with tumor mass, short LDT, diffuse bone marrow infiltration, early stage disease progression and reduced survival [60, 61].

1.5.5 Cytogenetic abnormalities

Some of the genomic aberrations as prognostic markers used in CLL are shown in Figure 2.

The most common cytogenetic abnormalities which can be used as prognostic factors are deletions of the long arm of chromosome 13 (del (13q)), long arm of chromosome 11 (del (11q)) and short arm of chromosome 17 (del (17p)) as well as trisomy 12q. These cytogenetic abnormalities can be assessed by interphase fluorescence in situ hybridization (FISH). Patients with del (17p) have the worst prognosis (median overall survival of 32 months), followed by del (11q) (79 months), trisomy 12q (114 months) and normal diploid karyotype (111 months) whereas patients with del (13q) have the best prognosis with a median overall survival of 133 months [62]. Patients with del (11q) and del (17p) are also related to other adverse prognostic factors such as IGHV mutational status, advanced stage and rapid disease progression, poor response to treatment and a short survival [63, 64]. The cytogenetic abnormalities have an important role for treatment decisions.

A TP53 mutation in CLL is related to short time to treatment and short overall survival (OS) regardless of 17p deletion [65-67]. TP53 mutations in CLL patients with mutated IGHV have demonstrated better survival than those with unmutated IGHV gene. TP53 mutations and 17p deletion have been associated with poor response to chemotherapy [65, 68] and are thus clinically important markers that should be assessed before every new line of treatment [45].

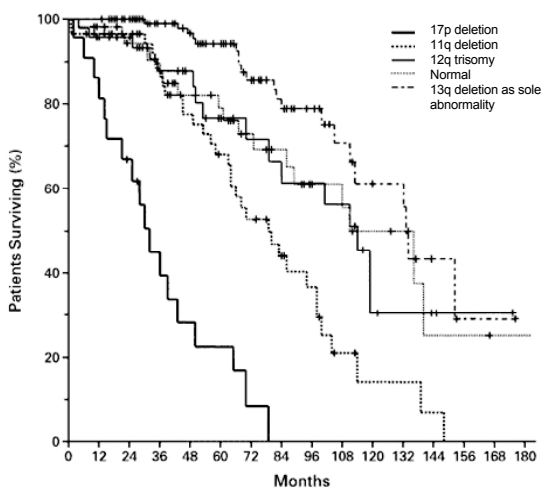

Figure 2. **Probability of survival from the date of diagnosis among patients in the five genetic categories**.

The median survival times for the groups with 17p deletion, 11q deletion, 12q trisomy, normal karyotype, and 13q deletion as the sole abnormality were 32, 79, 114, 111, and 133 months, respectively.

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1.5.6 IGHV mutation status

During development of normal B-lymphocytes, a number of changes take place in immunoglobulin genes including mutation of IGHV genes. CLL patients with somatic mutations in IGHV genes are usually associated with del (13q) and Binet stage A and have good prognosis [63, 64]. On the other hand, patients with un-mutated IGHV are more likely to be in advanced disease stage, to have an aggressive course and more prone to get cytogenetic aberrations [69, 70]. CLL patients with un-mutated IGHV genes have overall survival of approximately 8 years while patients with mutated IGHV gene have around 25 years. The IGHV mutational status for patients has been shown to be closely correlated to

the expression of ZAP70 and CD38 [35, 71]. The mutational status of CLL patients (whether mutated or unmutated) remains unchanged during disease evolution.

1.5.7 ZAP-70

ZAP-70 (Zeta-chain-associated protein kinase 70), a member of Syk-ZAP-70 protein tyrosine kinase family, normally expressed in T and NK cells and plays key role in initiation of T-cell signaling. ZAP70 protein is also expressed on CLL cells but not on normal B cells and its expression remains constant over the course of disease. The expression of ZAP70 may be used in predicting the treatment free survival and overall survival outcomes. The expression of ZAP70 is considered superior compared to expression of CD38 or IGHV mutation status in predicting time to first treatment even in patients with early stage asymptomatic disease [72]. The concordance between ZAP70 and IGHV mutational status lies in the range of 77-95%. A 5.5-fold higher ZAP70 expression was noted in those with un-mutated IGHV status compared to those with mutated IGHV [73]. However the expression of ZAP70 might not provide any further prognostic information when used in addition to CD38 and IGHV mutational status and is thus seldom used in routine health care.

1.5.8 CD38

CD38 is a transmembrane protein receptor that can function in cell adhesion, signal transduction and may act as an enzyme. Patients with elevated level of CD38 expression on the CLL cells are associated with adverse prognosis including advanced stage, hepatomegaly, elevated sβ2m, high-risk cytogenetics, short LDT, poor response to therapy and short overall survival [49]. CLL cells with un-mutated IGHV genes express high levels of CD38 but the association between CD38 expression and IGHV mutational status is not absolute. CD38 expression may vary during the course of the disease [49]. CD38 is thus an important prognostic factor but is not a perfect surrogate marker for IGHV mutation status and not recommended by Swedish CLL guidelines.

1.5.9 Other prognostic markers

There are a few other molecular markers that have recently been suggested as prognostic markers. Among them are NOTCH1, SF3B1, BIRC3, MYD88, lipoprotein lipase (LPL) and CLL up-regulated gene 1 (CLLU1) as well as miRNA.

Mutated NOTCH1 is associated with a shorter time to treatment and survival as well as resistance to chemotherapy [74-77]. However, mutated NOTCH1 was shown to be not an independent prognostic marker for time to treatment, PFS or OS [66, 68].

Patients with mutated MYD88 and 13q deletion may have better OS compared to wild-type patients [74, 78]. Mutated SF3B1 is also related to poor prognosis and advanced stage disease [68, 79, 80]. However, a recent study showed that mutated SF3B1 was only an independent marker for shorter PFS but not OS [68]. After TP53 mutation and IGHV mutational status, SF3B1 seems to be a strong prognostic marker in patients receiving first line treatment [68].

Mutated BIRC3 has been associated with short OS and resistance to chemotherapy [81]. Mutations in BIRC3 have been also linked to fludarabine refractoriness and are observed in 40% of fludarabine resistant CLL with TP53 wild type [78, 81].

The expression of LPL has been associated with poor prognosis and might be useful in evaluation of low risk patients [82].

Overexpression of CLLU1 has been associated with poor prognosis and advanced stage disease and shorter time to treatment but it is neither a predictive nor an independent prognostic marker [82]. CLLU1 may be a useful prognostic marker to identify younger patients who need treatment. miRNA expression has been shown to be different between prognostic subgroups and therefore may be used as prognostic markers [82].

Further studies are needed to identify novel markers that could predict the response to therapy.

1.6 Microenvironment

The tumor microenvironment in the lymph nodes and bone marrow is essential for survival of CLL cells. The CLL microenvironment is illustrated in Figure 3. Microenvironment provides a niche for CLL cells to interact and cross talk with cytokines, chemokine and other accessory cells. CLL cells isolated from patients undergo spontaneous apoptosis that could be prevented by culturing with cytokines, bone marrow stromal cells and accessory cells [83]. This implies that CLL cells need a favorable environment and an external stimulus for their proliferation and survival.

Proliferation of CLL cells occurs predominantly in lymph nodes [84]. Proliferation centers (PC) also known as pseudofollicles are the tissues sites in lymph nodes and bone marrow where proliferation takes place. PC presents an environment where CLL cells interact with antigens and accessory cells (mesenchymal stromal cells (MSC), monocyte derived nurse-like cells (NLC) as well T cells) [44, 85].

Adhesion molecules mediate migration of CLL cells to secondary lymphoid organs. Integrins are heterodimeric glycoproteins that mediate cell-cell and cell-matrix adhesion. High levels of integrin CD49d and CD38 in CLL are associated with potential for higher migration towards CXCL12 chemokine [44]. CLL cells from secondary lymphoid organs are then guided by chemokines into the tissues by a phenomenon known as homing [85]. Highly expressed chemokine receptor CXCR4 on CLL cells is attracted by a chemokine CXCL12 resulting in chemotaxis and migration [86]. The chemokine CXCL12 is secreted by MSC. Stimulated CXCR4 activates PI3K and MAPK pathways resulting survival effects for CLL cells [87]. PI3K, BTK and SYK inhibitors can inhibit the survival and stimulatory effects induced by CXCR4 in CLL cells [88-90]. Stimulated CLL cells secrete CCL3/4 chemokines, which attract monocytes and T cells [91]. High level of CCL3/4 in plasma has been associated with poor outcome in CLL [92]. BMSCs also appear to activate lymphoid-proto oncogene TCL1 (T-cell leukemia/lymphoma protein 1) and subsequent activation of NFκB and protein kinase C (PKC) [85].

NLCs can be found in spleen and lymphoid tissues but their mechanism of differentiation from blood monocytes is not clear. In vitro, CLL cells stimulate NLC differentiation by activating TLR-9 (toll-like receptor 9) [44]. NLCs attract CLL cells by secreting CXCL12 and CXCL13 and guide them to be in close contact with CXCR4 and

CXCR5. CXCL12 acts as co-stimulatory factor for CD4+ T cells in CLL cells [93]. NLCs also activate BCR signaling and NFκB pathways. NLC also expresses CD31, which by binding to CD38 on CLL cells stimulates survival [44]. CD4+ T cells secrete CD40L and IL-4 which are important for proliferation and survival of B cells via NFκB [44]. CD40L binding to CD40 may also activate PI3K pathway, which is important for survival and proliferation of CLL cells.

NFκB pathway can be activated by several ways including the stimulation of TLR and CD40 as well as BCR signaling. BMSC, NLC, cytokines, chemokines and activated CD4+ T cells provide a very favorable niche for survival of CLL cells and also protect them against immune-chemotherapy ensuing resistance to drugs [85, 93, 94]. Disruption of the interactions and cross talks in microenvironment may present a very good therapeutic option for the treatment of CLL.

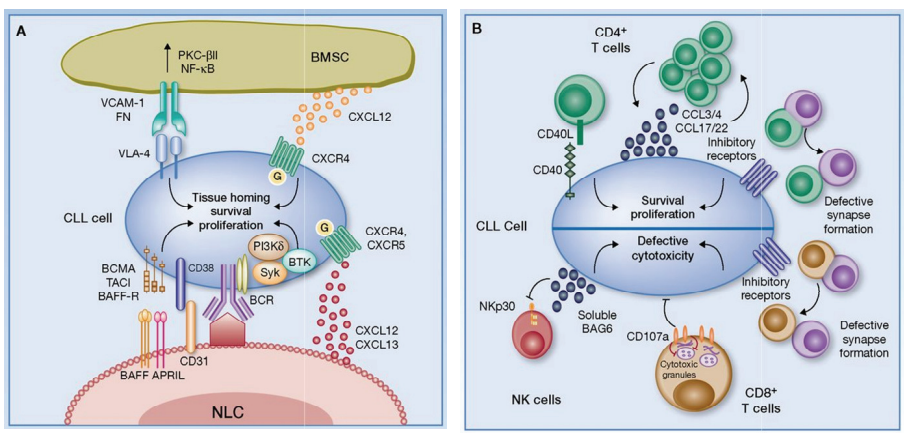

Figure 3. The CLL microenvironment.

A) In the CLL microenvironment, CLL cells interact with bone marrow stromal cells (BMSC) and nurse like cells (NLC) through adhesion molecules and chemokine receptors, expressed on CLL cells. These interactions, in addition to B-cell receptor engagement, promote CLL survival, proliferation, and homing to tissues.

B) $CD4^+$ T cells are recruited into the tissue microenvironment by CLL cell–derived chemokines, including CCL3 and CCL4, to support CLL cell survival and proliferation. Inhibitory receptors expressed by CLL cells induce defective immune synapse formation between CLL and T cells. Cytotoxic granule secretion by $CD8^+$ T cells is also defective, and production of soluble factors by CLL cells suppresses NK cell cytotoxicity, favoring immune evasion of CLL cells.

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1.7 Treatment

CLL is still an incurable disease but various treatment approaches are used to control symptoms and prolong survival of patients. With the application of Rai and Binet staging system advantages of various interventions can be acknowledged [46]. During last two decades, the discovery of increasing number of prognostic markers has led to a better understanding of the heterogeneity of the disease. Risk stratification is helpful in deciding a particular treatment approach [95]. Depending upon the clinical situation, different options of

treatments are considered, which may include the use of conventional agents or other recently developed combination approaches [96-99]. Recently, a number of new agents have shown promising clinical activity and some have been approved for the treatment of CLL. The increasing number of new agents offers the future potential to achieve long-term control and stabilization of the disease.

1.7.1 Cytotoxic agents

1.7.1.1 Alkylating agents

Alkylating agents, the initial therapy for CLL, were successful to control clinical manifestations and reduce CLL cell number in blood. Chlorambucil was the gold standard for CLL therapy for a long time and even today is an appropriate but moderately effective option for elderly CLL patients with co-morbidities. The advantages of chlorambucil are low cost, moderate side effects and its convenient oral use. However, this treatment has relatively low response rates when used as monotherapy (ORR up 30-70% and CR only 0-7%) and a median progression free survival (PFS) of 8-18 months [96, 100, 101]. With combination as alkylating agent based chemotherapy, significant numbers of complete responders were observed in various clinical trials but the disease was never cured as the patients eventually relapsed at some point [102-105]. Phase II studies of chlorambucil in combination with rituximab in elderly patients showed better ORR (84% and 82.4%), CR rates (10% and 16.5%) and PFS (23.5 and 34.7 months) than previously achieved with chlorambucil as monotherapy [100, 106]. The combination was well tolerated with comparable toxicities to chlorambucil alone. A phase III study combining obinutuzumab, a CD20 mAb, with chlorambucil in untreated CLL patients showed significantly better response with CR rates (20.7% vs. 7.0%), more minimal residual disease (MRD) negative (19.5% vs. 2.6%) and PFS (median PFS 27.6 vs. 16.3) compared to those treated with rituximab-chlorambucil combination [107]. Chlorambucil in combination with ofatumumab showed better clinical activity with manageable side effects in CLL patients who were not suitable for fludarabine treatment [108].

1.7.1.2 Purine analogs

Purine analogs are cytotoxic agents with established activity in indolent lymphoid malignancies. Deoxycoformycin also known as pentostatin was the first purine analog to enter clinical trials with promising results [109]. Pentostatin in combination with an alkylating agent (cyclophosphamide) and a CD-20 monoclonal antibody (PCR) is effective but not widely used in health care front-line therapy [110, 111]. The other two used purine analogs in CLL are fludarabine and cladribine. Fludarabine is the most studied purine analog in CLL. Fludarabine induced more remission and more complete remissions (CR) (7%-40%) than other conventional chemotherapies but as a single agent it did not improve overall survival (OS) [112-115]. Fludarabine in combination with cyclophosphamide [116] served as the basis and later combined with rituximab (FCR), which is now considered as the golden standard frontline therapy in CLL [116, 117]. FCR has shown ORR of about 90% and CR rates of 44% and has prolonged PFS of 57.9 months as well as improved OS versus FC alone [118, 119]. Even though FCR has shown better response rates and improved survival

but it is less well tolerated in patients older than 70 years of age [120, 121]. Therefore FCR is not a first line treatment for all patients as it is too toxic for elderly and fragile patients who constitute a large fraction of all CLL cases. Similarly, cladribine another purine analog showed promising results with complete and partial remissions of 38% and 37% respectively in untreated patients but did not prolong overall survival when used as monotherapy [122, 123]. It is today mainly used in hairy cell leukemia. Bendamustine was used in randomized trial resulting improved response but more with toxicity and no overall survival [96]. Bendamustine in combination with rituximab (BR) is an option for patients with borderline fitness status. BR has demonstrated response rates (ORR 88%, CR 23.1%) but with less neutropenia (19.7% vs. 34%) but severe infections (7.7% vs. 25%) when compared to FCR [124].

Even with more aggressive approach such as FCR, the eradication of the disease at the molecular stage is still not possible and side effects like immunosuppression with resultant infections and possibly increased risk of secondary malignancies have been reported [125, 126].

1.7.2 Allogeneic stem cell transplantation

Allogeneic stem cell transplantation has been employed for around three decades for the treatment of CLL and updates confirm its efficiency in the long-term control or even eradication of disease [127]. Recently, panel of experts has indicated that allogeneic stem cell transplantation is a reasonable treatment option for younger patients, non responsive to standard therapies and patients with p53 abnormalities who need treatment. However, its role has been questioned in patients who continue to respond to the new BTK inhibitor ibrutinib.

1.7.3 Monoclonal antibody therapies

The most specific treatment approach is to target structures and molecules that are limited only to malignant cells. The presence of immunoglobulin on B cells in non-Hodgkin lymphoma showed the practicability of this approach [128]. In this approach, using unique idiotypes convinced scientists to focus on producing antibodies utilizing less specific but more generalized tumor antigens as targets. Mainly unconjugated antibodies have been in use and the mechanisms of tumor cells cytotoxicity are complement dependent cytotoxicity (CDC), antibody-dependent cell cytotoxicity (ADCC) and signaling apoptotic pathways. In CLL, antibodies against target antigens CD20 and CD52 are used [129, 130]. In Table 1, a number of therapeutic monoclonal antibodies for various malignancies have been summarized [131].

Table 1. Therapeutic monoclonal antibodies targeting non-tyrosine kinase molecules.

Name	Trade name	Target	Antibody format	Targeted cancer (examples)
Alemtuzumab	Campath	CD52	Humanized IgG1	CLL, CTCL, TCL
Brentuximab vedotin (ADC)	Adcetris	CD30	Chimeric IgG1	Systemic anaplastic large cell lymphoma, Hodgkin's lymphoma
Catumaxomab	Removab	CD3 and EpCAM	Mouse biospecific mAb against	Malignant ascites generated by an EpCAM-positive tumor malignant lung cancer
Dacetuzumab	-	CD40	Humanized IgG1	NHL
Denosumab	Prolia	RANKL	Humanized IgG2	Non-metastatic prostate cancer
Gemtuzumab ozogamicin	Mylotarg	CD33	Humanized IgG4	AML
Ibritumomab tiuxetan (ADC)	Zevalin	CD20	Mouse IgG1	B-cell NHL
Ipilimumab	Yervoy	CTLA-4	Humanized IgG1	Metastatic melanoma
(MDX-010/ MDX-101)				
Labetuzumab	CEA-CIDE	CEA	Humanized IgG1	Colorectal carcinoma
Lumiliximab	-	CD23	Humanized IgG1	CLL
Milatuzumab	-	CD74	Humanized IgG1	Multiple myeloma, NHL, CLL
Obinutuzumab	Gazyva	CD20	Humanized IgG1	CLL
Ofatumumab (HuMax-CD20)	Arzerra	CD20	Humanized IgG1	CLL
Pembrolizumab	Keytruda	PD-1	Humanized IgG4	Metastatic melanoma
Rituximab	Rituxan	CD20	Humanized IgG1	B-cell NHL, CLL
	(MabThera)			
Tositumomab	Bexxar	CD20	Mouse IgG2	B-cell NHL, FL
Veltuzumab	-	CD20	Humanized IgG1	B-cell NHL, CLL
Vivatuxin	-	Intracellular DNA-associated antigens	Radiolabeled chimeric IgG1κ	Malignant lung cancer
^{90}Y-Ibritumomab tiuxetan/	Zevalin	CD20	^{90}Y labelled Anti-, deliver Y-90, murine IgG1	Follicular B-cell NHL

CLL: chronic lymphocytic leukemia, CTCL: cutaneous T-cell lymphoma, TCL: T-cell lymphoma, ADC: antibody-drug conjugate, EpCAM: epithelial cell adhesion molecule, NHL: non-Hodgkin's lymphoma, AML: Acute myeloid leukemia, CTLA-4: cytotoxic T-lymphocyte antigen-4, CLL: chronic lymphocytic leukemia, PD-1: programmed cell death 1 receptor, FL: follicular lymphoma.

1.7.3.1 Rituximab

Rituximab is directed against CD20 epitope, was the first monoclonal antibody approved for the treatment of lymphoproliferative disorders. It showed modest results in CLL after completions of phase I studies as compared to its use in non-Hodgkin lymphoma as single agent therapy [132]. Low expression of CD20 was perceived to be the primary reason for its weak activity as single agent [133]. Modulation of CD20 by bortezomib, epigenetic alteration of gene expression and use of higher doses may improve rituximab efficacy [134-136]. However, as mentioned above, rituximab (R) in combination with fludarabine and cyclophosphamide (FC) has become the standard front-line treatment in CLL after studies showing significant activity [118]. Rituximab has proved to be effective in treating complications of CLL including autoimmune hemolytic anemia (AIHA) and pure red cell aplasia (PRCA) [137-139]. New novel antibodies against CD20 challenging rituximab are now

being explored [140-142]. Rituximab maintenance after chemoimmunotherapy induction in CLL showed feasibility [143].

1.7.3.2 Ofatumumab

Ofatumumab is fully humanized antibody recognizing an epitope different than the one recognized by rituximab on CD20 molecules expressed on human B cells. Ofatumumab has stronger binding affinity to CD20 and results in more cytotoxicity due to greater complement-dependent cytotoxicity and similar antibody-dependent cellular cytotoxicity activity compared to rituximab, specially in cells with low CD20 expression [144]. Ofatumumab has shown activity in patients who are refractory to fludarabine and alemtuzumab [145, 146]. Ofatumumab in combination with fludarabine and cyclophosphamide shows significant activity in the initial treatment of patients with high risk [147] but has not become standard therapy. However, ofatumumab in combination with bendamustine was recently approved by EMA based on promising phase II results [148]. However, the overall activity of ofatumumab in patients with 17p deletion and bulky disease has been shown to be modest. Recent data indicate a potential role for ofatumumab as a maintenance therapy for relapsed CLL with no unexpected toxicities [149]. A recent retrospective follow up of ofatumumab in relapsed/refractory CLL patients treated in a community-based setting showed an ORR of only 23% and OS of 12 months [150]. A phase III study of ofatumumab in combination with chlorambucil demonstrated improved clinical results in patients with CLL who had not received prior therapy and who were considered inappropriate for fludarabine based therapy [108].

1.7.3.3 Alemtuzumab

Alemtuzumab is fully humanized monoclonal antibody directed against CD52 antigens expressed on lymphocytes as well as monocytes, granulocytes, eosinophil and macrophages. Alemtuzumab, as single agent treatment has produced response rates of 33% to 53% with duration of response ranging from 8.7 to 15.4 months in advanced stage CLL, who were previously treated with alkylating agent and had not responded or relapsed after second line fludarabine treatment [130, 151, 152]. Its mechanisms of cytotoxicity are ADCC, CDC and direct cell death and works independently of p53 abnormality seen in resistant CLL [153]. Alemtuzumab has shown effective responses in high-risk patients with deletion of 17p, 11q and p53 mutation [153, 154]. Therefore, alemtuzumab has been a reasonable single-agent treatment option for patients with poor prognostic (17p-) features. The antibody is today available for CLL patients at no cost through a named patient program after having been re-launched under a new name (Lemtrada™) for multiple sclerosis.

1.7.3.4 Other monoclonal antibodies and immunoconjugates

Obinutuzumab (GA101) is humanized and glycoengineered monoclonal antibody against CD20 which differs in its binding properties from rituximab and ofatumumab. It showed higher rates of apoptosis in malignant B cells in vitro as compared to rituximab [155]. The humanization and glycoengineering lead to higher affinity binding to CD20 type II epitope, increased ADCC, low CDC and increased direct cell death induction [156]. A phase I study with obinutuzumab in 13 CLL patients showed promising results with reported side effects including neutropenia, thrombocytopenia and tumor lysis syndrome [156]. Combination of

obinutuzumab with chlorambucil as compared to chlorambucil alone prolonged overall survival [107]. Obinutuzumab+chlorambucil as compared to rituximab+chlorambucil resulted in prolonged PFS and higher rates of CR (20.7% vs. 7.0%), though infusion related reactions and neutropenia were more common in obinutuzumab+chlorambucil but the risk of infection was not increased.

Other monoclonal antibodies that have shown some activity include HU1D10, humanized antibody against a variant epitope on HLA-DR B chain [157]. HU1D10 has demonstrated activity in clinical studies of CLL [158]. Examples of other monoclonal antibodies with potential activity include lumiliximab directed against CD23 induced ADCC and apoptosis in CLL cells [159], TRU-016 (otlertuzumab) directed against CD37 with phase I clinical trials [160], XmAb5574 against CD19 and epratuzumab against CD 22 [161].

Attempts are made to augment the therapeutic efficacy of monoclonal antibodies by conjugation (immunoconjugates) with other reagent like toxins. LMB-2, a conjugate using a pseudomonas exotoxin, directed against CD25 [162] and BL-22 against CD-22 have demonstrated significant responses in CLL [163]. Other antibodies under investigation are milatuzumab (CD74), cirmtuzumab (ROR1) and samalizumab (CD200).

1.7.4 BCR signaling and other kinase inhibitors

Many small therapeutic molecules are being explored currently in various types of cancer, and some of them are described below (Table 2) [131].

Table 2. Current inhibitors approved for cancer treatment.

Name	Trade name	Mol. mass (g/mol)	Target molecule/s	FDA approved or in phase III clinical trial for treatment
Anastrozole	Arimidex	293.366	Aromatase inhibitor	Breast cancer
Alitretinoin	Panretin	300.435	Retinoic acid receptors retinoid X receptors	Cutaneous lesions in patients with AIDS-related kaposi sarcoma
Aflibercept	Zaltrap	96900	VEGF	Metastatic colorectal cancer
Belinostat	ND	318.348	HDACs	T-cell lymphomas
Bexarotene	Targretin	348.478	Retinoic acid receptors	CTCL
Bortezomib	Velcade	384.237	Proteasome inhibitors	Multiple myeloma, MCL
Carfilzomib	Kyprolis	719.91	Proteasome inhibitors	Multiple myeloma
Deforolimus	ND	990.2	mTOR	Advanced soft tissue, bone sarcoma
Denileukin diftitox	Ontak	57647.3	IL-2 receptors	CTCL
Entinostat	ND	376.4	HDACs	Breast cancer, Hodgkin's lymphoma, lung cancer
Everolimus	Afinitor	958.224	mTOR	Advanced kidney cancer, subependymal giant cell astrocytoma, MBC, pancreatic neuroendocrine tumors, advanced renal cell carcinoma
Exemestane	Aromasin	296.403	Aromatase inhibitor	Breast cancer
Ibrutinib	Imbruvica	440.49	Btk (IMiD agent)	CLL, MCL, DLBCL
Idelalisib	Zydelig	415.42	PI3K (P110δ)	CLL, Follicular lymphoma, SLL
Iniparib	ND	292.03	PARP	Glioblastoma
Lasofoxifene	Fablyn	563.64	ER	ER-positive breast cancer
Lenalidomide	Revlimid	259.261	IMiD agent	Multiple myeloma, CLL, MCL
Letrozole	Femara	285.303	Aromatase inhibitor	Breast cancer
Mocetinostat	ND	396.44	HDACs	Follicular lymphoma, Hodgkin's lymphoma, AML
Olaparib	AZD-2281	435.08	PARP	Ovarian, breast, prostate cancers
Pladienolide	ND	536.7	spliceosome	Gastric cancer,
panobinostat	ND	349.426	HDACs	Hodgkin's Lymphoma, CTCL
Pralatrexate	Folotyn	477.47	Antifolate	TCL
Raloxifene	Evista	473.584	ER	ER-positive breast cancer
Romidepsin	Istodax	540.695	HDACs	CTCL
Rucaparib	ND	323.36	PARP	Ovarian, breast, prostate cancers
Sirolimus	Rapamune	914.172	mTOR	Hepatocellular carcinoma
Tamoxifen	Nolvadex/Istubal/ Valodex	563.638	ER	ER-positive breast cancer
Temsirolimus	Torisel	1030.28	mTOR	Renal cell carcinoma
Toremifene	Fareston	405.959	ER	ER-positive breast cancer, prostate cancer
Tretinoin	Vesanoid	300.4412	Retinoic acid receptors	Acute promyelocytic leukemia
Veliparib	ND	244.29	PARP	Melanoma, breast cancer, NSCLC
Vorinostat	Zolinza	264.32	HDACs	CTCL

AIDS: Acquired immunodeficiency syndrome, VEGFR: vascular endothelial growth factor receptor, HDAC: histone deacetylase, CTCL: cutaneous T-cell lymphoma, MCL: mantle cell lymphoma, mTOR: mammalian target of rapamycin, DLBCL: diffused large B-cell lymphoma, ND: not determined, AML: Acute myeloid leukemia, TCL: T-cell lymphoma, ER: estrogen receptor, PARP: poly ADP ribose polymerase, NSCLC; non-small cell lung carcinoma, SLL; small lymphocytic leukemia.

1.7.4.1 Idelalisib (CAL-101)

PI3K pathway regulates cellular functions related to oncogenesis. PI3K p110δ isoform plays an important role in the proliferation and survival of B cells [164]. In CLL, a PI3K pathway has been shown to be constitutively activated and dependent on PI3K p110δ isoform [88]. Idelalisib has been approved by FDA and EMA for the treatment of relapsed CLL and follicular lymphoma (FL). Idelalisib is a reversible selective inhibitor of PI3Kδ isoform that promotes apoptosis in primary CLL cells in time and dose-dependent manner without affecting normal T cells or natural killer (NK) cells [165]. Idelalisib induces caspase-dependent apoptosis, inhibits BCR survival signals and protective effect of stromal cells and with down-regulation of chemokine and cytokine secretion. In a phase I clinical trial, idelalisib showed reasonable toxicity with favorable clinical activity (lymph node regression) in heavily pretreated and high risk CLL patients [166]. 81% of the patients showed lymph node response. Side effects like fatigue, diarrhea, rash and respiratory tract infections were observed. In relapsed patients, idelalisib monotherapy achieved an ORR of 54% and PFS of 5 months in those with TP53 mutations to 72% and 41 months those without TP53 mutation [167]. In a phase 3 study, the efficacy and safety of idelalisib in combination with rituximab in relapsed CLL patients were assessed [168]. The combination of idelalisib and rituximab as compared to rituximab and placebo significantly improved progression-free survival, response rates and overall survival in relapsed patients who were not appropriate to undergo chemotherapy. Preclinical data suggests that idelalisib may be a treatment choice for patients with progressive disease undergoing ibrutinib treatment [169].

IPI-145 (duvelisib) is another orally available PI3K inhibitor against both δ and γ isoforms. IPI-145 showed activity in CLL patients with advanced stage disease in phase I study with reasonable toxicity. The ORR was similar for patients with and without TP53 mutations. Phase III study of IPI-145 in relapsed CLL patients is currently ongoing.

1.7.4.2 Ibrutinib (PCI-32765)

Bruton's tyrosine kinase (BTK) is involved in the activation of downstream survival signaling pathways such as NF-κB and MAPKs through Src family kinases. Ibrutinib is an orally active small molecule inhibiting BTK irreversibly that plays an important role in the transduction of BCR signaling [170]. This inhibitor has also been shown to inhibit the microenvironmental stimuli [171]. FDA approved Ibrutinib in the US for the treatment of mantle cell lymphoma (MCL) in November 2013 and for CLL in January 2014. Inhibition of BTK with ibrutinib induced apoptosis in B-cell lymphoma and CLL cells [171]. Ibrutinib showed significant activity in patients with relapsed CLL or other B –cell malignancies [172]. Phase 1b-2 multicenter study with ibrutinib as a single agent on relapsed or refractory CLL and small lymphocytic leukemia, with mostly high-risk disease, was conducted [173]. Side effects including diarrhea, fatigue, respiratory tract infection and nausea were noted. The response was independent of clinical and genetic risk factors including advanced stage disease. The ORR was 71% and mostly with PRs. At 26 months, the estimated PFS rate was 74% and the OS was 83%. Studies of combining ibrutinib with monoclonal antibodies and other agents are ongoing. A phase III randomized trial of relapsed/refractory CLL compared ibrutinib with ofatumumab [174]. Ibrutinib showed better responses as compared to ofatumumab (43% vs. 4%) and a significantly improved PFS and OS at a median follow up of 9.4 months. Ibrutinib monotherapy as a first line treatment produced an ORR of 71%

and 13% CR in untreated older patients [175]. Combination of ibrutinib and rituximab resulted in 95% ORR and 8% CR in patients with 17p deletion [176]. Three-year follow-up of previously treated and naïve CLL and SLL patients receiving ibrutinib as monotherapy showed improved responses and durable remissions [177]. Ibrutinib was well tolerated and toxicities with longer follow up diminished. In a recent study, Ibrutinib with chemoimmunotherapy showed responses. BR-ibrutinib demonstrated ORR of 93.3% including 16.7% CR whereas patients treated with ibrutinib-FCR exhibited CR [178]. Despite of these impressive responses with ibrutinib in CLL, minimal residual disease-negative status has not been achieved and a recent study has identified a mutation in BTK that results in an autonomous BCR signaling and could explain the resistance of these patients to ibrutinib [179]. Further studies on long-term follow up and utilizing combinations of different therapeutic agents with ibrutinib are currently undergoing.

1.7.4.3 Fostamatinib (R406)

Syk is another intracellular kinase of BCR signaling pathway affecting downstream proteins that are involved in the survival and proliferation of cell. Fostamatinib is a reversible inhibitor of Syk and induce apoptosis of CLL cells in vitro [90, 180]. Fostamatinib has shown modest clinical activity with minimal toxicity [181]. However, fostamatinib was found to have limited specificity to Syk and showed activities against other kinases too [182].

GS-9973 is another selective and orally available inhibitor of Syk that has been used in previously treated patients with CLL or lymphoma [183]. Another selective inhibitor of Syk is P505-15 that has shown activity in vitro as well as in vivo and enhances the activity of fludarabine in CLL [184].

1.7.4.4 Dasatinib (Sprycel)

Dasatinib is an oral broad-spectrum BCR/Abl and Src family kinase inhibitor approved for up-front therapy of chronic myelogenous leukemia (CML), also shows modest activity in CLL with myelosuppression as the main side effect [185, 186].

1.7.5 Bcl-2 inhibitors

The Bcl-2 family proteins are known to regulate apoptosis. The family comprises of Bcl-2, Bcl-xL and Mcl-1, they may induce or inhibit apoptosis [187, 188]. Bcl-2 is regarded as a key antiapoptotic protein and damage to Bcl-2 has been identified in several cancer and cause of resistance to cancer treatments [189-191]. Bcl-2 is up-regulated in CLL cells, possibly due to deletion of miRNA regulators [192].

1.7.5.1 Oblimersen sodium

The first such Bcl-2 inhibitor to enter clinical trial in CLL was oblimersen sodium that showed modest activity as a single agent with myelosuppression as a predominant side effect [193]. Oblimersen sodium has been used in combination with rituximab and cyclophosphamide, showing an increase in CR and PR rates [194]. Tumor lysis syndrome was reported when used as a single agent. Currently there are no reports on plans for their further development.

1.7.5.2 ABT-263 (Navitoclax)

ABT-263 is another agent that inhibits Bcl-2, Bcl-xL and Bcl-w, causing apoptosis in CLL cells [195]. ABT-263 demonstrated activity in fludarabine refractory patients in clinical trials [196]. This agent had however dose-limiting thrombocytopenia as a major side effect.

1.7.5.3 ABT-199 (GDC-0199)

ABT-199 is another specific Bcl-2 inhibitor with higher affinity for Bcl-2 than Bcl-xL [197]. Recently a phase I study of ABT-199 in relapsed patients showed an ORR of 77% with 23% CR and 59% PFS with two years [198]. Neutropenia was the most severe side effect but less thrombocytopenia was observed than with ABT-263. Diarrhea, nausea and fatigue were the non-hematological side effects affecting one third of patients. Recent data from phase Ib study of ABT-199 in combination with rituximab in relapsed patients demonstrated 84% objective responses, 36% CRs, MRD negativity and better tolerated toxicities [199]. Further studies of ABT-199 as a single agent as well as in combinations are currently underway.

1.7.5.4 Homoharringtonine

Homoharringtonine is an inhibitor of Mcl-1, an antiapoptotic protein of Bcl-2 family, which is upregulated in CLL cells. This inhibitor was originally developed for treatment of acute myeloid leukemia and currently used for the treatment of CML, has shown significant activity in CLL cells in vitro and will be entering to clinical trials [200].

1.7.6 CDK inhibitors

Cyclin-dependent kinases (CDK) are important regulatory proteins of cell cycle. CDKs can be targeted by inhibitors resulting in the induction of programmed cell death in CLL cells [201]. CDK inhibitors flavopiridol, dinaciclib, P1446A and CDK-73 have shown preclinical activity against CLL cells [202-204]. The CDK inhibitors appear to be active both as single agent as well in combinations in relapsed CLL cases but they may not be as effective and more toxic than ibrutinib [205].

1.7.7 HDAC inhibitors

Histone deacetylases (HDACs) have been shown to be overexpressed in CLL cells. Inhibition of HDAC has resulted in apoptosis of CLL cells by down regulation of Mcl-1 antiapoptotic protein [206]. HDAC inhibitors may also induce autophagy, which is pro-apoptotic mechanism in CLL. However, the HDAC inhibitors mocetinostat and vorinostat have not induced clinical responses in high-risk CLL patients [207-209].

1.7.8 Immunomodulation

The introduction of immunomodulating drugs represents a major progress in the treatment of multiple myeloma and myelodysplastic syndrome (MDS) [210, 211].

1.7.8.1 Lenalidomide

Lenalidomide is an analog of thalidomide, which in addition to its significant activity in multiple myeloma, has also demonstrated to downregulate the production of growth factors that are important in the survival and prolongation of CLL cells [212]. Lenalidomide showed activity in refractory or relapsed CLL patients with significant overall response rate but no evidence of specificity for the 17p resistant patients [213]. Tumor lysis syndrome and other life threatening significant toxicities were noticed at a dose of 25 mg/d for three weeks [214]. Combination of lenalidomide with monoclonal antibodies and chemotherapeutic agents deems another approach but high risk of toxicity remains a major challenge. Phase III study of lenalidomide as a potential maintenance agent in CLL is currently undergoing.

1.7.8.2 CAR-T cells

Another approach is to use the therapeutic effect of the T cell immune response in eliminating CLL cells [215, 216]. The patient's own T cells are modified to target the malignant cells. The T cells out of inhibitory microenvironment may be used to reverse the T-cell dysfunction and this has been achieved by using single-chain variable fragment from antibody fused with an internal signaling domain to form chimeric antigen receptor (CAR). CD19 is the most commonly targeted tumor associated antigen in CLL which is restricted only to B cells [217].

The first trial of three relapsed CLL patients with anti-CD19 CAR-T cells has shown impressive results [218]. In a recent update, 14 relapsed patients showed responses. The responses were independent of age, TP53 abnormalities and no dose: toxicity or dose: response relationships were noted at the dose ranges used. The expanded CAR-T cells were able to last for at least three years [219]. Fever, hypotension, deterioration in mental status occasionally and neurological changes were the observed side effects.

Many challenges are yet to be addressed including better efficacy, less toxicity and capability to produce large number of CAR-T cells. Reason for CAR-T cells failure is not completely understood but could be associated with down-regulation of tumor associated antigens and may be the inability of CAR-T cells to recycle effector functions owing to tumor environment [217]. Acceptable toxicity and producing CAR-T cells are the main targets in future to achieve.

2 RECEPTOR TYROSINE KINASES (RTKs)

RTKs are cell surface glycoproteins with enzymatic activity which regulate various important functions [220, 221]. The receptors play a vital role in cellular processes as differentiation, cell-cell interactions, survival, proliferation, metabolism, migration and signaling. RTKs are classified into 20 different receptor tyrosine kinase families which consist of 58 members [222] as shown in the Figure 4.

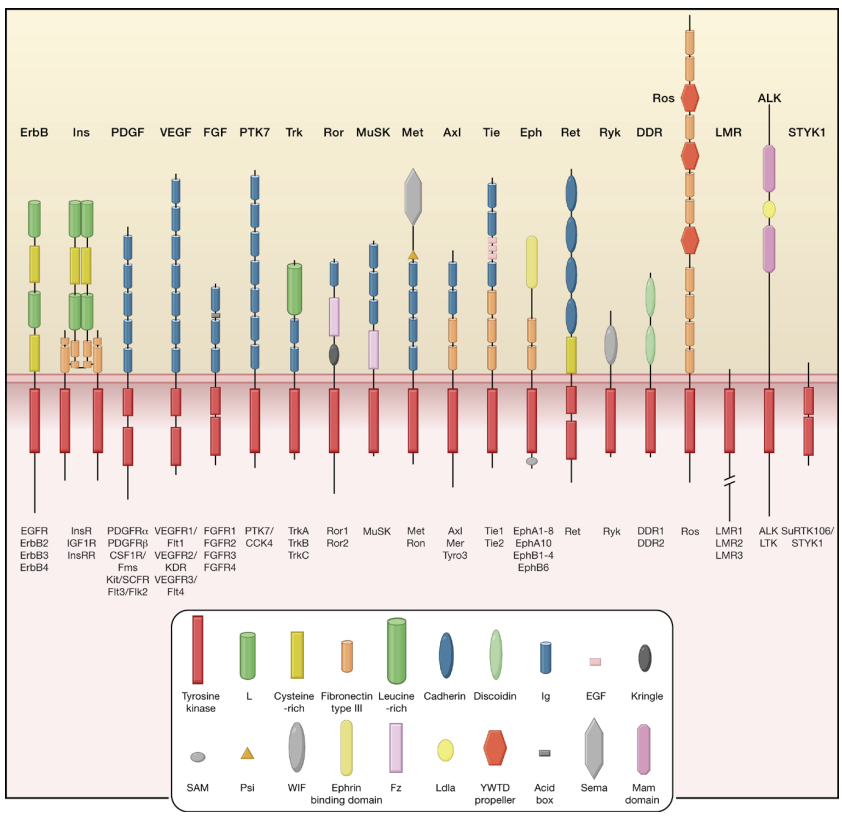

Figure 4. **Cell signaling by receptor tyrosine kinases.** Human receptor tyrosine kinases (RTKs) contain 20 subfamilies, shown here schematically with the family members listed beneath each receptor. Structural domains in the extracellular regions, identified by structure determination or sequence analysis, are marked according to the key. The intracellular domains are shown as red rectangles.

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The structure of RTKs consists of three parts, the extracellular part containing the ligand binding domain, a transmembrane part and an intracellular part containing the TK domain involved in downstream signaling.

Figure 5 shows RTKs expressed in CLL.

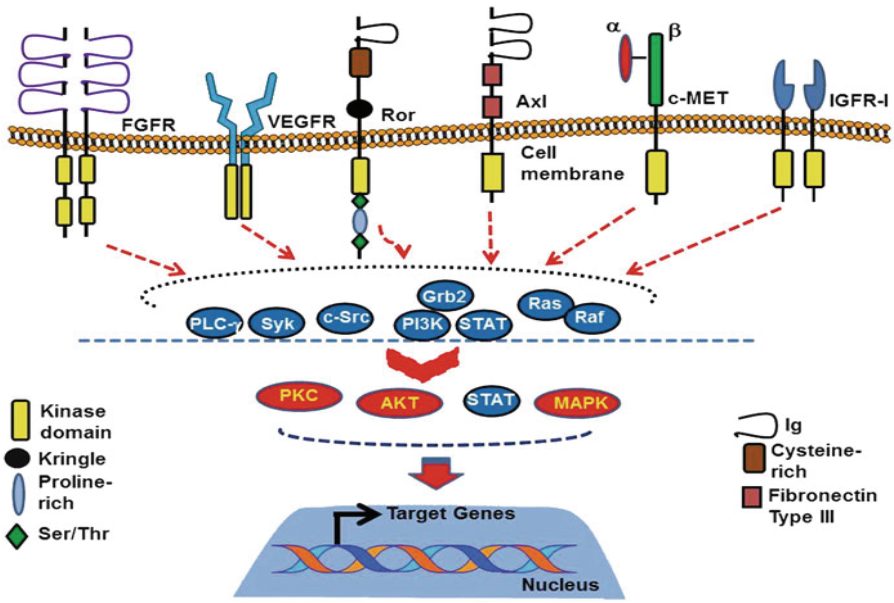

Figure 5. Tyrosine kinase network in CLL. Leukemic B cells from CLL patients express multiple RTKs, which may directly or indirectly participate in the "Cell Survival" signaling network. As most of the RTK signaling pathways share common intermediate signaling components, for example, Src and PI3K/AKT, we believe that in this "RTK Network," one RTK plays the role of the "Predominant RTK" while others play a secondary role, likely depending on the risk factors of the cells. In CLL, upon binding specific ligands, these RTKs may activate multiple signaling intermediates, including Src, Syk, Grb2/PI3K, Ras/Raf, and PLC-γ, leading to activation of the downstream effector signaling components: for example, AKT, MAPK, PKC, or STATs, which ultimately activates various specific target genes, resulting in cell survival, proliferation, and apoptosis resistance. However, expression of constitutively active RTKs in CLL B cells results in uncontrolled activation of the downstream signaling molecules, leading to increased cell survival and apoptotic resistance to therapeutic agents. One such constitutively active RTK in CLL we detected was Axl.

Springer and Advanced in Experimental Medicine and Biology; Chronic Lymphocytic Leukemia, Malek S, 2013, Chapter:" Critical Signal Transduction Pathways in CLL", Ghosh AK et al, pp 215-239, Figure 10.3. Reproduced with kind permission from Springer Science and Business Media.

Activation of the RTKs by ligand binding and dimerization of the extracellular part initiates signaling that regulates various cellular functions [223]. Aberrant expressions of different RTKs in various malignancies are summarized in Table 3 [224]. Mutations of RTKs or their overexpression have been described in many human cancers and are considered as therapeutic targets [220]. Abnormal RTK activation involved in carcinogenesis e.g. may result from overexpression, amplification, mutations or chromosomal translocation [225-227]. Some of the deregulated RTKs that have been identified in cancers as epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and receptor tyrosine kinase orphan receptor 1 (ROR1). These deregulated RTKs might be targeted by monoclonal antibodies against the extracellular part and by kinase inhibitors against the intracellular kinase-containing domain [228].

Table 3. Expression of oncogenic RTKs in cancer

RTK	Chromosome location	Normal function	Malfunction leading to overexpression	Malignancy (examples)
ROR1	1p31.3	ED, NSD	Unknown	CLL, ALL, AML, MCL, HCL, melanoma, prostate, lung, breast, pancreas, colon, ovarian, uterus cancers
ROR2	9q22.31	ED, NSD	Mutations	Melanoma, medulloblastoma, testicular cancer, gastrointestinal stromal tumor, hepatocellular carcinoma, colon cancer, renal cell carcinoma, osteosarcoma, prostate carcinoma
ALK	2p23	NSD	Translocation (t2:5) Overexpression	NSCLC, colorectal cancer, breast cancer, oesophageal cancer (squamous cell), renal cell cancer
ROS1	6q22	NSD	Deletion, inversion, translocation	NSCLC, cholangiocarcinoma, ovarian cancer, gastric cancer, colorectal cancer
RET	10q11.2	NSD	Mutations	NSCLC, medullary thyroid carcinoma
NTRK1 (TrkA)	1q21-22	Development and maturation of central and peripheral nervous system	Translocation	Colorectal cancer, papillary thyroid cancer, lung adenocarcinoma, breast cancer, oral squamous cell carcinoma
NTRK2 (TrkB)		Development and maturation of central and peripheral nervous system	Translocation	Neuroblastoma, astrocytoma, oral squamous cell carcinoma
NTRK3 (TrkC)	15q25	Development and maturation of central and peripheral nervous system	Translocation	Neuroblastoma, breast cancer
PDGFRA	4q12	ED	Mutations	Lung adenocarcinoma, gastrointestinal stromal tumors
PDGFRB	5q32	Regulation of embryonic development, cell proliferation, survival, differentiation, chemotaxis, migration and blood vessel development	Mutations	Gastrointestinal stromal tumors, glioblastoma
FGFR1	8p12	Regulation of embryonic development, cell proliferation, differentiation and migration	Mutations	Squamous cell lung cancer, breast cancer
FGFR2	10q26	ED	Mutations, overexpression	Squamous cell lung cancer, lung adenocarcinoma, breast cancer, thyroid cancer, prostate cancer, cholangiocarcinoma, astrocytoma
FGFR3	4p16.3	Normal skeleton development	Mutations	Bladder cancer, squamous cell carcinoma (lung, head and neck)
MET	7q31.2	Gastrulation, development and migration of muscles and neuronal precursors, angiogenesis and kidney formation	Mutations	Hepatocellular carcinoma, CLL, breast cancer, pancreatic cancer, lung cancer, gastric adenocarcinomas
Axl	19q13.1	ND	Unknown	Lung cancer, colon cancer, breast cancer, AML, CML, esophageal, thyroid cancer, gastrointestinal stromal tumors, astrocytoma-glioblastoma
IGF1R	15q26.3	Embryonic and fetal development	Mutations	CLL, breast cancer, oral squamous cell carcinoma cells. gastrointestinal stromal, squamous-cell laryngeal cancer tumors, hepatocellular carcinoma, pancreatic cancer
IGF2R	6q25.3	Embryonic and fetal development	Mutations	Squamous cell carcinoma, breast cancer, prostate cancer, hepatocellular carcinoma, colorectal carcinoma, NSCLC, pancreatic cancer
EGFR1 (ERBB1)	7p11.2	ED	Mutations	Breast cancer, hepatocellular carcinoma, head and neck squamous cell carcinoma
EGFR2 (ERBB2)	17q12	ED	Mutations	Breast cancer, gastric adenocarcinomas
EGFR3 (ERBB3)	12q13.2	ED	Mutations	Breast cancer
EGFR4 (ERBB4)	2q34	ED	Mutations	Breast cancer, melanoma
VEGFR1 (FLT1)	13q12.3	ED	Mutations	Ovarian cancer, NSCLC, colorectal carcinoma

RTK	Chromosome location	Normal function	Malfunction leading to overexpression	Malignancy (examples)
VEGFR2 (KDR)	4q12	ED	Mutations	Renal cell carcinoma, hepatocellular carcinoma
VEGFR3 (FLT4)	5q35.3	ED	Mutations	
FLT3	13q12.2	Hematopoiesis	Mutations	AML, acute promyelocytic leukemia
KIT	4q12	Hematopoiesis, stem cell maintenance, gametogenesis, mast cell development, migration and function, and in melanogenesis	Mutations	AML, melanoma, ovarian carcinoma, gastrointestinal stromal tumors
RON (MST1R)	3p21.31	Regulates many physiological processes including cell survival, migration and differentiation	Mutations	Pancreatic cancer, breast cancer, NSCLC, laryngeal squamous cell carcinoma, head and neck squamous cell carcinoma
INSR	19p13.2	Metabolic actions of insulin	Mutations	Colorectal cancer, prostate cancer
INSRR	1q23.1	Metabolic actions of insulin	Mutations	Neuroblastomas
CCK4 (PTK7)	6p21.1	ED	Mutations	Squamous cell carcinoma, small cell lung cancer, breast cancer, gastric and colon cancer, AML
EPHA1	7q35	NSD	Mutations	NSCLC, prostate cancer, esophageal squamous cell carcinoma
EPHA2	1p36.13	NSD	Mutations	Hepatocellular carcinoma. colorectal cancer, osteosarcoma, breast cancer
EPHA3	3p11.1	NSD	Mutations	Glioblastoma, lung cancer, melanoma, ALL, T-cell leukemia, hodgkins lymphoma
EPHA4	2q36.1	NSD	Mutations	NSCLC, gastric cancer
EPHA5	4q13.1	NSD	Mutations	Breast cancer, hepatocellular carcinoma, ALL
EPHA6	3q11.2	NSD	Mutations	-
EPHB1	Xq13.1	NSD	-	NSCLC, cervical cancer, ovarian cancer
EPHB2	13q33.3	NSD	-	Cervical cancer, breast cancer
EPHB3	3q27.1	NSD	-	NSCLC, breast cancer, colorectal cancer
EPHB4	7q22.1	NSD	-	Breast cancer, melanoma, glioma
MER	2q13	Survival, migration, differentiation, and phagocytosis of apoptotic cells	Mutations	Glioblastoma, hepatocellular carcinoma, astrocytoma
TYRO3	15q15.1	Cell survival, migration and differentiation	Mutations	Colon cancer, melanoma, thyroid cancer, breast cancer
TIE	1p34.2	Regulation of angiogenesis	Mutations	Glioblastoma
TEK	9p21.2	Regulates angiogenesis, endothelial cell survival, proliferation, migration, adhesion and cell spreading, reorganization of the actin cytoskeleton, but also maintenance of vascular quiescence	Mutations	Bladder cancer, glioblastoma, AML
RYK	3q22.2	Neuron differentiation, axon guidance, corpus callosum establishment and neurite outgrowth	Translocation, mutations	CML, ovarian cancer
DDR1	6p21.33	Regulates cell attachment to the extracellular matrix, remodeling of the extracellular matrix, cell migration, differentiation, survival and proliferation	Mutations	NSCLC, breast cancer, AML, ovarian cancer, hepatocellular carcinoma
DDR2	1q23.3	Regulates cell differentiation, remodeling of the extracellular matrix, cell migration and cell proliferation	Mutations	Head and neck squamous cell carcinoma, NSCLC, lung cancers, CML, breast cancer
LTK	15q15.1	ND	Mutations	Gastric cancer, lymphomas, leukemias
MUSK	9q31.3	NSD	Mutations	Ovarian cancer

ROR: receptor tyrosine kinase-like orphan receptor, ED: embryonic development, NSD: nervous system development, CLL: chronic lymphocytic leukemia, ALL: acute lymphoblastic leukemia, AML: acute myeloid leukemia, HCL: hairy cell leukemia, NSCLC: non-small cells lung carcinoma, NTRK: neurotrophic tyrosine kinase, PDGFR: platelet- derived growth factor receptor, FGFR: fibroblast growth factor receptor, ND: normal development, CML: chronic myeloid leukemia, INSR: insulin receptor, EGFR: epidermal growth factor receptor, VEGFR: vascular endothelial growth factor receptor, CCK: colon carcinoma kinase, RYK: receptor related to tyrosine kinases, DDR: discoidin domain receptor, LTK: leukocyte tyrosine kinase, MuSK: muscle-specific kinase..

2.1 ROR family of receptor tyrosine kinase

The receptor tyrosine kinase orphan receptor (ROR) family consists of ROR1 and ROR2 previously known as neurotrophic tyrosine kinase receptors 1 and 2 (NTRKR1) and (NTRKR2) respectively [229]. ROR1 and ROR2 belong to type I transmembrane proteins that are evolutionary highly conserved among different species including humans, mice, rats, chickens, zebra fish, roundworms and fruit flies.

The structure of ROR family RTKs contains the extracellular part with the ligand binding domains, a transmembrane part that anchors the receptor in plasma membrane, and an intracellular part expressing a kinase domain for signal transmission as shown in Figure 6. The extracellular ROR part consists of the immunoglobulin (Ig)-like domain, Frizzled-like cysteine-rich domain (CRD) and a membrane-proximal kringle (KNG) domain. The intracellular part of ROR contains a tyrosine kinase (TK) domain, proline-rich domain (PRD) and a serine/threonine rich domain [230].

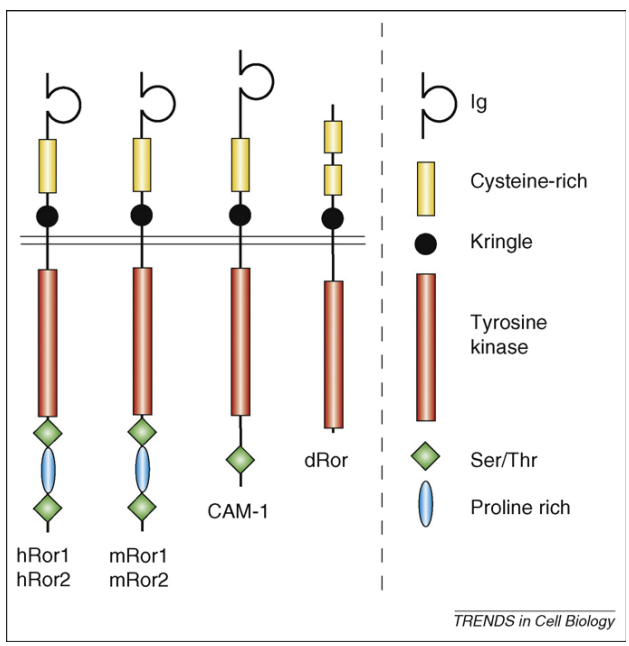

Figure 6. Structure of ROR receptor tyrosine kinases (RTKs) in different species. Domain organization of ROR proteins in human (hROR1, hROR2), mouse (mRor1, mRor2), C. elegans (CAM-1) and Drosophila (dROR). The N-terminal extracellular domain (ECD) is above and the intracellular domain (ICD) is below the double line representing the plasma membrane.

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2.2 Structure of ROR1

Human ROR1 and ROR2 (hROR1 and hROR2) share 58% identity in amino acids sequence and 68% in the kinase domains. Amino acids sequence identity is highly conserved among different species within the ROR1 and ROR2 subgroups respectively. A 97% amino acid sequence identity of human and mouse ROR1 (hROR1 and mROR1) has been noted and 92% for human and mouse ROR2 [231, 232]. Other mammalian ROR proteins have the same extracellular region containing Ig-like domain, CRD and KNG domains,

transmembrane and an intracellular part containing TK domain, proline-rich and two serine/threonine motifs.

Human ROR1 is located on chromosome 1 (1p31.3) with a protein size of 937 amino acids and molecular weight of approximately 105 kDa. The human ROR2 is located on chromosome 9 (9q22) with a protein size of 943 amino acids and a molecular weight of approximately 104.7 kDa.

2.2.1 Extracellular part of ROR1

2.2.1.1 Signal peptide

ROR family of RTKs possesses a short peptide sequence of about 20-30 amino acids at the N-terminal. The signal peptide directs the protein to the endoplasmic reticulum for further maturity and secretion. Then it is recognized by signal recognition particle (SRP) and cleaved by signal peptidase [232, 233].

2.2.1.2 Immunoglobulin-like domain (Ig)

The Ig-like domain is at the far end of the extracellular part consisting of 106 amino acids residues in human ROR1 and 91 in ROR2. The precise role of the Ig-like domain is unknown but may be involved in protein and ligand interactions as well as interfering with the CRD and KNG domains [234].

2.2.1.3 Cysteine-rich domain (CRD)

The CRD domain consists of 135 amino acids sequence, which is located between the Ig-like and KNG domains. 10 conserved cysteine residues that form five disulfide bonds characterize the CRD domain. The CRD domain is similar to the Wnt binding domain of Frizzled receptors and is suggested as one of the ligand binding domain of ROR1. ROR1 has been shown to physically interact with Wnt5a, resulting in increased survival of CLL cells in vitro by activating NF-κB [235].

2.2.1.4 Kringle domain (KNG)

The KNG domain is located in close proximity to the plasma membrane consisting of 79 amino acids and is highly conserved among different species. The KNG domain might serve as a recognition binding site for Wnt regulatory proteins and other ROR1 ligands [229]. The ROR family is the only RTK family that contains a KNG domain with the exception of Torpedo Musk [232].

2.2.2 Intracellular part of ROR1

2.2.2.1 Tyrosine kinase (TK) domain

ROR family proteins are the most divergent within the RTK family containing only 21 of the 40 consensus residues in the TK compared to other RTKs [236]. The functional role of the ROR1 TK domain is a matter of intense debate. Early studies showed that ROR1 possessed weak to a very moderate kinase activity [230, 237] but recently a study showed that ROR1 TK domain was sufficient to phosphorylate c-SRC in NIH3T3 cells [238]. Now we

know that ROR1 is constitutively phosphorylated at tyrosine residues in CLL cells, which is dephosphorylated after treatment with ROR1 monoclonal antibodies against CRD and KNG domains [239].

2.2.2.2 Serine/threonine-rich domains 1 & 2 (S/TRD1 & S/TRD2) and proline-rich domain (PRD)

Two serine/threonine-rich domains (S/TRD), which are separated by a proline-rich domain (PRD) have been described in ROR RTK. S/TRDs or PRD have not been found in other RTKs. These cytoplasmic domains are important as they contain potential phosphorylation sites. The SH2 and SH3 recognition motifs interact with mediators of different downstream signaling molecules [240, 241]. ROR1 is also phosphorylated at 786, 789, 822, and 836 tyrosine residues that contribute to phosphorylation of tyrosine in the kinase domain by SRC kinases. The consensus SH3 motifs facilitate binding of SRC to the proline-rich domain, which ultimately results in ROR1 phosphorylation. Deletion of PRD as well as SRC prevented phosphorylation of ROR1 within the kinase domain [242].

2.3 ROR tyrosine kinase activity

ROR kinase activity has been discussed. Earlier studies indicated a strong autocatalytic kinase activity for ROR2 but a weak to moderate for ROR1 [230, 237]. In a recent study, ROR1 TK domain was shown to possess kinase activity [238]. However, in another study ROR1 was suggested to be a pseudokinase without kinase activity in COS-7 cells [242]. A pseudokinase is a kinase with no or very limited enzymatic activity and may not be capable to phosphorylate the substrate due to amino acid substitutions in critical residues that regulate the enzymatic activity of tyrosine kinases [243]. However, specific ROR1 silencing and targeting ROR1 by monoclonal antibodies resulted in the apoptosis of CLL cells [244, 245]. This indicates the crucial role of ROR1 in survival of CLL cells.

2.4 Expression and functions of ROR1 during development

Several studies using in situ hybridization and mutant knockout mice models indicated the involvement of ROR during skeletal, cardiorespiratory and neurological development. mROR1 seemed to be limited to the cephalic mesenchyme and neural crest cells whereas mROR2 expression was noted in both neural and non-neural cells during development [246]. ROR1 was tightly down regulated after birth [230, 237].

Human ROR1 expression was found in fetal heart, lung, kidney and low expression in placenta, pancreas and skeletal muscle [231]. ROR1 expression at mRNA level was not found in various normal hematopoietic and non-hematopoietic tissues but a low expression was seen in pancreas and adipose tissues. Normal adult tissues tested for ROR1 protein did not show expression in majority of samples except testis, uterus, lung, bladder and colon, where a low expression was noticed [247].

ROR1 protein was detected at an intermediate stage of the normal B-cell maturation in the bone marrow (pre-BII stage), where cells proliferated following internalization of the pre-B-cell receptor complex. This process was essential for the development to an immature B-cell stage [248].

Knockdown studies for ROR1 and ROR2 have been conducted in mice to evaluate their functions. Mice with homozygous knockout of mROR2 resulted in shortened limbs, cyanosis, septal defects of the heart. The mice died within six hours after birth due to respiratory defects [249]. Mice with homozygous knockout of mROR1 developed perinatally lethal defects because of respiratory dysfunction but these mice did not show pronounced heart or skeletal abnormalities [250]. ROR1 and ROR2 double deficient mice died shortly after birth due to respiratory problems associated with incomplete expansion of lung alveoli [234, 249, 250]. The expression of ROR2 was sustained in hippocampus and caudate putamen in the late stages of embryonic development but mROR1 was not detectable [251].

2.5 ROR1 and cancer

ROR1 expression has been seen in various blood and solid malignancies. The low ROR1 expression in normal adult tissue and high expression in cancer have encouraged researchers to investigate the functional advantage conferred by ROR1 and to explore ROR1 for targeted therapy in cancer [245, 247, 252, 253]. In Table 4, the expression of ROR1 and its association with progression in different malignancies is described [224].

Table 4. Overexpression of ROR1 in malignancies.

Malignancy	Constitutively phosphorylated ROR1	Association to disease progression
CLL	+	+
B-ALL	NI	+
CML	NI	NI
AML	NI	NI
Hairy cell leukemia	NI	NI
Mantle cell lymphoma	NI	NI
Pancreatic cancer	NI	+
Prostate cancer	NI	NI
Colon cancer	NI	NI
Bladder carcinoma	NI	NI
Ovarian cancer	NI	+
Testicular cancer	NI	NI
Uterus	NI	NI
Adrenal carcinoma	NI	NI
Breast cancer	+	+
Lung cancer	+	+
Melanoma	+	NI

CLL: chronic lymphocytic leukemia, B-ALL: B-cell acute lymphoblastic leukemia, NI: no information,
CML: chronic myeloid leukemia, AML: acute myeloid leukemia.

2.5.1 ROR1 in hematological malignancies

ROR1 expression was initially identified in chronic lymphocytic leukemia and later on in other malignancies. In 2001, two independent gene-profiling studies identified high ROR1 expression (45-fold increase) in CLL as compared to normal mature B-lymphocytes [37, 254].

ROR1 protein expression has been shown in CLL [235, 247, 255] and also in other malignancies such as acute lymphocytic leukemia (ALL) [256, 257], breast cancer [258], renal cell carcinoma [259], melanoma [260], lung adenocarcinoma [238] and other lymphoid and myeloid malignancies [255, 257, 258, 260-263].

The number of ROR1 receptors on the surface of CLL cells was estimated to be in the range of 10,000 per cell [235, 255] which should be enough to be targeted by monoclonal antibodies [239, 245]. ROR1 expression in CLL increased during disease progression. STAT3 is constitutive phosphorylated in CLL and has been shown to bind to the promoter region of ROR1 in CLL [264]. Maybe STAT3 is involved in the expression of ROR1. ROR1 expression could also be induced by IL-6 in a STAT3 dose-dependent manner [265].

Wnt5a is suggested to be one of the ligands for ROR1. Wnt5a was shown to bind to ROR1 in the HEK cells inducing activation of NF-κB. Co-culturing of Wnt5a significantly increased survival of CLL cells compared to those without Wnt5a. The Wnt5a dependent survival of CLL cells was inhibited by ROR1 antisera [235].

ROR1 has been shown to be phosphorylated at tyrosine and serine residues in CLL and also in other malignancies. Wnt5a could maintain phosphorylation of ROR1 [258]. ROR1 phosphorylation varied from patient to patient in CLL. High ROR1 phosphorylation intensity was found in progressive compared to non-progressive CLL. The same pattern was observed in other malignancies including breast, lung and ovarian cancer cells with an aggressive course [239]. Furthermore, ROR1 expression at the protein level was significantly higher in aggressive tumors [253]. Collectively these data may suggest that the expression pattern of ROR1 is related to aggressiveness.

ROR1 was highly expressed in some but not in all cases of multiple myeloma, diffused large B-cell lymphoma (DLBCL), marginal zone lymphoma (MZL), follicular lymphoma (FL) and mantle cell lymphoma (MCL) at gene level as well as at the protein level [252]. High ROR1 expression has also been shown in ALL and specifically those with t(1;19)(q23;p13) translocation. ROR1 was important for survival of ALL cells with t(1;19)(q23;p13) translocation when screened for critical tyrosine kinases in the pathogenesis of ALL [266].

2.5.2 Expression of ROR1 in solid tumors

ROR1 expression has been shown in various solid tumors. The expression pattern varied from moderate to high depending on the type of cancer. Moderate ROR1 staining was observed in majority of prostate, testicular, uterine, ovarian, lymphoma, adrenal and melanoma cancers [253]. Strong ROR1 staining was found in 30% or greater of primary samples in the lung, colon and pancreatic cancers [246]. In lung adenocarcinoma, TITF1 has been shown to regulate ROR1 expression. ROR1 up-regulation was linked with the potentiation of EGF ligand-induced EGFR signaling, phosphorylation and activation of c-SRC [238].

ROR1 expression was also detected in all melanoma cell lines at mRNA level as well as protein level as assessed by Western blot and RT-PCR in addition to surface ROR1 staining by flow cytometry. Knockdown of ROR1 in melanoma cell lines induced apoptosis [261]. ROR1 was shown to be phosphorylated at both tyrosine and serine residues. Monoclonal antibodies against ROR1 induced apoptosis [261]. ROR1 and ROR2 were shown

to be inversely expressed in melanoma cells indicated that they may negatively regulate each other [260]. Hypoxia resulted in a shift of ROR1 positive cells to an aggressive ROR2 phenotype in melanoma cells. The switch to the ROR2 positive phenotype resulted in 10-fold decrease in sensitivity to BRAF inhibitors.

In breast cancer, ROR1 was shown to be expressed in human neoplastic cells but absent in stromal cells. ROR1 overexpression in breast cancer was linked to aggressive disease. Breast cancer cell lines with strong ROR1 expression were more aggressive and invasive but declined in non-migrating cells [258]. ROR1 silencing through specific ROR1 siRNA resulted in down-regulation of ROR1 expression in human breast cancer cell lines and impairment of growth in immunodeficient mice. ROR1 was also shown to activate PI3K mediated AKT and CREB signaling pathways by interacting with casein kinase 1 (CK1) [258]. Wnt5a increased the survival of ROR1 expressing breast cancer cells, confirming the notion of Wnt5a as a ligand for ROR1 [258, 267].

High levels of ROR1 expression in patients and cell lines were linked to genes contributing to epithelial-mesenchymal transition (EMT) such as ZEB1 and vimentin and inversely related to adherent junction proteins. ROR1 expression was high in breast adenocarcinomas with high level of EMT related genes and with a high capacity to metastasize. Silencing of ROR1 in the triple negative breast cancer cell line MDA-MB321 by small hairpin (sh) RNA reduced in vitro cell migration as well as bone and lung foci xenografts [267]. Knockdown of ROR1 in triple negative breast cancer cells also reduced the EMT genes like SNAI1, SNAI2, ZEB1 and vimentin [267]. Similarly, ROR1 transfected MCF-7 cell lines showed low level expression of adherent junction proteins like E-cadherin and CK-19 which contribute to homing of cells at proliferation sites but ROR1 transfection did not change expression levels of SNAI1, SNAI2 and vimentin [267].

2.6 Roles of ROR1

ROR1 localization to the nucleus may suggest that it might act as transcription factor to activate genes involved in tumorigenesis [239, 268]. Stimulation of multiple myeloma cell lines by IL-6 resulted in phosphorylation of signal transducer and activator of transcription factor 3 (STAT3) and up-regulation of ROR1 indicating a possible role of STAT3 in the activation of ROR1 [269]. The ROR1 promoter region contains γ-interferon activation sequence like elements, which are activated by STAT3 [270]. STAT3 has also been shown to induce Wnt5a expression [271, 272]. Based on these findings, it is suggested that STAT3 may activate ROR1 and Wnt5a, which may promote cell survival, growth and migration through Wnt5a binding to the ROR1 promoter region.

A schematic depiction of ROR1 signaling is shown in Figure 7.

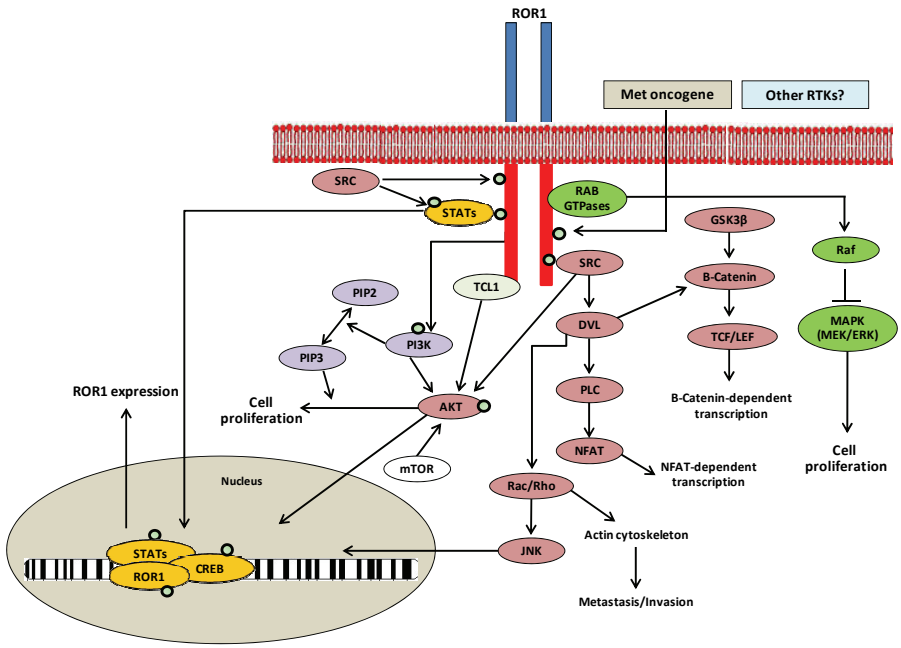

Figure 7. A schematic proposed model for ROR1 signaling. The ROR1 receptor tyrosine kinase recruits canonical and non-canonical signaling pathways for cell survival and invasion. A central pathway is the PI3K/AKT/mTOR pathway which activates the CREB transcription factor for nucleus translocation. ROR1 kinase-dependent SRC activation is a key initiating event. Proteins like ROR1, STATs and CREB might act as transcription factors and bind to ROR1 promoter region to enhance the expression of the ROR1 gene. Phosphate groups are denoted as green circles.

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In CLL, ROR1 has been shown to undergo extensive N-linked glycosylation modifications. Several ROR1 isoforms with different electrophoretic mobility in the range of 100-130 kDa have been identified probably as a result of posttranslational modifications [273]. Prevention of ROR1 glycosylation resulted in the altered surface expression of the fully mature 130 kDa ROR1 isoform and formation of filopodia in HEK cell lines suggesting a role of ROR1 in cell migration and metastasis.

ROR1 signaling pathways have not been fully recognized in cancer. PI3K/AKT pathway was shown to be activated and related to ROR1 after stimulation with Wnt5a in breast cancer cell. Knockdown of ROR1 by siRNA or absence of Wnt5a inhibited growth of breast cancer cells [258]. Our preliminary data showed that treatment of ROR1 positive pancreatic carcinoma cell lines with anti-ROR1 mAbs or tyrosine kinase inhibitors against ROR1 dephosphorylated ROR1 and downstream signaling proteins as SRC, PI3K, AKT, mTOR and CREB subsequently leading to apoptosis.

ROR1 has been shown to be related to BCR signaling in a counterbalance manner for the survival of ALL cells [266]. Downregulation of pre-BCR signaling and ROR1 induced permanent dephosphorylation of AKT and enhanced cell cytotoxicity. However,

downregulation of either ROR1 or BCR signaling alone did not cause the same effect, suggesting a link between ROR1 and BCR signaling.

ROR1 may also play a role in leukemogenesis of CLL cells by interacting with the T-cell leukemia antigen (TCL1), which is an activator of AKT. Co-expression of ROR1 and TCL1 enhanced leukemogenesis resulting in phosphorylation of AKT, cell proliferation and resistance to apoptosis. Down-regulation of ROR1 by anti-ROR mAbs reduced AKT activity and loss of tumorigenic characteristics in a syngeneic mice model [274].

Our preliminary data showed involvement of MET and the SRC oncogene in phosphorylation of ROR1. Phosphorylation of ROR1 at tyrosine residues 641 and 646 in the TK domain of ROR1 which was important for survival of the cells [239]. SRC dependent phosphorylation of ROR1 was crucial for cell invasion and specific inhibition of SRC activity by saracatinib induced impaired cell invasion but not proliferation [275].

Based on current data, ROR1 is suggested to be involved in recruiting different signaling proteins, transcription factors and activate signaling pathways, which ultimately result in high survival, proliferation and metastasis. The initial activation of ROR1 may be different in various malignancies but signaling through the PI3K/AKT/mTOR axis seems to be important. This pathway may be switched on by GTPases followed by SRC phosphorylation and subsequently binding of SRC to ROR1. Axl, MET, EGFR but other tyrosine kinases may also be involved in the activation of ROR1 [238, 275, 276]. Activation of ROR1 may then activate and upregulate various genes involved in growth, migration and metastasis [267]. Further studies are needed to fully understand the signaling pathways affected by ROR1.

2.7 Isoforms of ROR1 in cancer

The full length ROR1 with a molecular weight of 105-130 kDa has been identified in all malignancies positive for ROR1 [239, 242, 245]. In addition, a truncated isoform of ROR1 (t-ROR1) without extracellular and transmembrane parts was also identified in neural tissues [231]. mRNA encoding the t-ROR1 was found in fetal and adult central nervous system (CNS), human leukemia, lymphoma cell lines and other human cancers originating from the neuroectoderm. ROR1 encoded by 6kb mRNA was also noted in normal human lung, kidney and heart [231]. A neurogenic teratocarcinoma cell line (NTera2) showed the expression of a 2373 nucleotide transcript translating to a 388 amino acids protein that was identical to the cytoplasmic C-terminal of ROR1 (ROR1-201, ENSP00000441637). A splice variant of a 50 kDa ROR1 was also found [247]. Another ROR1 isoform with a molecular weight of 260 kDa has also been identified by our group which may represent dimerized ROR1 [239]. The 260 kDa ROR1 isoform could represent homodimerized ROR1 or heterodimerization with ROR2 or other receptors. ROR1 and EGFR were shown physically bound to each other in lung adenocarcinoma [238]. We have also identified a 64 kDa ROR1 in CLL cells which was mainly localized to the nucleus [239] suggesting that ROR1 may act as transcription factor [268]. The predominant full length ROR1, glycosylated and mature isoform 130 kDa was found to be more abundant in progressive than in non-progressive CLL [239]. Different isoforms of ROR1 need further investigation to assign them to different functions.

2.8 ROR1 as therapeutic targets in cancer

ROR1 like other oncogenic RTKs might be targeted in cancer. There are two main strategies; targeting the extracellular part of the receptor by monoclonal antibodies or by tyrosine kinase inhibitors directed against the intracellular kinase domain (Figure 8). In Table 5, current tyrosine kinase inhibitors targeting different RTKs are summarized [277]. Targeting the extracellular region of RTK by mAbs may disrupt the cytoplasmic kinase signaling by neutralization of the ligand, hampering the ligand binding, internalization of the receptor or by interacting with immune effectors targeting the tumor cells. Different RTKs HER-2, EGFR, VEGFR and VEGF and their ligands have been targeted in various cancers by mAbs. Trastumuzab was the first approved antibody against HER-2 for the treatment of breast cancer patients [278]. Pertuzumab is another antibody approved against HER-2 to prevent dimerization of HER-2 with members of EGFR family. Similarly, tyrosine kinase inhibitors against the intracellular kinase domain in various cancers have been designed and approved for clinical use; gefitinib [279] and erlotinib [280] against EGFR and lapatinib [281] against HER2.

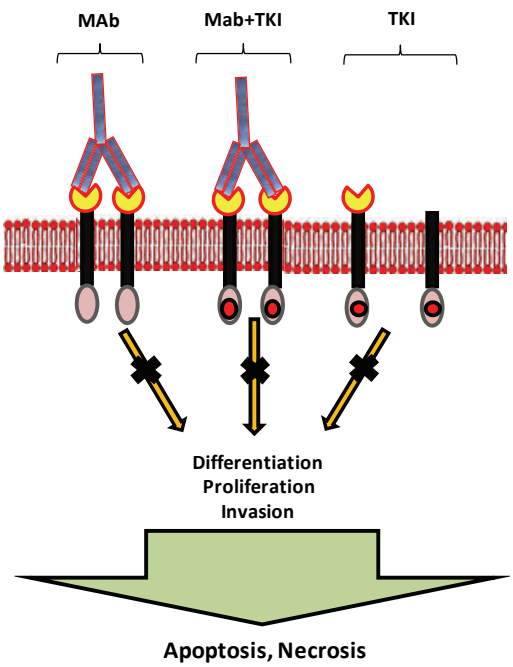

Figure 8. Strategies to target the receptor tyrosine kinase ROR1. Monoclonal antibodies (mAbs) and small molecules (tyrosine kinase inhibitor TKI) (red circles) may interfere with cell proliferation, differentiation, migration, metastasis and invasion as well as induction of cell death by apoptosis or necrosis. Dual blockage by mAbs and TKI may further augment the anti-tumor effects as has been shown for anti-EGFR mAb/gefitinib and anti-HER2 mAb/lapatinib combinations.

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Combination approach, where the extracellular part of RTK is targeted by mAbs and the intracellular kinase domain by tyrosine kinase inhibitors, has shown synergistic effects in preventing the tumor growth and proliferation of cancer cells. Treatment of xenograft mice expressing HER-2 with trastuzumab and lapatinib resulted in significant tumor growth inhibition [282]. Similarly treatment of colon cancer cell lines expressing EGFR with cetuximab and gefitinib prevented proliferation and induced apoptosis [283]. Targeting of HER-2 by trastuzumab and lapatinib has shown better clinical activity than either alone in HER-2 positive breast cancer patients [284, 285].

Table 5. Tyrosine kinase inhibitors (TKIs) targeting RTKs.

Name	Developed by	Trade name	Chemical formulation	Mol. mass (g/mol)	Selective target	IC_{50} (nM/L)*	Appr. for treatm.	Malignancies (examples)
Afatinib	Boehringer Ingelheim Pharmaceuticals	Gilotrif	$C_{24}H_{25}ClFN_5O_3$	485.94	HER2, EGFR, HER4	**14, 0.5, 1**	+	NSCLC, squamous cell carcinoma of the head and neck, breast cancer
Canertinib	Pfizer	-	$C_{24}H_{25}ClFN_5O_3$	485.94	EGFR, HER2, HER4	**0.8,19, 7**		Head and neck, breast, and NSCLC, ovarian cancer
Cediranib	AstraZeneca Pty Ltd	Recentin	$C_{25}H_{27}FN_4O_3$	450.505	VEGFR	**1**	-	NSCLC, kidney and colorectal cancer
CP-673451	Pfizer	-	$C_{24}H_{27}N_5O_2$	417.5	PDGFR	**1**	-	NSCLC, colon carcinomas, glioblastoma
Crizotinib	Pfizer	Xalkori	$C_{21}H_{22}Cl_2FN_5O$	450.337	MET	**11**	+	NSCLC, anaplastic large cell lymphoma, neuroblastoma
Crenolanib	Pfizer	-	$C_{26}H_{29}N_5O_2$	443.54	FLT3, PDGFRα/β	**0.74, 1, 0.4**	-	AML, gastrointestinal stromal tumor, glioma
Dacomitinib	Pfizer	-	$C_{24}H_{25}ClFN_5O_2$	469.94	EGFR	**6**	-	NSCLC, gastric, head and neck/glioma
Erlotinib	OSI Pharmaceuticals	Tarceva	$C_{22}H_{23}N_3O_4$	393.436	EGFR	**2**	+	NSCLC, pancreatic cancer
Gefitinib	AstraZeneca Pty Ltd	Iressa	$C_{22}H_{24}ClFN_4O_3$	446.902	EGFR	**<57**	+	NSCLC, AML
Icotinib	Zhejiang Beta Pharma	Conmana	$C_{22}H_{21}N_3O_4$	391.15	EGFR	**5**	+	NSCLC
KW-2449	Kyowa Pharma	-	$C_{20}H_{20}N_4O$	332.4	FLT3	**6.6**	-	AML
Lapatinib	GlaxoSmithKline	Tykerb	$C_{29}H_{26}ClFN_4O_4S$	581.058	HER-2, EGFR	**9.2, 10.8**	+	Breast cancer
Lenvatinib	Eisai	-	$C_{21}H_{19}ClN_4O_4$	426.853	VEGFR2/3	**<4**	+	Approved for thyroid cancer in Japan
LY2801653	Eli Lilly and Company	-	$C_{30}H_{22}F_2N_6O_3$	552.53	Met, RON	**<2**	-	NSCLC
Neratinib	PharmGKB	HKI-272	$C_{30}H_{29}ClN_6O_3$	557.04	EGFR, HER2	**92, 59**	-	NSCLC, breast cancer
PD-173074	Pfizer	-	$C_{28}H_{41}N_7O_3$	523.67	FGFR	**25**	-	NSCLC, gastric carcinoma, breast cancer
Quizartinib	Ambit Biosciences	-	$C_{29}H_{32}N_6O_4S$	560.67	FLT3	**<4.2**	-	AML
R428 (BGB-324)	BerGenBio AS	-	$C_{30}H_{34}N_8$	506.64	AXL	**14**	-	AML, NSCLC, breast cancer
Tandutinib	Millennium Pharmaceuticals	-	$C_{31}H_{42}N_6O_4$	562.7	FLT3	**<100**	-	RCC, CML
Tivantinib	ArQule	ArQule	$C_{23}H_{19}N_3O_2$	369.42	MET	**4**	-	RCC, breast cancer
Tivozanib	AVEO Pharmaceuticals	AV-951	$C_{22}H_{19}ClN_4O_5$	454.86	VEGFR1, 2, 3	**0.21, 0.16, 0.24**	-	RCC, breast cancer
Vatalanib	Bayer Schering and Novartis	-	$C_{20}H_{15}ClN_4$	346.813	VEGFR2	**37**	-	NSCLC, DLBCL, colorectal adenocarcinoma

* IC50 (half maximal inhibitory concentration) values are the measure of the effectiveness of TKIs in inhibiting the RTKs in biochemical assays, Appr: approved. treatm: treatment, HER: human epidermal receptor, EGFR: epidermal growth factor receptor, NSCLC: non-small cells lung carcinoma, VEGFR: vascular endothelial growth factor receptor, PDGFR: platelet-derived growth factor receptor, AML: acute myeloid leukemia, CML: chronic myeloid leukemia, RCC: renal cell carcinoma

2.8.1 Monoclonal antibodies against ROR1

The extracellular region of ROR1 contains Ig-like, CRD and KNG domains, which could be targeted by monoclonal antibodies. Monoclonal antibodies directed against these extracellular domains have been developed as potential therapeutic agents [245, 247, 286]. The anti-ROR1 mAbs may kill cells by direct apoptosis or by activation of complement or

immune effector cells. Most effective anti-ROR1 mAbs to induce significant apoptosis in CLL cells were those against the CRD and KNG domains [245]. Anti-ROR1 CRD mAb also induced apoptosis in pancreatic cancer cell lines. De-phosphorylation of ROR1, the PI3Kδ, AKT and mTOR was also observed prior to apoptosis by the treatment of anti-ROR1 CRD mAb suggesting inhibition of downstream signaling but there was no effect on ERK and PKC proteins. These findings may suggest that ROR1 signaling occurs via PI3K/AKT/mTOR axis in pancreatic cancer cell lines.

The anti-ROR1 mAbs also resulted in killing of melanoma cell lines by direct apoptosis as well as complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC) [287]. ESTDAB081 and ESTDAB094 melanoma cell lines were resistant to direct apoptosis by anti-ROR1 mAbs alone but sensitive to apoptosis by anti-ROR mAbs mediated through CDC and ADCC. Knockdown of ROR1 through specific siRNA resulted in apoptosis of melanoma cell lines.

The effects of anti-ROR mAbs in TCL1 transgenic mice expressing ROR1+/CD5+ /B220low leukemic B cells as a model for in vivo studies were analyzed [288]. Two anti-ROR1 antibodies, D10 and 4A5 against different epitopes produced different effects in vivo. D10 anti-ROR1 mAb reduced phosphorylation of AKT but 4A5 mAb did not. Leukemic cells were cleared from the blood and spleen of transgenic mice following intravenous injections of D10 mAb but not by 4A5 [288, 289]. Cirmtuzumab, a humanized anti-ROR1 mAb killed tumor cells and was internalized by malignant cells. Cirmtuzumab with ADC (antibody drug conjugates) cleared ROR1 expressing CLL cells in xenografted mice as well as induced apoptosis of breast and pancreas cell lines in vitro.

Anti-ROR1 antibodies may also be used to deliver toxins. Immunotoxin (BT-1) from Pseudomonas exotoxin (PE38) conjugated with the variable fragments of an anti-ROR1 mAb showed a dose-dependent and selective binding to leukemic cells from CLL and MCL patients, followed by internalization of the immunotoxin and subsequent apoptosis in vitro [290].

ROR1 expression has been shown to be associated with epithelial-mesenchymal transition of tumor cells as indicated above [267]. Treatment with anti-ROR1 mAbs prevented metastasis by down-regulation of proteins involved in cell motility and metastasis of breast cancer cell lines [267].

2.8.1.1 Immunotherapeutic strategies targeting ROR1

ROR1 is highly expressed in many malignancies but not in healthy adult tissues. ROR1 as an oncofetal antigen might thus be recognized by the immune system. A humoral immune response against ROR1 in CLL patients was observed after vaccination with Ad-CD154 transduced CLL cells [235]. Antibodies induced against ROR1 blocked the interaction of Wnt5a and ROR1 and reduced proliferation of CLL cells. Lenalidomide, an immune modulating drug treatment induced ROR1 antibodies in CLL patients indicating that ROR1 may be an immunodominant epitope [291-293]. CLL patients may also mount a type 1 T cell response against ROR1 and a humoral response against ROR1 [294, 295].

Transgenic leukemic ROR1+ mice immunized with a ROR1 peptide produced high titers of anti-ROR1 antibodies which inhibited the engraftment of ROR1+ CLL cells [296].

ROR1 may thus act as a tumor antigen for vaccination in a similar way as HER2 derived vaccine in breast cancer patients [297].

T cells from healthy donors or CLL patients have also been genetically modified to express ROR1-CART targeting ROR1+ tumor cells [248]. CD8+ T cells engineered to express specific ROR1-CART lysed CLL and MCL cells but not mature normal cells. ROR1-CARTs were shown be as potent as CD19-CARTs in a immunodeficient mice model of human MCL [298]. Clinical trials on ROR1-CART are just to be started.

2.8.2 Tyrosine kinase inhibitors against ROR1

Most oncogenic RTKs are highly up-regulated and activated in malignant cells but have no or low expression and activity in normal tissues [299]. RTKs in tumors might also be targeted by TKIs against the intracellular kinase domain [300]. Axl is an RTK constitutively expressed in CLL and by targeting the intracellular kinase domain by a specific inhibitor, R428, a robust apoptosis of CLL cells was induced in a dose- and time-dependent manner [276]. The VEGFR inhibitors vatalanib and pazopanib decreased phosphorylation of the VEGF receptor and induced apoptosis of CLL cells in clinically achievable concentrations with a mild cytotoxic effect on healthy B cells [301].

Tyrosine kinase inhibitors might also target the intracellular kinase domain of ROR1. TKIs against ROR1 have been produced and developed by Kancera (www.kancera.com). Our preliminary results indicated that one of the promising ROR1 TKI induced apoptosis both in progressive and non-progressive CLL cells but not in normal PBMCs. Our own study, which is part of this thesis (Paper V) showed robust cytotoxic activity of this ROR1 TKI in CLL cells. ROR1 TKI treatment of CLL cells induced dephosphorylation of ROR1 and subsequent apoptosis. Progressive and non-progressive CLL cells were sensitive to ROR1 TKI. ROR1 TKI also showed activity in fludarabine resistant CLL cells. Treatment of CLL cells with ROR1 TKI dephosphorylated ROR1. ROR1 TKI induced apoptosis in CLL cells via caspase activation, PARP cleavage and downregulation of Mcl-1 and Bcl-2. Oral administration of ROR1 TKI in xenografted transplanted NOD SCID mice reduced leukemic cells. Our preliminary data also show that ROR1 TKI could induce apoptosis of pancreatic, breast and lung cancer cell lines in a dose and time-dependent manner.

In conclusion, ROR1 may be promising therapeutic target for both ROR1 TKI and mAbs in CLL and other malignancies.

3 AIMS OF THE STUDY

- Structurally and functionally characterize the receptor tyrosine kinase ROR1 in chronic lymphocytic leukemia as a basis for the development of ROR1 directed targeted therapy.

- Develop prototype ROR1 directed unconjugated monoclonal antibodies as a basis for development of therapeutic monoclonal antibodies.

- Develop ROR1 tyrosine kinase inhibitor as a drug candidate.

4 RESULTS AND DISCUSSION

4.1 Paper I

Monoclonal antibodies against ROR1 induce apoptosis of CLL cells

Leukemia, 26:1348-1355, 2012

Aim and background of the study: ROR1 is overexpressed in CLL cells both at gene and protein levels. The extracellular part of ROR1 consists of an immunoglobulin (Ig) like domain, a cysteine-rich domain (CRD) and a kringle domain (KNG) while the intracellular part contains a kinase domain, which is constitutively phosphorylated in CLL cells.

The aim of the study was to produce monoclonal antibodies against different epitopes of the external part ROR1 and analyze their apoptotic activity in CLL cells.

Results: Five anti-ROR1 mAbs were produced; one against Ig-like domain (Ig 3B8 mAb), two against CRD domain (CRD IC11 and CRD ID8 mAbs) and two against KNG domain (KNG 4A7and KNG 4C10 mAbs). All 5 anti-ROR1 mAbs were tested for staining of ROR1 surface expression and showed a strong ROR1 reactivity of CLL cells from 20 patients. The frequency of ROR1+ cells were as follow; Ig 3B8: 92±1% (mean±SEM)(range: 77-98%), CRD 1C11: 94±1% (72-98%), CRD ID8: 88±1% (72-94%), KNG 4A7: 93±1 (74-98%), KNG 4C10: 94±1 (84-99%). No ROR1 expression was detected on PBMC of healthy donors. The expression of ROR1 was slightly higher in progressive compared to non-progressive disease.

Functional activity of these anti-ROR1 mAbs was assessed for apoptosis measured by the annexin V/PI assay. 18h incubation of CLL cells with anti-ROR1 mAbs was chosen as optimal. All five anti-ROR1 mAbs induced significant apoptosis in CLL cells as compared to controls. Three anti-ROR1 mAbs i.e. CRD 1C11, CRD 1D8 and KNG 4C10 were the best to induce apoptosis. Apoptosis induction was confirmed by PARP cleavage. These three anti-ROR1 mAbs induced higher apoptosis than the murine or chimeric rituximab (anti-CD20) ($p<0.0001$). Cross-linking of anti-ROR1 antibodies with $F(ab')_2$ fragments significantly enhanced apoptosis. CRD 1D8 and KNG 4A7 induced apoptosis by complement dependent cytotoxicity in a similar way as rituximab. KNG 4A7 also induced significant killing via antibody-dependent cellular cytotoxicity (ADCC) of CLL cells.

Discussion: The main goal of the study was to produce a panel of monoclonal antibodies against different domains of extracellular part of ROR1 and analyze their cytotoxic capacity.

ROR1 has been shown to be constitutively phosphorylated at the intracellular kinase domain and silencing of ROR1 by siRNA induced specific apoptosis of CLL cells [239, 244]. ROR1 is not expressed on adult lymphoid and non-lymphoid cells. Thus, ROR1 might be a suitable candidate for targeted therapy. A set of mouse mAbs against different ROR1 epitopes was produced to explore the functional roles of different epitopes. The antibodies raised were against different peptide sequences, Ig-like domain(23aa), CRD(15aa) and KNG(19aa) derived of extracellular part of ROR1.

Different staining pattern of the same patient by different ROR1 mAbs may suggest structural heterogeneity in the expression of ROR1 and/or difference in the binding affinity of mAbs for different epitopes on ROR1. The antibodies against CRD and KNG were

highly effective in inducing apoptosis. This finding may be interesting as the CRD and KNG regions are assumed to contain binding sites for Wnt-family and non-Wnt ligand binding proteins [229, 231, 302, 303]. Apoptosis induction was confirmed by activation of caspase 8 and caspase 9, covering both intrinsic and extrinsic apoptotic pathways [304]. Apoptosis induced by ROR1 mAbs alone was not a result of immune cells as more than 90% of PBMCs were CD19/CD5+ B cells and only 3-4% natural killer cells (NK) and monocytes. In vitro, 10-50 times more effector cells compared to target cells are needed for a detectable ADCC activity [305]. The IgG1 ROR1 mAbs also induced complement lysis, which may suggest a highly conserved CH2 domain of IgG compatible with complement activation [306]. It is also known that all CLL cells may not express detectable target molecules recognized by a given mAb [307, 308], which is in line with our studies, as we could not detect ROR1 on all leukemic cells. This could be due to either low surface expression of ROR1 or presence of a subpopulation of ROR1-negative leukemic cells.

The expression of ROR1 on CLL cells increased when disease progressed from indolent to aggressive phase. A high density of RTKs on tumor cells is linked to high proliferative potential as well as survival advantage of the tumor cells as an indicator of poor clinical prognosis [309, 310].

Three of our ROR1 mAbs could induce higher cytotoxicity than murine and chimeric anti-CD20 mAbs. Rituximab alone is known to induce a low degree of apoptosis [311], also confirmed by our study but cross-linking of rituximab, CDC and ADCC was required to enhance the cytotoxicity [312]. The difference in the apoptosis induction by ROR1 mAbs could be explained by different binding regions, mAbs of different isotypes and also difference in the expression of ROR1 between patients. However, we could not find any correlation between ROR1 expression and apoptosis induction by ROR1 mAbs, which has been reported for mAbs against CD20 and other RTKs [313, 314].

In summary, mAbs were raised against different epitopes of ROR1. These ROR1 mAbs alone induced apoptosis of CLL cells.

4.2 Paper II

The tyrosine kinase receptor ror1 is constitutively phosphorylated in chronic lymphocytic leukemia (CLL) cells

PLoS One, 8: e78339, 2013

Aim and background of the study: The intracellular part of ROR1 contains a tyrosine kinase domain, which is presumed to be involved in intracellular signaling. ROR1 silencing by specific siRNA and anti-ROR1 mAbs against extracellular domains induced apoptosis.

The aim of the study was to analyze different isoforms of ROR1 and their phosphorylation pattern.

Results: A polyclonal anti-ROR1 antibody (pROR1Ab) was produced against the intracellular phosphorylated kinase domain of ROR1. The specificity of anti-pROR1 pAb was confirmed by treatment with a lambda phosphatase enzyme that resulted in disappearance of phosphorylated bands but not the total protein. ROR1 was shown to be constitutively phosphorylated at tyrosine and serine residue in all tested CLL patients.

ROR1 proteins of 105 and 130 kDa were identified in CLL cells by Western blot. The 105 kDa is considered to be the native and unglycosylated whereas 130 kDa band is supposed to be the fully matured and glycosylated protein. The native and immature (non-glycosylated) 105 kDa ROR1 isoform was found to be more frequently expressed in non-progressive as compared to progressive CLL patients, which may indicate a correlation between glycosylation and disease progression as have been shown for other diseases [315]. In addition to 105 and 130 kDa, other bands of 260 kDa and 64 kDa respectively were also noted. The 260 kDa band may represent dimerized ROR1 and the 64 kDa a truncated ROR1. The expression of different ROR1 isoforms was different among patients. The unglycosylated ROR1 105 kDa isoform was observed more frequently in non-progressive as compared to progressive patients.

There was no difference in the phosphorylation intensity for 105 kDa ROR1 isoform between progressive and non-progressive CLL patients but was significantly higher for the 130 kDa ROR1 isoform in progressive as compared to non-progressive CLL patients. Phosphorylated 105 and 130 kDa were localized both to nucleus as well as cytoplasm while 64 kDa was only detected in nucleus. ROR1 was shown to be constitutively phosphorylated at serine and tyrosine residues in all tested CLL patients.

Discussion: Similar to other RTKs, ROR1 could be found of different isoforms, the most prominent being the 105 and 130 kDa bands, localized in cytoplasm and nucleus. We could also detect a 260 kDa band presumably a dimerized form of the ROR1, similar to ROR2 which has been shown to be homodimerized [316, 317] as well as heterodimerized [318].

Posttranslational modification of ROR1 might be of functional significance. ROR1 was shown to display non-unique glycosylation [273], similar to our study. There are potentially seven asparagine residues for N-linked glycosylation and more than 24 serine and threonine residues for O-glycosylation that may contribute to different glycosylation patterns. The glycosylation pattern may vary from patient to patient and also between cells of an individual CLL clone. Tunicamycin and brefeldin induced deglycosylation resulting in 100 and 115 kDa bands compared to untreated 130 kDa [273]. Our finding also confirmed that non-glycosylated immature ROR1 105 kDa isoform was predominantly expressed in

non-progressive patients, suggesting that glycosylation might be associated with disease activity as has been shown for other diseases [315].

Truncated ROR1 containing 388 amino acids (40 kDa) has been detected in fetal CNS tissues and lymphoma and leukemic cell lines [231]. The truncated ROR1 could be due to alternative splicing [231] or a cleaved C-terminal fragment (CTF) produced by surface proteolytic enzymes [319]. We found that the phosphorylated truncated ROR1 was 64 kDa and present in nucleus, suggesting to act as a transcription factor [268]. The cytoplasmic domain of ROR1 may play an important role in cell migration and cytoskeleton remodeling by activating genes involved in actin cytoskeleton [320]. Truncated HER2 (P95 HER2) has been associated with poor prognosis in breast cancer [321].

ROR1 and ErbB2 or HER2/neu are members of type I RTKs that contribute to malignant phenotype of several cancers. High expression of HER1/2, VEGFR2 / KRD and estrogen receptors and their tyrosine phosphorylation in breast cancer was found to correlate to poor prognosis [322-325]. Fully mature and glycosylated ROR1 may be preferentially expressed on the surface, suggesting a relation between ROR1 maturity, phosphorylation and disease progression. Mouse ROR1 was shown to be phosphorylated at serine position 652, located in the activation segment [326]. NetPhosK software revealed two tyrosine residues at 641 and 646 with the probability to be phosphorylated by tyrosine kinase. The peptide used for the production of anti-pROR1 was within the activation segment of two tyrosine and one serine residues.

Constitutive phosphorylation of RTKs including ligand-dependent or ligand-independent plays an important role in cell signaling [327, 328]. Tyrosine phosphorylation triggered by other tyrosine kinases is a general mechanism for RTKs. Whether the constitutive phosphorylation of ROR1 at tyrosine [329-331] and serine [332, 333] residues is auto phosphorylated or not is not clear. ROR1 was shown to have a low degree of auto phosphorylation (low catalytic activity), similar to ErbB3 but weaker than ErbB2 [275]. Met oncogene which is highly overexpressed in CLL cells [334] has been suggested to phosphorylate ROR1 at tyrosine residue by trans-phosphorylation [275]. However, the Met oncogene might not be the only one to activate ROR1 as cell lines with low Met level and activity could still show ROR1 phosphorylation. No mutational activations have been shown for ROR1.

Binding of antibodies against the CRD and KNG domains induced dephosphorylation of ROR1 preceding apoptosis. In case of ligand-independent ROR1 phosphorylation, ROR1 mAbs may induce structural conformation that might activate phosphatases causing dephosphorylation of ROR1 and subsequently affect downstream signaling pathways [329-331, 333]. Wnt5a has also been suggested to be ligand for ROR1 [258] and CLL cells have been shown to produce Wnt5a [335]. In case of ligand dependent (Wnt5a) activation, the ROR1 mAbs may disrupt and prevent ligand binding.

In summary, ROR1 could be detected in different isoforms that were constitutively phosphorylated both at tyrosine and serine residues. The intensity of phosphorylation was significantly higher in progressive than non-progressive CLL cells. Inhibition of ROR1 phosphorylation was followed by apoptosis in CLL cells.

4.3 Paper III

The PI3K/AKT/mTOR pathway is involved in direct apoptosis of CLL cells induced by ROR1 monoclonal antibodies

British Journal of Haematology, 169(3):455-458, 2015

Aim and background of the study: ROR1 is a RTK expressed in many malignancies. It is a surface receptor that is involved in cell-cell interactions, proliferation, signaling and survival, and has been suggested to be a "survival factor" in CLL [235]. High ROR1 expression was detected in CLL patients with progressive disease compared to non-progressive disease [245]. Knockdown of ROR1 by siRNA as well as inhibition of ROR1 by monoclonal antibodies (mAb) induced apoptosis in CLL cells [244, 245].
The aim of the study was to analyze the cytotoxicity of ROR1 monoclonal antibodies and the effect on PI3K/AKT/mTOR signaling pathway.

Results: We have previously demonstrated that anti-CRD ROR1 mAb induced dephosphorylation of ROR1 and subsequently apoptosis of CLL cells [239]. Treatment of CLL cells with anti-CRD ROR1 monoclonal antibody in addition to apoptosis induced dephosphorylation of ROR1 as well as of the downstream proteins SRC, PI3Kδ, AKT, mTOR, CREB. However, an antibody against the Ig-like domain of ROR1 did not induce apoptosis.

Discussion: Anti-CRD ROR1 mAb inhibited the PI3K/AKT/mTOR pathway, which plays an important role in the tumorigenesis [336]. This pathway has been used as validated target for EGFR and HER-2, suggesting that ROR1 like the EGFR and HER-2 RTKs might be an interesting therapeutic target for anti-ROR1 mAbs in malignancies positive for ROR1. Recently, another study also has shown that anti-ROR1 D10 mAb which is directed against an epitope close to the CRD region induced apoptosis [337] whereas an anti-ROR1 mAb against the Ig-like domain did not induce apoptosis [338] similar to the present study. Our anti-CRD ROR1 mAb also induced ADDC and CDC, suggesting a multifunctional antibody.

In summary, an anti-CRD ROR1 mAb induced direct apoptosis of CLL cells associated with inhibition of PI3K/AKT/mTOR pathway. Further studies are needed to explore ROR1 as therapeutic target monoclonal antibodies in the treatment of malignant diseases. The selection of epitope of the external part recognized by the monoclonal antibody for apoptosis inductions seems to be very important to obtain a therapeutic effective monoclonal antibody.

4.4 Paper IV

Dishevelled proteins are significantly upregulated in chronic lymphocytic leukemia

Experimental Hematology, accepted, pending revision 2015.

Aim and background of the study: Dishevelled (DVL) proteins are important proteins of Wnt signaling pathways and have been shown to be upregulated in several cancers. Three DVL homologs have been identified in human and mouse, which consist of about 750 amino acids and show high sequence homology. They are expressed during early embryogenesis [339] and knockdown mice (DVL2 -/- and DVL3 -/-) die prenatally [340, 341].

The aim of this study was to analyze the expression of DVL proteins (DVL1, DVL2, and DVL3) in chronic lymphocytic leukemia (CLL).

Results: DVL1, DVL2 and DVL3 proteins were found to be highly upregulated in both progressive and non-progressive CLL patients but low or undetectable in peripheral blood of healthy donor. DVL1 and DVL3 were significantly more expressed in progressive than non-progressive CLL disease whereas expression of DVL2 was more pronounced in non-progressive than progressive cases. All three DVL proteins were phosphorylated both at tyrosine and serine residues. Inhibition of ROR1 with anti-CRD mAb induced apoptosis of CLL cells and dephosphorylated tyrosine and serine residues of DVL2 and DVL3. Knockdown of DVL1, DVL2 and DVL3 with siRNA in CLL cells and CLL derived cell line (EHEB) was successfully carried out but no apoptosis was detected.

Important mediators of canonical and non-canonical pathways were also analyzed. GSK-3β and β catenin of the Wnt canonical pathway were detected both in progressive and non-progressive CLL disease. GSK-3β was found to be statistically more phosphorylated in progressive than in non-progressive cases whereas expression of β catenin was more pronounced in non-progressive compared to progressive CLL. The difference was statistically significant. AKT was found to be phosphorylated in CLL patients, however the intensity of phosphorylation was significantly higher in non-progressive cases than in progressive CLL cases. PKC and GSK-3α of Wnt non-canonical pathway were also detected but there was no significance difference in the intensity of phosphorylation between progressive and non-progressive CLL patients.

Discussion: The aim of the study was to analyze the expression of DVL proteins, their phosphorylation status and downstream proteins of Wnt signaling pathways in CLL cells.

DVL proteins are important mediators of Wnt signaling pathway. Wnt signaling pathways are upregulated in several tumors and play a role in cell proliferation and migration [342]. Wnt signaling pathways are activated by binding of Wnt ligands to a Frizzled receptor inducing in phosphorylation of DVL proteins, which then activate downstream signaling pathways involved in cell survival and proliferation [343]. We found that DVL1, DVL2 and DVL3 were highly upregulated in both progressive and non-progressive CLL patients and we could also show phosphorylation both at tyrosine and serine residues. Knockdown of DVL proteins were performed to assess their role in survival, however, knockdown of DVLs did not induce apoptosis, suggesting they may not have a direct role in survival of CLL cells.

Activation of Wnt signaling in the presence of the LRP6 co-receptor inhibited degradation of β- catenin by GSK-3β [344]. High level of β-catenin and phosphorylated GSK-3β were detected in CLL cells, suggesting accumulation of β-catenin in the cytoplasm followed by translocation to nucleus where it may activate target genes involved in cell cycle progression, differentiation and adhesion.

ROR1 may also be involved in the activation of non-canonical pathways regulating downstream signaling proteins related to cytoskeletal formation and cell adhesion. Inhibition of ROR1 by anti-ROR1 antibody induced dephosphorylation of DVL2 and DVL3 proteins. Non–canonical proteins such as AKT, PKC and GSK-3α are upregulated in CLL cells. Activation of both canonical and non-canonical pathways may not only prolong cell survival but could also support cell proliferation and migration [345]. In non-progressive CLL, high level of DVL2 and activated AKT may inhibit GSK-3β resulting in the accumulation of β-catenin and subsequently contributing to cell survival, whereas in progressive CLL, high levels of DVL1 and DVL3 may trigger Wnt/PCP pathway resulting in cell proliferation and migration. This observation is in agreement with studies by Kaucka M et al [346].

In summary, all three DVLs were highly upregulated and phosphorylated in CLL patients but not detectable in peripheral blood of healthy donors. ROR1 inhibition induced dephosphorylation of DVL2 and DVL3. Knockdown of DVL1, DVL2 and DVL3 did not induce apoptosis in CLL derived cell line suggesting no direct role in the cell survival. More studies are needed to further explore the role of different DVL proteins and the mechanism of action in CLL.

4.5 Paper V

On-target effect of a ROR1 tyrosine kinase small molecule inhibitor by apoptosis mediated death in chronic lymphocytic leukemia cells in vitro and in vivo

Submitted

Aim and background of the study: Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is expressed in chronic lymphocytic leukemia (CLL) and other cancers both at gene and protein levels. ROR1 belongs to type 1 of receptor tyrosine kinases (RTKs) family. ROR1 consists of extracellular, transmembrane and intracellular parts. The extracellular part contains an immunoglobulin-like (Ig) domain, the kringle domain (KNG), and a cysteine-rich domain (CRD). The intracellular part of ROR1 contains a tyrosine kinase (TK) domain that plays an important role in transducing signals downstream. Serine/threonine and proline-rich motifs are also present on the intracellular cytoplasmic part of ROR1. ROR1 is expressed during embryogenesis and plays a crucial role in skeletal, lung and neural organogenesis but is repressed in adult tissues [347]. ROR is involved in survival, differentiation, proliferation and polarity as well as tumor like behavior such as migration and invasion [348, 349].

The main goal of the study was to develop a specific tyrosine kinase inhibitor against the intracellular kinase domain and assess the apoptotic effect in CLL cells.

Results: A biochemical-screening assay was used to select a potential series of small molecules from a chemical library and then modifications were made for a better mode of action and activity. KAN0439834 was chosen as a promising small molecule tyrosine kinase inhibitor drug candidate with specific anti-ROR1 activity.

Treatment of CLL cells with KAN0439834 (24h) induced equally well apoptosis of CLL cells from progressive as well as non-progressive disease. KAN0439834 had the same activity on fludarabine resistant CLL cells regardless of 17p- status. The cytotoxic activity for peripheral blood mononuclear cells (PBMC) from healthy donors was 60-fold less. Apoptosis was measured by annexin V/PI as well as the MTT assay and confirmed by PARP cleavage, caspase-3 and downregulation of the anti-apoptotic proteins Bcl-xL and Mcl-1. KAN0439834 treatment of CLL cells dephosphorylated ROR1 in a dose-dependent manner as early as after 15 minutes. ROR1 downstream signaling proteins including SRC, PI3K, AKT and CREB seemed to have also reduced their degree of phosphorylation intensity upon treatment with KAN0439834 after 2h.

Cytotoxicity and specificity of KAN0439834 were compared to other kinase inhibitors, which are in clinical trials. KAN0439834 was found to be more potent and highly specific compared to other kinase inhibitors in vitro as idelalisib, ibrutinib, dasatinib, sunitinib, gefitinib and others.

NOD-SCID mice were xenografted with human leukemic CLL cells. Oral treatment with KAN0439834 significantly reduced the number of leukemic cells and the degree of ROR1 phosphorylation. Toxicity was minimal.

Discussion: Biochemical assay was used to screen a large library of 80,000 small molecules for anti-ROR1 activity. High-throughput screening (HTS) generated a set of 222 primary hits tested in dose-response experiments. The hits were subjected to a single point

redox assay for identification of redox active compounds. 77 hits showed redox activity and were removed. Data on identity, purity, chemical stability and solubility was collected. 6 chemical clusters and 4 singletons were identified as promising. A chemistry-based program on one promising hit (KAN0173631) was initiated. 900 analogs have been synthesized, resulting in the present lead series. KAN0439834 is one of the promising candidates in the lead series with a molecular weight of 530 kDa. KAN0439834 contains no apparent toxicophores and is metabolically stable in human hepatocytes.

ROR1 has been shown to be highly expressed at gene and protein levels in CLL cells as well as other malignancies [235, 247, 252, 255]. Therapeutic approaches are underway to target ROR1 in different malignancies. In paper I, we showed that the CRD and KNG domains of the extracellular part of ROR1 could be targeted by monoclonal antibodies inducing apoptosis in CLL cells. We have also shown that ROR1 is constitutively phosphorylated in the TK domain of CLL cells [239]. Our own developed drug KAN0439834 against the intracellular kinase domain of ROR1 was tested in CLL cells. KAN0439834 induced specific apoptosis of CLL cells in a time and dose dependent manner. Since KAN0439834 is against the kinase domain, phosphorylation status of ROR1 was assessed after treatment with the drug. KAN0439834 reduced phosphorylation of ROR1 within 15 minutes of treatment with CLL cells. KAN0439834 also triggered higher cytotoxicity of leukemic B cells but not of normal T cells from the same CLL patients. Specificity index for KAN0439834 was in the range of 60, i.e. killing CLL cells 60 times more than healthy PBMCs. Apoptosis was confirmed by PARP cleavage and downregulation of anti-apoptotic proteins as Mcl-1 and Bcl-xL. Treatment of CLL cells with KAN0439834 reduced the phosphorylation of SRC, PI3K, AKT and CREB but longer exposure time was needed as compared to dephosphorylation of ROR1.

KAN0439834 was found to be equally effective against fludarabine resistant CLL cells with or without 17p- as indolent CLL cells. Other kinase inhibitors currently in preclinical and clinical studies were compared with KAN0439834. The specificity index for KAN0439834 was about 60 (median value) as compared to < 5 for other kinase inhibitors. Ibrutinib (PCI-32765), which has been approved for CLL and MCL, showed lesser cytotoxic activity and specificity than KAN0439834 in our in vitro assays.

In summary, KAN0439834 was developed as a completely new drug and showed high potency and specificity for CLL cells. KAN0439834 was equally effective in leukemic cells from all CLL groups irrespective of disease stage. KAN0439834 dephosphorylated ROR1. In a mouse model KAN0439834 confirmed the antitumor effect. More studies are warranted to fully understand the therapeutic value of ROR1 TKI. However, this is the first candidate of a novel class of anti-cancer drugs.

5 CONCLUSIONS

ROR1 is a receptor tyrosine kinase like orphan receptor 1, located on chromosome 1 (1p31.3), 2814bp and 937 amino acids sequence in human. Initially ROR1 was thought to be exclusively expressed in CLL but now we know that ROR1 is expressed in other cancers including hematological malignancies as well as solid tumors.

ROR1 exists in different isoforms due to posttranslational modifications. The native immature and unglycosylated ROR1 is present as a 105 kDa protein and the fully mature, glycosylated ROR1 as a 130 kDa. We also showed a 64 kDa truncated ROR1 localized to the nucleus. The truncated 64 kDa ROR1 might act as transcription factor, although the target genes are not yet identified. A band of 260 kDa was also detected presumably representing a dimerized form of ROR1. All isoforms of ROR1 were shown to be constitutively phosphorylated at tyrosine and serine residues. Phosphorylation of ROR1 might be important in transducing downstream signaling which may contribute to cell survival.

Monoclonal antibodies (mAbs) were produced against different extracellular domains of ROR1. The expression of ROR1 was higher in progressive as compared to non-progressive disease. All mAbs showed cytotoxicity against CLL cells in a dose and time dependent manner. mAbs against the CRD and KNG domains were the most effective in inducing apoptosis of CLL cells. CRD and KNG domains contain binding modules for Wnt and non-Wnt ligand family proteins. Cross-linking of ROR1 mAbs with (Fab') fragments enhanced apoptosis of CLL cells. The mAbs were also active in ADCC and CDC. CRD and KNG ROR1 mAbs showed higher cytotoxicity than rituximab.

An anti-phospho ROR1 polyclonal antibody (pROR1 pAb) was produced against the intracellular kinase domain of ROR1. ROR1 was found to be constitutively phosphorylated in CLL. The anti-pROR1 pAb could detect phosphorylation of different isoforms. Phosphorylation intensity of the 130 kDa ROR1 isoform was higher in progressive than in non-progressive CLL. Treatment with anti-ROR1 CRD and KNG mAbs dephosphorylated ROR1 and subsequently induced apoptosis. ROR1 dephosphorylation was accompanied by downregulation of downstream proteins as SRC, PI3K, AKT, mTOR and CREB.

DVL proteins are important proteins of Wnt signaling pathways and have been shown to be highly expressed in various cancers. DVL1, DVL2 and DVL3 were highly upregulated in CLL cells. The expression of DVL1 and DVL3 was found to be higher in progressive than in non-progressive CLL cells while DVL2 was more pronounced in non-progressive than in progressive CLL. DVL proteins were phosphorylated both at tyrosine and serine residues. ROR1 inhibition with anti-ROR1 mAb induced dephosphorylation of DVL2 and DVL3. Knockdown of DVL proteins in the CLL cells and EHEB cell line did not induce apoptosis, suggesting that DVL proteins may not have a direct role in the survival of CLL cells.

A biochemical assay was performed to screen a library of small molecules for anti-ROR1 activity and a synthesizing program was initiated. A completely novel molecule (KAN0439834) was produced with a molecular weight of 530 kDa. KAN0439834 induced apoptosis of CLL cells in a dose dependent manner and dephosphorylated ROR1 within 15 minutes at a dose of 50 nM. Apoptosis was confirmed by PARP cleavage and downregulation of anti-apoptotic proteins as Mcl-1 and Bcl-xL. KAN0439834 was equally

effective in CLL cells derived from patients irrespective of disease stage. KAN0439834 was found to be more active than other tyrosine kinase inhibitors in vitro, which are approved, including ibrutinib and idelalisib. KAN0439834 showed a high specificity against CLL cells. KAN0439834 significantly reduced the number of CD45+CD19+ ROR1+ cells in non-SCID mice xenografted with human CLL cells. KAN0439834 has also showed high cytotoxic capability against pancreatic, breast and lung cancer cell lines (data not published).

In summary, ROR1 is expressed in CLL cells as well as in other malignancies. Anti-ROR1 mAbs against KNG and CRD domains as well as a small molecule TKI, KAN0439834 induced specific apoptosis of CLL cells. KAN0439834 is the first drug candidate of a novel class of anti-cancer drugs.

6 FUTURE PERSPECTIVES

CLL is the most common leukemia in the Western world. During the last decade, an enormous progress has been seen in the understanding of the molecular and cellular biology of the disease as well as in the treatment of the patients. These achievements have provided opportunities to design and develop innovative therapeutic strategies for CLL. Despite these developments, CLL still remains an incurable disease although an improvement in survival is noted. The availability of new active agents has improved the outcome for CLL patients but elimination of the malignant cells and normalization of the immune system has not yet been reached. Still most patients with active CLL die from the disease or its complications.

ROR1 has during the last decade shown up to be an interesting structure for targeted therapy. There are several reasons for that, the unique expression in malignant cells compared to normal tissues, the importance of ROR1 for several functions of malignant cell survival, proliferation, metabolism and metastasizing capacity. However, it is urgently needed to have a better understanding on the biology of ROR1 to be able to optimize therapeutic approaches, in spite of that promising drug candidates have been produced.

Many RTKs have been implicated of significant importance in various human malignancies [220, 350]. The ROR1 RTK has been shown to be highly expressed in many cancers but not in normal tissues. The role of ROR1 in cancer is not fully known and needs to be further explored.

Targeting of ROR1 with small molecules and mAbs induced apoptosis of CLL cells in vitro and in vivo. Humanized antibody (cirmtuzumab) against ROR1 has already entered clinical trials in CLL (https://clinicaltrials.gov/ct2/show/NCT02222688). However, it is important to select an optimal epitope for the external part of ROR1 to utilize maximum apoptotic effect. In this regard cirmtuzumab may not be the best and ongoing clinical trial has been halted due to insufficient clinical effect. Several other malignancies may also be candidates for ROR1 mAb therapy as ALL, breast cancer, lung cancer, pancreatic cancer, ovarian cancer and non-Hodgkin's lymphoma of the B cell type. Clinical trial selecting mAbs against the CRD and KNG region might be of preference. In this regard, it is also of interest to note that ROR1-CART is in clinical development for CLL and pancreatic carcinoma.

ROR1 TKI has shown promising results in a mouse CLL model and pancreatic carcinoma animal studies are in progress. Step by step pharmacological characteristics of ROR1 TKI will be optimized with improved efficacy. The first clinical trials are expected to be initiated within 1-2 years. In addition to CLL ROR1 TKI might also be tested in breast cancer, lung cancer, pancreatic cancer, ovarian carcinoma and different ROR1 expressing hematological malignancies.

ROR1 as a uniquely expressed antigen of tumor cells might also be of interest to be a target for active specific immunotherapy. ROR1 seems to be spontaneously recognized by the patient's immune system. Pre-clinical animal studies are in progress. A ROR1 therapeutic cancer vaccine might be optimally used when cytoreduction has been achieved with ROR1 TKI and/or mAb to maintain disease control.

To be able to optimize ROR1 therapy, biomarker tests should be developed (companion diagnostics). Such an approach should facilitate selection of patients and the

therapeutic evaluation of early clinical trials. Our pROR1 antibody in combination with ROR1 antibodies might be a valuable tool.

Targeting ROR1 is a new principle with other mechanisms of action than existing anti-cancer drugs. ROR1 directed drugs represent a novel class of anti-cancer drugs and might add to the therapeutic arsenal and as such improve the prognosis for several types of malignancies, hematological diseases as well as solid tumors.

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