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ROR1 – a druggable target: preclinical studies of ROR1 and combinatorial partners in malignancies

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ROR1 – a druggable target: preclinical studies of ROR1 and combinatorial partners in malignancies

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By

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“What you seek is seeking you.”

— Mawlana Jalal-al-Din Rumi—

To my family

ABSTRACT

Cancer cells are masters of adaption, and their ability to sustain proliferative signaling presents a complicated challenge to cancer treatment. Therefore, novel drugs that attack key drivers of cancer cell growth are urgently needed. Targeted cancer therapy aims to selectively kill tumor cells and block their growth by interfering with specific molecular survival mechanisms. Tyrosine kinase orphan receptor 1 (ROR1) is abundantly expressed in numerous human cancers, whereas it has lower expression in most normal adult tissues. ROR1 is essential for oncogenesis and tumor cell survival. Based on these characteristics, ROR1 is considered to be an attractive target for a targeted drug strategy. The aim of the thesis was to understand how ROR1 activity is regulated in malignancies and identify the mechanism of action (MOA) of the small molecule inhibitor of ROR1 in killing tumor cells.

The **first study** investigated the *in vitro* activity of the first-in-class small molecule ROR1 inhibitor (KAN0439834) against a number of human pancreatic carcinoma cell lines. The result showed that the combination of KAN0439834 with ibrutinib or erlotinib had a significant additive apoptosis effect, which supports further investigation as both drugs are currently in clinical trials concerning pancreatic carcinoma.

The **second study** investigated the functional and clinical inhibitory properties of ROR1 in diffuse large B-cell lymphoma (DLBCL). Our data show that a second-generation small-molecule of ROR1 inhibitor (KAN0441571C) in combination with venetoclax achieves the desired outcome in apoptosis of DLBCL tumor cells. Furthermore, KAN0441571C caused significant tumor reduction in zebrafish transplanted with a $ROR1^{+}$ DLBCL cell line.

The **third study** assessed the result of KAN0441571C treatment in chronic lymphocytic leukemia (CLL) cells from six patients collected before and after the acquisition of resistance to ibrutinib. The result showed an induction of apoptosis in both ibrutinib-sensitive and -resistant CLL cells by ROR1 inhibitor. However, the combination of ROR1 inhibitor and venetoclax had a synergistic cell death in ibrutinib-resistant cells.

The **fourth study** examined the inhibition of ROR1 in human MCL cell lines and primary MCL cells. We described a combinatorial approach of KAN0441571C with ibrutinib, venetoclax, idelalisib, everolimus, and bendamustine which had an additive or mostly synergistic apoptotic effect in MCL cells.

The **fifth study** investigated the clinical and functional expression of ROR1 in non-small cell lung cancer (NSCLC) cases and the outcomes of ROR1 inhibition in NSCLC cell lines. Dephosphorylation of ROR1 by KAN0441571C results in time- and dose-dependent apoptosis of NSCLC cell lines. In addition, targeting NSCLC cells by using ROR1 and EGFR inhibitors showed synergistic or additive effects on lung cancer cells.

In conclusion, these studies reveal novel functions for ROR1 tyrosine kinase in cancer cells and demonstrate that blocking its function by a small molecule ROR1 inhibitor results in a cancer-specific apoptotic effect, both alone and in combination with conventional therapies, which could lead to more effective cancer therapy.

LIST OF SCIENTIFIC PAPERS

I. Daneshmanesh A.H, Hojjat-Farsangi M, **Ghaderi A**, Moshfegh A, Hansson L, Schultz J, Vågberg J, Byström S, Olsson E, Olin T.H, Österborg A, Mellstedt H. A receptor tyrosine kinase ROR1 inhibitor (KAN0439834) induced significant apoptosis of pancreatic cells which was enhanced by erlotinib and ibrutinib. *PLoS ONE* 2018,13(6): e0198038.

II. **Ghaderi A**, Daneshmanesh A.H, Moshfegh A, Kokhaei P, Vågberg J, Schultz J, Olin T, Harrysson S, Smedby KE, Drakos E, Rassidakis G.Z, Österborg A, Mellstedt H, Hojjat-Farsangi M. ROR1 is Expressed in Diffuse Large B-Cell Lymphoma (DLBCL) and a Small Molecule Inhibitor of ROR1 (KAN0441571C) Induced Apoptosis of Lymphoma Cells. *Biomedicines* 2020; 8(6):170.

III. **Ghaderi A**, Okhovat M.A, Sekar Sih Wikanthi L, Svensson A, Palma M, Schultz J, Olin TH, Österborg A, Mellstedt H, Hojjat-Farsangi M. A ROR1 small molecule inhibitor (KAN0441571C) induced significant apoptosis of ibrutinib-resistant ROR1^{+} CLL cells. *eJHaem* 2021; 2:498–502.

IV. **Ghaderi A**, Aschan J, Okhovat M.A, Mozaffari F, Svensson A, Sander B, Schultz J, Olin TH, Österborg A, Mellstedt H, Hojjat-Farsangi M. A ROR1 small molecule inhibitor (KAN0441571C) induced significant apoptosis of mantle cell lymphoma (MCL) cells. *Manuscript*

V. **Ghaderi A**, Lehto J, Okhovat M.A, De Petris L, Manuchehri E, Kokhaei P, Daneshmanesh A.H, Moshfegh A, Rassidakis GZ, Schultz J, Olin TH, Österborg A, Mellstedt H, Hojjat-Farsangi M. ROR1 targeting tyrosine kinase inhibitor induced significant apoptosis of non-small cell lung cancer (NSCLC) cells in combination with erlotinib and ibrutinib. *Manuscript*

LIST OF ABBREVIATIONS

ADC	Antibody-dependent cellular cytotoxicity
ADCC	Antibody-dependent cellular cytotoxicity
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
Akt	Protein kinase B
ATP	Adenosine triphosphate
B-ALL	B-Cell acute lymphoblastic leukemia
BAX	BCL2 associated X protein
Bcl-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
BCR	B cell receptor
BTK	Bruton's tyrosine kinase
CAR-T	Chimeric antigen receptor T
CDC	Complement-dependent cytotoxicity
CK1	Casein kinase 1
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
CRD	Cysteine-rich domain
CREB	cAMP response element-binding protein
DLBCL	Diffuse large B cell lymphoma
EC_{50}	Half maximal effective concentration
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
EMT	Epithelial–mesenchymal transition
ERK	Extracellular signal-regulated kinase
FGFR	Fibroblast growth factor receptor
FISH	Florescent *in situ* hybridization
GSK3ß	Glycogen synthase kinase 3 beta
HER	Human embryonic kidney cells
Ig	Immunoglobulin
KDa	Kilodalton
KNG	Membrane-proximal kringle
KRAS	Kirsten rat sarcoma virus
LRP6	Low density lipoprotein receptor-related protein 6
mAB	Monoclonal Antibody
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex

MCL	Mantle cell lymphoma
Mcl-1	Myeloid leukemia cell differentiation protein 1
miRNA	Micro RNA
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MZL	Marginal zone lymphoma
NFκB	Nuclear factor kappa-light chain enhancer of activated B cells
NHL	Non-Hodgkin lymphoma
NK cells	Natural killer cells
NRTK	Nonreceptor tyrosine kinases
NTRKR1	Neurotrophic tyrosine kinase receptors 1
NTRKR2	Neurotrophic tyrosine kinase receptors 2
OS	Overall survival
p53	Tumor Protein P53
PARP	Poly (ADP-ribose) polymerase 1
PBMC	Peripheral blood mononuclear cells
PDGFR	Platelet-derived growth factor receptor
PFKFB	Phosphoinositide 3-kinase
PI	Propidium iodide
PI3K	Phosphoinositide-3 kinase
PRD	Proline-rich domain
ROR1/2	Receptor tyrosine kinase-like orphan receptor 1/2
RTK	Receptor tyrosine kinases
scFv	Single-chain variable fragment
SH2	Src homology 2
siRNA	Small interfering RNA
SRC	Proto-oncogene tyrosine-protein kinase
S/TRD	Serine/threonine rich domain
SYK	Spleen tyrosine kinase
TITF1	Thyroid-specific-enhancer-binding protein
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TNBC	Triple-negative breast cancer
TP53	Tumor protein p53
VEGFR	Vascular endothelial growth factor receptor
Wnt	Wingless/integrated

CONTENTS

1 RECEPTOR TYROSINE KINASES (RTKs)

Survival rates for some cancers have improved dramatically over the past 40 years, thanks to revolutionary treatment strategies and screening methods for the early diagnosis of tumor cells. Even though some cancers are now treatable, many malignancies do not yet have desirable therapies.

Cancer is a genetic disease that disturbs the perfect balance in cell division with the ability to metastasize. The hallmarks of cancer constitute an organizing system for reasoning the complexities of neoplastic disease development from initiation to metastatic spread [1] (Figure 1). One mechanism that allows tumor cells to proliferate is by maintaining proliferative signals and bypassing growth suppressors that lead to uncontrolled proliferation of tumor cells. The future of cancer therapy is moving towards a highly personalized treatment approach defined on the basis of the molecular pathway of the individual patient [2]. As cancer is a heterogeneous disease, patients usually carry multiple genetic driver mutations that make cancer treatment extremely difficult and resistant to conventional therapeutics. RTKs are transmembrane receptors that are of great clinical interest due to their role in cancer. Following their discovery, many mechanisms of RTK dysfunction were identified, leading to several tumor types exhibiting "oncogenic addiction" on RTKs [3]. Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is an essential oncofetal protein that is present only during fetal development but is a survival factor for malignant cells.

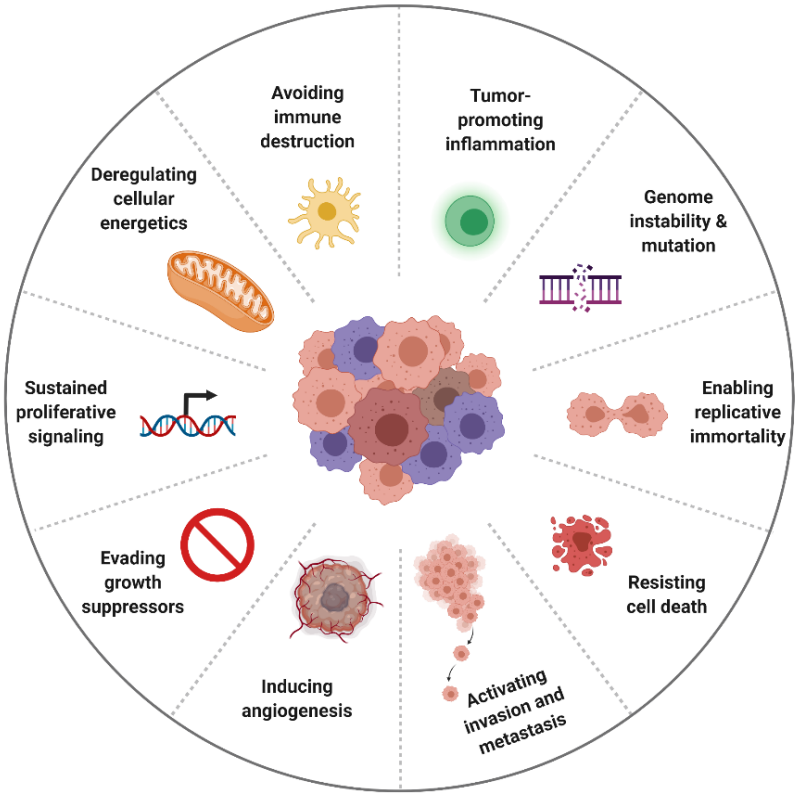

Figure 1. The hallmarks of cancer. *Figure modified from Hanahan & Weinberg 2011. Created with BioRender.com*

1.1 RTKs function and structure

RTKs is one of the main subgroups of protein kinases. RTKs are transmembrane glycoproteins expressed on the cell surface [4]. They have enzymatic activity and transduce the extracellular signal into the cytoplasm by binding their cognate ligands, autophosphorylating their receptors on tyrosine residues and downstream signaling proteins [5, 6]. All RTKs share a comparable molecular composition: an extracellular part as a ligand-binding transmembrane region and a conserved intracellular kinase domain associated with downstream signal transduction. There are 20 different families of RTKs with a total of 58 members [7]. One of the primary communication mechanisms between multicellular organisms is the binding of polypeptide ligands to maintain the catalytic activity of tyrosine kinases [8]. RTKs are activated by ligand binding and dimerization of their extracellular section and initiate signal transduction that controls various cellular functions. [9]. Moreover, RTKs activate various cell signaling pathways, leading to cell proliferation, migration, differentiation, or metabolic alterations [6]. During the binding of signal molecules to RTKs, the neighboring RTKs bind together and create cross-linked dimers [9]. The cross-linking activates tyrosine kinase expression through a phosphorylation − particularly, each RTK in the dimer phosphorylates multiple tyrosine from the other RTKs, which is referred to as cross-phosphorylation. RTKs are expressed as single/homodimers or heterodimers as ligand-binding receptors for the initiation of signaling cascades [3, 9].

Overexpression or mutations of RTKs in various cancers are considered attractive targetable properties [10]. Abnormal RTK activation may result from gain-of-function mutations, amplification, overexpression, or chromosomal translocation [11-13]. Overexpression of RTKs has been detected in many human diseases, especially cancer: Vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptor (FGFR), and ROR1. Dysregulation of RTKs can be targeted either by monoclonal antibodies against the extracellular region or a small-molecule inhibitor against the intracellular kinase domain [14]. Disruption of more than 30 RTKs in malignancies has been described [15].

However, a subset of tyrosine kinases (TK) known as non-receptor tyrosine kinases (NRTKs) regulates a variety of cellular functions such as cell survival, division/proliferation, and adhesion, gene expression, and immune response. Examples of NRTKs include proto-oncogene tyrosine-protein kinase (SRC), the Janus kinases (Jaks) and Abl [5].

1.2 ROR family of receptor tyrosine kinase

The ROR family includes ROR1 and ROR2, formerly known as neurotrophic tyrosine kinase receptors 1 and 2 (NTRKR1) and (NTRKR2), respectively [16]. ROR1and ROR2 are among the highly conserved type I transmembrane proteins in various species such as humans, chickens [17], mice [18], rats, zebrafish, fruit flies and roundworms [19-21]. The

molecular features of ROR family RTKs include the extracellular region containing the immunoglobulin (Ig)-like domain, the frizzled-like cysteine-rich domain (CRD), and a membrane-proximal kringle (KNG), domain ligand-binding. The intracellular part contains a tyrosine kinase (TK) domain, a proline-rich (PRD) domain, and a serine- and threonine-rich region for signal transduction. A schematic representation of the potential targeting option for ROR1 is shown in Figure 2 [6, 22, 23].

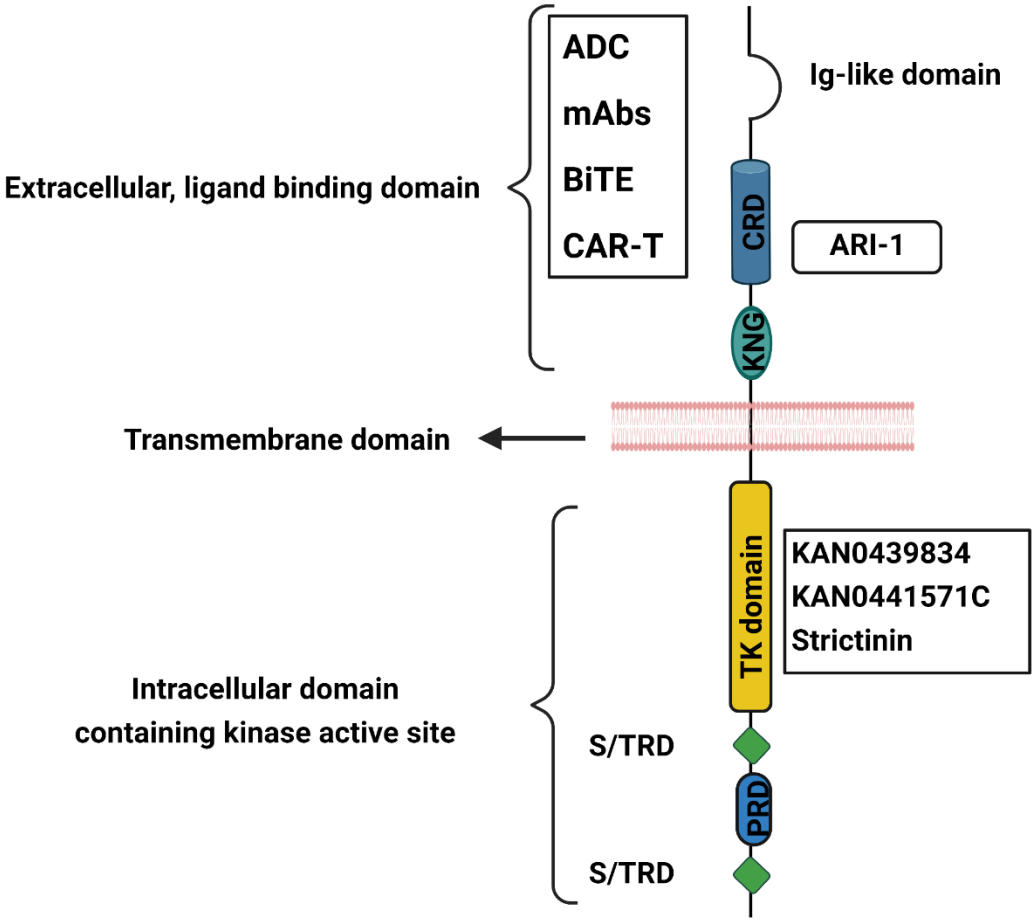

Figure 2. Schematic representation of the ROR1 structure and possible targets in cancer therapy. ADC : antibody-drug conjugates, mAb: monoclonal antibodies, BiTE: bi-specific T- cell engagers, CAR - T: chimeric antigen receptor T cells. *Reprinted and modified by permission from Springer Nature Customer Service Centre GmbH: Springer Nature. Targeting the receptor tyrosine kinase ROR1 by small molecules by Hojjat-Farsangi M, Moshfegh A, Schultz J, Norin M, Olin T, Österborg A, Mellstedt H. © 2021.* Created with BioRender.com

1.3 ROR1 structure and function

The ROR family is a type 1 transmembrane protein expressed on the plasma membrane of the cell during fetal development. The extracellular domain of ROR1 functions as ligand binding and signal transduction. Human ROR1 with 937 amino acids is located on chromosome 1 (1p31.3) and has a molecular weight of around 105 kDa [7]. The human ROR2 protein with 943 amino acids is located on chromosome 9 (9q22) and has a molecular weight of about 104.7 kDa. ROR1 and ROR2 are 58% similar in amino acid

sequence and 68% similar in the kinase domains. Other non-human ROR proteins have the same extracellular and intracellular regions [24].

The function of the Ig-like region is still unclear, but it may be related to protein and ligand interactions in the CRD and KNG domains [11]. The CRD domain is located between the Ig-like and KNG domains, in which ten cysteine residues are conserved and form five disulfide bonds. The domain of the CRD resembles the Wnt-binding domain of frizzled receptors which is proposed as a ligand-binding region of ROR1. ROR1 has been reported to be associated with Wnt5a, leading to enhanced survival of CLL cells in vitro through activation of NF-κB. The highly conserved KNG domain is located near the plasma membrane and consists of 79 amino acids. The KNG domain serves as a binding region for regulatory Wnt proteins and other ROR1 ligands. The KNG domain mediates as a binding site for regulatory Wnt proteins. The ROR family is the only RTK with a KNG domain, except for MuSk tyrosine kinase [16].

The TK domain of ROR1 consists of a phosphorylation site containing tyrosine residues. The cytoplasmic domains of ROR1 are important possible phosphorylation sites [25]. The Src homology 2 (SH2) and SH3 identification motifs are associated with various downstream signaling molecules [26, 27]. ROR1 is also phosphorylated at tyrosine residues 786, 789, 822 and 836, which serve as phosphorylation site for SRC kinases. The SH3 motifs operate as SRC binding promoters to the PRD domain, which sequentially phosphorylates ROR1. The deletion of PRD can inhibit the phosphorylation of ROR1 by SRC at the tyrosine kinase domain [28].

1.4 ROR1 expression during embryogenesis and in normal adult tissues

Knockdown mouse models have revealed the role of the ROR family in the development of skeletal, cardiorespiratory, and neurological diseases. Double knockdown of ROR1 and ROR2 in mice results in death shortly after birth due to respiratory disease because of incomplete alveoli development [24, 29, 30]. ROR1 is highly expressed during embryogenesis and tightly downregulated after birth [18, 22].

Expression of ROR1 has been detected in the lung, kidney and fetal heart, while low expression has been discovered in the pancreas, placenta, and skeletal muscle [31]. Furthermore, mRNA expression of ROR1 was not detected in several tissues, but low expression was found in adipose tissue and pancreas [32]. The ROR1 protein was detected at an intermediate stage of normal B-cell maturation in the bone marrow (pre-B II stage), where cells proliferated after internalization of the pre-B-cell receptor complex. This process is crucial for the development of the immature B-cell [33]. The function of the ROR1 receptor has been studied by *in situ* hybridization and knockout mouse models, showing that ROR plays an important role in skeletal, cardiac, and respiratory development as well as neurology [34].

The ROR receptor is mainly expressed in mesenchymal cells and in the migrating neural crest [35]. Mutation of ROR2 leads to Robinow syndrome [36], a skeletal disorder characterized by shortened limbs, segmental defects of the spine, and dysmorphic facial

appearance. Also, ROR2 mutation leads to brachydactylic B1 [37], a disorder characterized by a terminal deficiency of fingers and toes, depending on whether the mutation was homozygous or heterozygous. ROR1 homozygous knockout mice showed no skeletal abnormalities but severe respiratory failure, growth retardation, and death shortly after birth. Seventy-five knockout mice lacking both ROR1 and ROR2 died shortly after birth due to respiratory failure [30].

In adult humans, expression of ROR1 is absent, or less than 10%. Baskar et al.[32] examined ROR1 expression in 28 healthy adult tissues by Western blot and found that the expression was completely absent in most tissues. However, very low levels were detected in testis, uterus, lung, bladder and colon [38]. Expression of ROR1 on B-cell precursors at the intermediate stage of maturation in the bone marrow has been reported. Interestingly, both early and late B-cell precursors do not express ROR1, proposing that therapies directed against ROR1 would not harm healthy mature B cells [33].

1.5 ROR1 and cancer

The overexpression of ROR1 in hematologic and solid malignancies and the low expression of ROR1 in healthy adult tissues has prompted researchers to further explore the functional advantage of ROR1 for targeted therapy in cancer [32, 39-41]. To evaluate the targetability of ROR1 as a unique therapeutic strategy, it is important to define the pattern and level of ROR1 expression in normal tissues, in which ROR-targeted therapies may induce adverse off-target toxicity effects. Besides, previous studies showed that ROR1 is not uniformly expressed in all cancer tissues [42] and that its function may change in different tumor types. Table 1 reviews the current knowledge on the expression of ROR1 in cancer [39, 43, 44].

In drug resistance, ROR1 and ROR2 may interfere in different ways. The WNT/ROR1 pathway has been reported to be a rescue pathway by inducing NF-κB, PI3K, AKT, and ERK signaling proteins that activate BTK kinase through the formation of the ROR1 and CD19 complexes in MCL and CLL [45-47]. This activation represents a novel resistance mechanism for treatment approaches involving BTK/BCR inhibition. Indeed, targeting ROR1 and the BCR or BCL-2 proteins together has a synergistic effect in CLL and MCL drug resistance [46, 48]. Moreover, the expression of ATP-dependent translocase ABCB1 is upregulated by ROR1, which acts as a multidrug efflux pump to facilitate the clearance of drugs from tumor cells [49].

Table 1. Overexpression of ROR1 in malignancies

Cancer type	Expression of ROR1	Correlation to disease progression
ALL	+	+
AML	NI	+
B-ALL	NI	+
CLL	+	+
CML	NI	+
DLBCL	+	+
MCL	+	+
Hodgkin lymphoma	+	+
Follicular lymphoma	+	+
Marginal zone lymphoma	+	+
Breast cancer	+	+
Colorectal cancer	+	+
Gastric cancer	+	NI
Lung cancer	+	+
Melanoma	+	+
Ovarian cancer	+	NI
Pancreatic cancer	+	+

ALL: Acute lymphocytic leukemia, AML: Acute myeloid leukemia, B-ALL: B-cell acute lymphoblastic leukemia, CML: chronic myeloid leukemia, NI: no information [23, 25, 39, 42]

1.5.1 Wnt/ROR pathway

The Wnt signaling pathway is associated with the regulation of several important cellular processes, and dysregulation of this pathway has been discovered to correlate with the progression and growth of several malignancies in humans. Similarly, dysregulation of Wnt co-receptors ROR1 and ROR2 expression has been linked to numerous cellular features that promote malignancy, particularly cell proliferation, survival, migration/invasion, and stem cell formation. In fact, most studies have identified ROR1 as an oncogene, whereas the role of ROR2 in cancer remains unclear [42]. Studies in osteosarcoma, melanoma, CLL, breast, and kidney cancer have shown that ROR2 acts as an oncogene in these tissues. However, some studies describe a tumor-suppressive function of ROR2 in prostate, colon, and uterine cancers [38]. The signaling pathway of ROR2 in colorectal cancer is still controversial, whether it is controlled by the B-catenin-dependent canonical Wnt pathway or other distinct repertoires of FZDs and alternative Wnt co-receptors.

While possible interactions with WNT5B, WNT11, and WNT16 need to be verified, binding a WNT ligand alone is not adequate to activate the downstream signaling. For

example, the binding of WNT5A triggered phosphorylation of ROR family tyrosine kinase [50]. In addition, when ROR2 was overexpressed, the protein phosphorylation of cells was stimulated by WNT11, suggesting that the ligand may be able to induce activation of AKT, ERK, and GSK3B, which are known downstream regulators of the ROR2 pathway. Moreover, WNT/ROR signaling has been shown to trigger cell migration through activating downstream RAC by targeting JUN, JNK and ATF2 [51-54].

ROR1 appears to mediate between WNT signaling and other key signaling pathways involved in the control of important cellular processes, particularly the PI3K/AKT/mTOR pathway. Activation of ROR1 in numerous tumor entities has been shown to lead to phosphorylation of PI3K, AKT, CREB, and mTOR, as well as its downstream targets S6 and 4EBP1 [41, 45, 55, 56]. In another study, ROR1 was found to be associated with phosphorylation of CREB and AKT in human patients with gastric cancer [57]. In CLL cells, inhibition of ROR1 by cirmtuzumab decreased mTOR-induced genes [58].

Many studies have linked ROR family activation to β-catenin-independent WNT signaling responses through binding of the non-canonical WNT ligand WNT5A. The Wnt/ROR signaling is reviewed in Figure 3. If the expression of one of the ROR co-receptors is modulated to investigate downstream signaling events, it should be carefully considered that this could trigger upregulation of the other ROR co-receptor, which could compensate for the loss. For example, in melanomas, knockdown of ROR1, the expression of ROR2 and Wnt5a was increased, whereas knockdown of ROR2 stimulated the expression of ROR1 [59], confirming a reciprocal regulation.

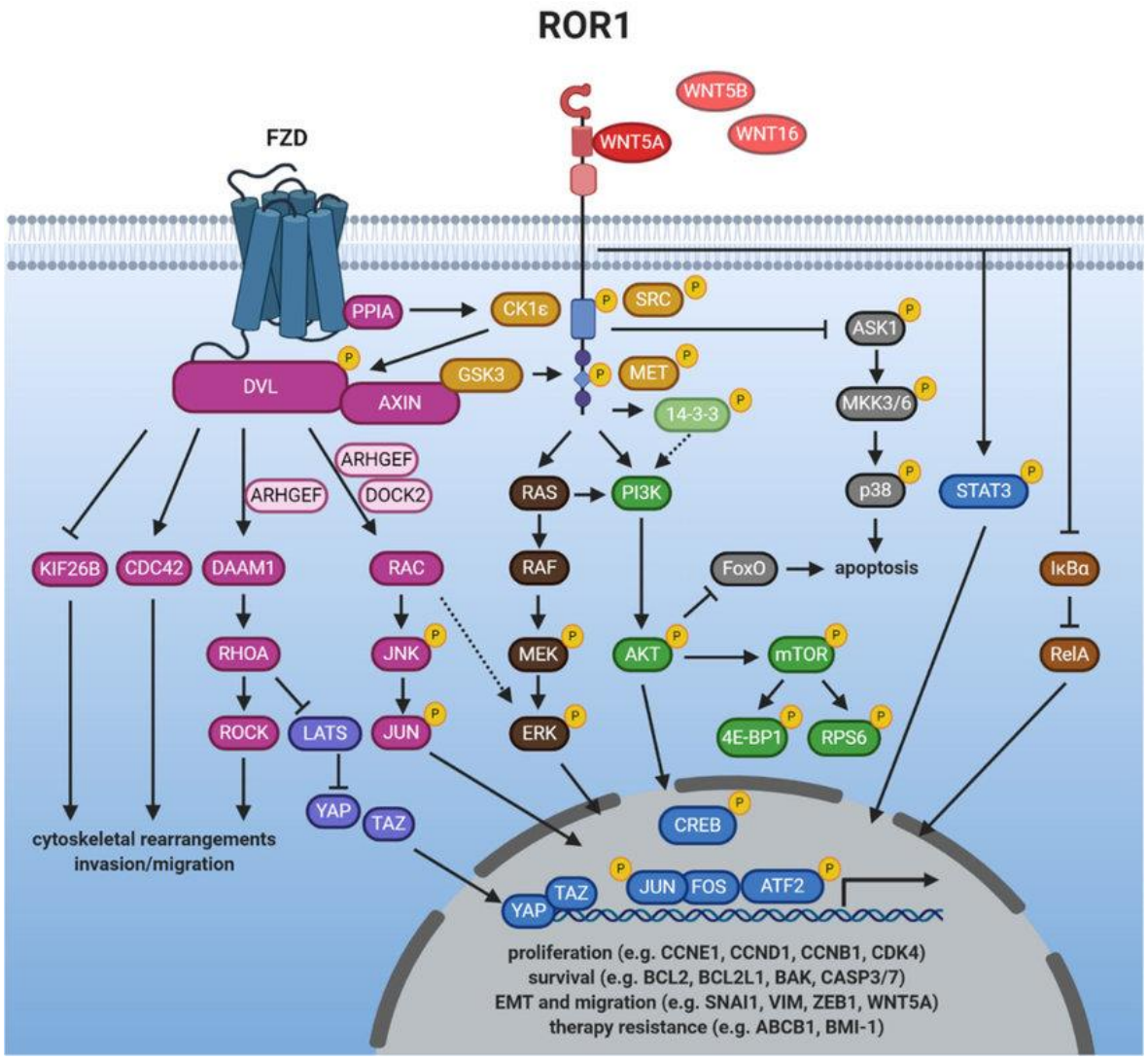

Figure 3. WNT/ROR1 signaling pathway. Signal transduction is mediated by ROR1 phosphorylation via multiple kinases (orange), leading to inhibition of anti-apoptotic pathways (gray), while downstream pathways such as WNT/PCP (magenta), MAPK/ERK (dark brown), PI3K/AKT (green) or NF-KB (light brown) are activated. These either trigger cytoskeletal rearrangements linked to increased tumor cell migration or induce a transcriptional response (blue), leading to the expression of genes that promote cell proliferation, survival, EMT, or therapy resistance. *Reprinted under the Creative Commons Attribution 4.0 International License, http://creativecommons.org/licenses/by/4.0/ from Cells 10(1):142, 2021. Menck K, Heinrichs S, Baden C, Bleckmann A. The WNT/ROR Pathway in Cancer: From Signaling to Therapeutic Intervention.*

1.5.2 ROR1 in hematological malignancies

A gene expression analysis detected ROR1 as a major element that separates CLL tumor cells from normal and other B-cell malignancies. In addition, the gene expression of ROR1 is increased 19-fold in CLL [60, 61]. High expression of ROR1 was reported to correlate with disease progression [62] and significantly shorter overall survival (OS) [63]. In addition to CLL, overexpression of ROR1 has also been found in other hematologic malignancies such as non-Hodgkin lymphoma (NHL), mantle cell lymphoma (MCL) [33, 64] and pediatric acute lymphatic leukemia (ALL) [65]. Suppression of ROR1 expression

in ALL primary cells and cell lines increased apoptosis in several clinically used small molecule inhibitors [66].

1.5.3 Expression of ROR1 in solid tumors

The expression of ROR1 in solid tumors ranges from low to high depending on the type of cancer, including lymphoma, uterine, prostate, lung, ovarian, testicular, melanoma, adrenal cancers [41]. For example, in lung adenocarcinoma, thyroid-specific-enhancer-binding protein (TITF1) has been found to regulate ROR1 expression, and overexpression of ROR1 has been associated with c-SRC activation and phosphorylation of the EGFR pathway [67]. Moreover, inhibition of ROR1 expression in lung cancer cells results in suppressed expression of the cell cycle regulators, CDK4 and CCNE1, along with two important anti-apoptotic proteins, BCL-2 and BCL-XL [68]. In epithelial ovarian tumors, ROR1 and ROR2 were reported to have a synergistic effect on cell proliferation after only a double knockdown has caused a remarkable decrease in cancer cell proliferation [69].

Silencing of ROR1 expression in melanoma cell lines induced apoptosis [70]. In breast cancer, overexpression of ROR1 is associated with aggressive disease. Breast cancer cell lines with high ROR1 expression were more aggressive and invasive [71]. In ROR1-positive breast tumor cells, Wnt5a increased malignant cell survival, confirming the role of Wnt5a as a ligand for ROR1 [71, 72]. ROR1 has also been shown to activate PI3K-mediated protein kinase B (AKT) and cAMP response element-binding protein (CREB) signaling pathways by interacting with casein kinase 1 (CK1) [71].

1.5.4 ROR1 isoforms in cancer

Protein isoforms are a set of very similar proteins derived from a single gene or gene family due to genetic variations. While most of them have similar biological functions, some isoforms hold unique roles and may arise from post-transcriptional modifications or alternative splicing of a single gene [73].

Different isoforms of ROR1 may correlate with disease activity similar to what was previously described for HER2 isoforms in breast cancer [74]. Moreover, glycolysis ROR1 inhibits the expression of a fully mature ROR1 isoform in the embryonic kidney cell line HEK293, changing the functional characteristic of the cell line in terms of metastatic and migration ability [75]. ROR1 might dimerize with ROR2 or EGFR, as has been described in lung adenocarcinoma [67].

A 64 kDa isoform of ROR1 has been identified in CLL cells, localized mainly in the nucleus, suggesting that ROR1 is a transcription factor [23]. However, in a number of tumors, a ROR1 protein of 260 kDa has been identified, which could be homo- or hetero-dimerized ROR1 [75]. In CLL, ROR1 has been reported to go through a significant N-linked glycosylation alteration, with various patterns reported. However, the predominant full-length (130 kDa) glycosylated ROR1 isoform was detected in progressive CLL [23].

Inhibition of ROR1 glycosylation modified the surface expression of the fully mature 130 kDa ROR1 isoform and filopodia formation in human embryonic kidney cell lines, which suggests that ROR1 may be involved in cell metastasis and migration. Isoforms of ROR1 include the full-length protein expressed on the cell surface (100-105 kDa, unglycosylated), an intracellular variant (~44 kDa) and the third variant with as yet unknown localization of ~40 kDa [42]. The full-length ROR1 of 105-130 kDa has been identified in ROR1-positive tumor cells [23, 28, 39]. Depending on the progression status of the CLL patient, different ROR1 isoforms of 64, 100-105, 130 and 260 kDa are phosphorylated at the tyrosine and serine residues [23, 75].

However, a ROR1 isoform lacking the transmembrane and extracellular part has also been described in neuronal tissues. In addition, ROR1 mRNA encoding a truncated isoform has been detected in fetal and adult normal human cells and tumor cells such as human leukemia, lymphoma cell lines, and other human neuroectoderm-derived cells [31].

Two isoforms of ROR1, 64 and 130 kDa, have also been detected in the nucleus of CLL cells, suggesting that ROR1 is a transcription factor for the activation of other genes [76]. Finally, another ROR1 isoform of 50 kDa has been described from a splice variant in CLL [32].

2 TARGETED ANTI-CANCER THERAPY

Surgical intervention with chemotherapy and radiation are the main strategies for treating cancer. For a long time, chemotherapy was the only approach for drug treatment of cancer. However, the main problem with chemotherapy is its inability to differentiate between cancer or normal cells, leading to severe toxicities and side effects. As a result, cancer therapy has undergone a major transformation in recent decades, from broad-spectrum cytotoxic drugs to targeted drugs [77].

The first tyrosine kinase inhibitor (TKI), imatinib, was approved by the US Food and Drug Administration (FDA) in 2001. As of December 2020, approximately 89 small-molecule targeted drugs have been approved to treat malignancies [78]. Figure 4 summarizes the small-molecule drugs approved by the FDA, the European Medicines Agency (EMA), and the National Medical Products Administration (NMPA) in China since 2001 [44]. However, despite the significant development, small-molecule anti-cancer drugs have faced several difficulties, such as low response rates and drug resistance [44].

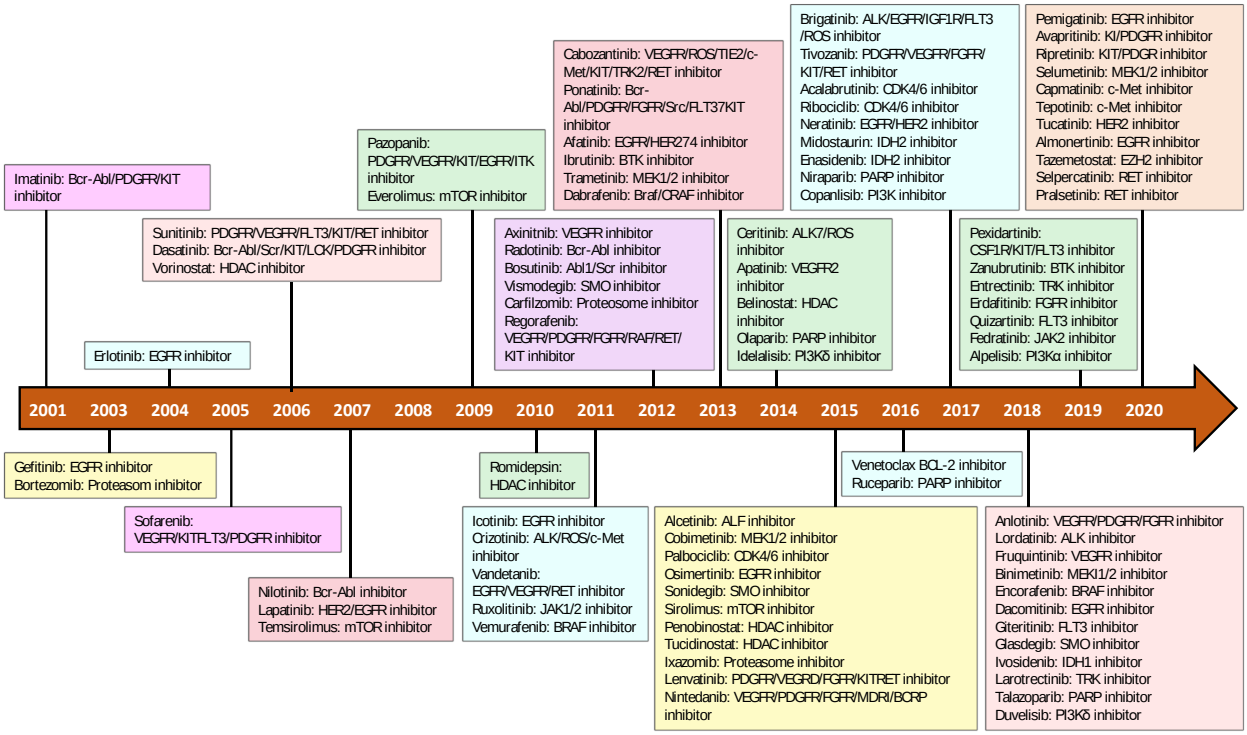

Figure 4. Timeline for the approval of small-molecule targeted anti-cancer drugs. *Reprinted under the Creative Commons Attribution 4.0 International License,http://creativecommons.org/licenses/by/4.0/. Zhong, L., Li, Y., Xiong, L. et al. Small molecules in targeted cancer therapy: advances, challenges, and future perspectives. Sig Transduct Target Ther 6, 201 (2021).*

Targetable drugs can be categorized into two classes: small molecules and macromolecules (e.g., monoclonal antibodies, antibody-drug conjugates, nucleic acids, and polypeptides) [79, 80].

There are two main groups of targeted therapies: The first group is small-molecule inhibitors (SMIs), engineered to target tumor-specific (TSA) or tumor-associated

antigens (TAA) on cancer cells, leaving healthy cells unharmed. TKIs are a major group of SMIs that target the domain of tyrosine kinases. TKIs can be divided into five groups: Type 1 inhibitors are the most common type, which binds to the adenosine triphosphate (ATP)-binding site of the kinase and inhibits tyrosine phosphorylation. This type of SMI is referred to as an ATP competitor. Type II binds to the inactive part of the kinase enzymes and occupies an additional hydrophobic pocket formed by a conserved amino acid sequence (DFG sequence). These inhibitors are more selective and specific compared to type I. Type III binds to the allosteric portion of the enzyme-substrate, located near the ATP-binding site. Type III inhibitors are highly selective and specific. Type IV inhibitors form an irreversible bond to a cysteine residue through the active part of the target. Accordingly, a cysteine residue within the active site of the target is necessary to develop this type of inhibitor, which is very specific for the target. The Bruton's tyrosine kinase (BTK) inhibitor, ibrutinib and HKI-272 as an EGFR inhibitor are type IV inhibitors. The last type has been classified recently, which they are a few TKIs have categorized in this group. Type V inhibitors are bivalent compounds that bind to different regions of the target [81, 82].

SMIs have a low molecular weight and therefore can penetrate the cell membrane, allowing oral administration and targeting both intracellular and membrane-bound antigens [83]. Some of the SMIs used in cancer are crizotinib, erlotinib, gefitinib and imatinib in non-small-cell lung cancer (NSCLC), gastrointestinal stromal cancer and CML, respectively. However, some non-tyrosine kinase inhibitors such as olaparib and vemurafenib are also involved in metastatic/malignant melanoma and prostate, ovarian, and breast cancer, respectively [84].

The second group is monoclonal antibodies (mAbs), which act on the cell surface, where they bind to a specific target on the surface of the cells. Many studies are increasingly demonstrating the molecular differences between different types of cancer and the different therapeutic approaches required for each type. For example, mAbs (cetuximab, trastuzumab, and rituximab) have been used in wild-type Kirsten rat sarcoma virus (*KRAS*) colorectal cancer, *HER2*-positive breast cancer, and CD20-positive non-Hodgkin's lymphoma [84]. The efficacy of different targeted drugs in such diverse tumors suggests that we are at the dawn of an era in which treatment strategy is based on the molecular profile or "signature" of the tumor rather than the type of tumor tissue or anatomical site of origin, which will improve diagnosis and patient quality of life [83].

2.1 Monoclonal antibodies against ROR1

Targeting of RTKs can be achieved by mAbs against membrane-bound epitopes such as Her2/neu or epidermal growth factor receptors [39]. mAbs against RTKs alone or combined with chemotherapy shift towards standard treatment in many cancer treatments [85, 86]. RTK antibody-dependent causes cancer cell apoptosis either through RTK-dependent signaling pathways or immune-mediated regulatory functions [87, 88]. Drug-conjugated antibodies are targeting the extracellular region of ROR1 that are in early clinical development [89], bispecific antibodies targeting ROR1 and T cells or natural

killer (NK) cells [90] or CD19/CD20, and other CAR –T [91] are some of the examples shown in Figure 2.

The extracellular region of ROR1 can be directed against the Ig-like, CRD and KNG domains. Monoclonal antibodies targeting the extracellular domain of RTKs have been developed as potential therapeutics for various cancers [32, 39, 92].

In CLL cells, anti-ROR1 mAbs targeting the CRD and KNG domains of ROR1 are most effective in causing tumor cell death [39]. In melanoma cell lines, anti-ROR1 mAbs induce direct apoptosis of cancer cells as well as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) [70]. Anti-ROR1 mAbs inhibit metastasis in breast cancer cell lines by downregulating proteins involved in cell motility and metastasis [72].

Cirmtuzumab is the first humanized mAb with a high affinity that binds to an epitope on ROR1, blocks Wnt5a activation, and consequently inhibits tumor cell proliferation, migration, and survival [93]. Cirmtuzumab binds to cancer cells but does not recognize most normal adult tissues. Preliminary clinical data suggest that cirmtuzumab synergizes with ibrutinib as a potential combination treatment for CLL and MCL [48].

In addition to mAbs, several therapeutic options are currently being developed to activate the humoral immune response to halt tumor growth, known as conjugates and bispecific antibodies. Bispecific antibodies (biAbs) consist of two monoclonal antibodies linked by a flexible peptide linker and bind to two different antigens [42, 94]. Such bispecific T cell engagers (BiTEs) represent a subclass of biAbs, characterized by a CD3-specific arm and a tumor cell-specific arm. BiTEs can redirect endogenous polyclonal T cells to tumor sites, promoting the formation of immunological synapses upon contact with tumor antigen. Subsequently, perforins, granzymes B, and cytokines are released to selectively kill tumor cells independent of major histocompatibility complex (MHC), co-stimulatory molecules, and antigen presentation [95]. Blinatumomab, the first-in-class BiTE, targets CD19 and is used to treat chemotherapy-resistant relapsed/refractory B-ALL patients [96-98]. Because CD19 is expressed only on B lymphocytes, blinatumomab cannot be used to treat other cancers with significant unmet need, such as pancreatic cancer. Therefore, BiTEs with wide applications in a variety of cancers are needed. Gohil et al.[90] developed a bispecific ROR1 T-cell engager from a series of rat anti-human ROR1 antibodies. They then selected a single-chain variable fragment (scFv) targeting the CRD domain of ROR1, as it consistently exhibited better, and reproducible cytotoxicity compared to scFvs targeting the ROR1 immunoglobulin-like domain. The result demonstrated that ROR1-BiTE promoted efficient T cell-mediated killing of pancreatic and ovarian cancers *in vitro* and *in vivo* and a variety of solid tumor cell lines of different histological origins.

2.2 Small molecule inhibitors of ROR1 tyrosine kinase

Targetable treatment strategies with small molecule inhibitors are often ≤500Da in size. Thus, compared to mAbs, which typically have proteins with a large molecular weight of about 150 kDa, small molecule anticancer agents are considerably smaller and

consequently able to cross plasma membranes and communicate with the cell-surface receptor of the cytoplasmic domain and intracellular signaling molecules [99, 100]. In addition, small-molecule drugs can target any part of a molecule, regardless of where the target is located in the cell. In contrast, mAbs can solely function on the cell surface. Small-molecule agents are relatively cost-effective with oral administration, whereas mAbs are often administered intravenously [101].

Both mAb and TKI small molecule induced protein dephosphorylation and associated with downstream signaling molecules of ROR1. A collection of approximately 110 000 chemical compounds has been selected to develop ROR1 small molecule inhibitors and those that inhibit ROR1 activity *in vitro* [25].

2.2.1 KAN0439834 and KAN0441571C

The first production of a ROR1 drug-like agent tested in preclinical studies was KAN0439834. The small ROR1 molecule is orally available and has an *in vivo* half-life in mouse plasma of 6 hours and a low toxicity profile. The second-generation ROR1-TKI, KAN0441571C, is also orally available, with low toxicity and a half-life of 11.2 hours in mice, as well as improved tumor cell killing activity compared to KAN0439834. With the structural alignment of both KAN0439834 and KAN0441571C, the docked conformation fits well into the ATP-binding pocket of the ROR1 TK domain and forms an h-bound in the hinge region at the central scaffold of the ROR1 inhibitor [25]. A graphic description of the small molecule ROR1 inhibitors (ROR1-TKIs) development is shown in Figure 5. Both KAN0439834 and KAN0441571C kill CLL cells with high specificity [25]. The EC_{50} for KAN0439834 was 250 nM and for KAN0441571C was 50-100 nM.

Dephosphorylation of ROR1 is inhibited by ROR1 small molecule in a dose- and time-dependent pattern [25]. ROR1 inhibitors dephosphorylated the co-receptors LRP-6 and SRC, and dephosphorylation of the PI3K/AKT/mTOR pathway, regarded as an important cancer target therapy. In addition to inactivating the non-canonical pathway, molecules of the canonical pathway were also altered by reducing the expression and phosphorylation of GSK3β and β-catenin [9].

A single oral dose (50 mg/kg) of KAN0439834 in mice resulted in a plasma concentration of 800 nM after 6 hours, which might be enough to induce apoptosis of CLL cells. Moreover, in xenografted NOD-SCID mice with primary CLL cells (wild-type *TP53*), there was a significant decrease in ROR1+ cells in a spleen with a high dose of the drug where it was less noticeable at a lower dose. A similar significant reduction in tumor cell was also observed in CLL cells with deletion of (17q) but not in CLL cells without (17q). Blood chemistry analysis revealed no abnormalities besides for a slight elevation of the enzyme alanine transferase. Histopathological examination of the tissue detected small necrotic foci in the liver and insignificant variation in the regeneration of the tubular epithelium of the kidney [75].

Ibrutinib is usually used in CLL patients. Resistance may occur due to a BTK mutation, so patients may need to switch to venetoclax (Bcl-2 inhibitor) or a non-

covalently binding BTK used in trials (portobrutinib). The previous study in CLL patients with ibrutinib-naive and -resistant cells showed that tumor cells died equally in both groups. The ROR1 inhibitor has a significant cytotoxicity effect in ibrutinib and venetoclax double-refractory CLL cells [102]. Therefore, a new effective drug option is beneficial where previous drugs have failed, and ROR1 inhibitors may be such an alternative.

Combining different drugs with different MOAs could improve clinical outcomes, especially those that have a synergistic effect. For example, in CLL, combining venetoclax and ROR1 inhibitors has been proved to have a synergistic cell death *in vitro* in both ibrutinib-sensitive and ibrutinib-resistant CLL cells [103]. Moreover, it has been reported that a combination of ROR1 siRNA or mAbs with venetoclax is more effective than either drug alone in MCL [46].

In DLBCL, expression of ROR1 was more prevalent in progressed stage and in relapsed/refractory patients. Therefore, there is a correlation between ROR1 expression and poor survival. KAN0441571C has a significant apoptotic effect in ROR1+ DLBCL cell lines, comparable to venetoclax but better than ibrutinib. The combination of KAN0441571C and venetoclax resulted in the total death of the tumor cells of the cell lines. In zebrafish transplanted with ROR1+ tumor cells for DLBCL, KAN0441571C resulted in a dose- and time-dependent reduction in tumor size [43]. Preliminary data in MCL show similar results to DLBCL [103]. ROR1 inhibitors may therefore serve as a promising new strategy in various B-cell malignancies.

However, a second-generation ROR1 inhibitor (KAN0439834) had a significant apoptotic effect in a series of ROR1+ pancreatic cancer cell lines with an EC_{50} that varied between 250-680 nM. As EGFR inhibitors (erlotinib) and ibrutinib are currently being tested in a clinical trial for pancreatic cancer, the combination of these anticancer drugs with ROR1 inhibitors was tested and a significant increase in tumor cell killing was observed compared to either drug alone [43].

Although breast cancer prognosis has improved, there are still subgroups with a poor prognosis for which new therapeutic strategies are required. In a transplanted mouse model with chemotherapy-resistant tumor cells, cirmtuzumab inhibited the expression of genes correlated with breast tumor stem cell formation and prevented the ability of cancer cells to metastasize. In addition, combining paclitaxel with cirmtuzumab was more effective in destroying breast tumor cells than either treatment alone [104].

In conclusion, a range of hematological and non-hematological malignancies overexpress ROR1. In many experimental tumor models, small molecule ROR1 TKIs have effectively induced apoptosis and downregulated both non-canonical and canonical Wnt signaling pathways, and combination with other targeted drugs appears to be either synergistic or additive in inducing tumor cell death.

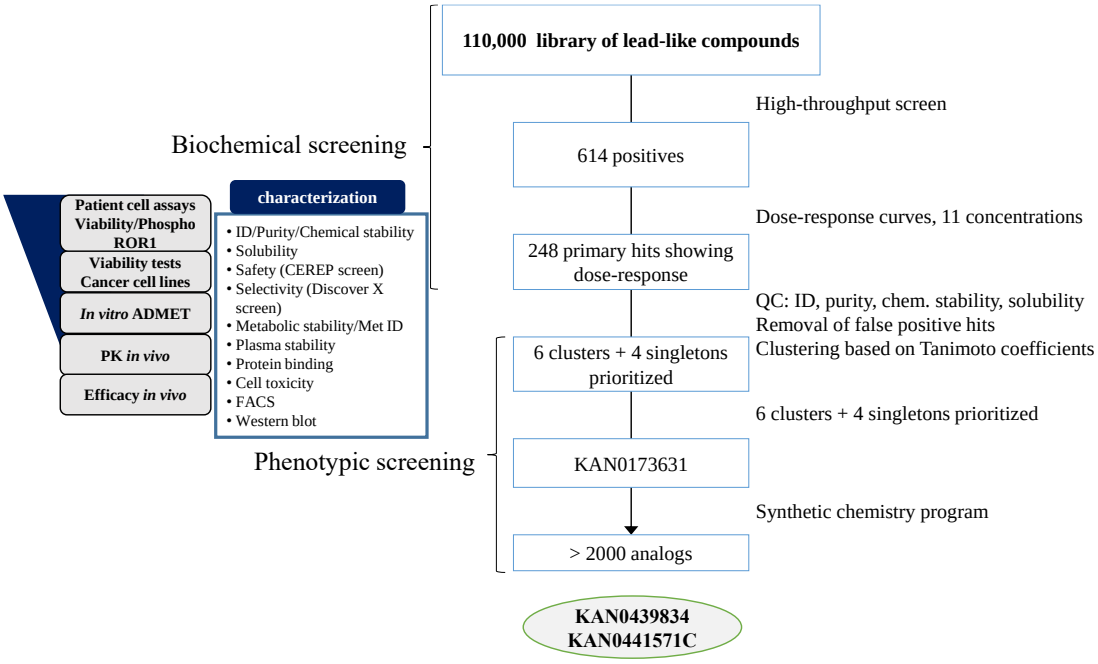

Figure 5. A schematic representation of the development of small molecule ROR1 inhibitors. *Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature. Targeting the receptor tyrosine kinase ROR1 by small molecules by Hojjat-Farsangi M, Moshfegh A, Schultz J, Norin M, Olin T, Österborg A, Mellstedt H. © 2021*

2.2.2 ARI-1 and Strictinin

Recently, a novel ROR1 inhibitor, (R)-5,7-bis(methoxymethoxy)-2-(4-methoxyphenyl) chroman-4-one (ARI-1), was reported [104]. The ARI-1 inhibitor targeted the extracellular frizzled part of ROR1-RTK and inhibited proliferation and migration of NSCLC cells via the PI3K/AKT/mTOR pathway [104].

Strictinin is another type of ROR1 inhibitor isolated from Myrothamnus flabellifolius that attaches to the intracellular section of ROR1. In a study by Fultang et al, the compound induced the killing effect of TNBC cells and prevented the activity of ROR1 by reducing AKT phosphorylation at serine residue 473 and suppressing GSK3β phosphorylation. Inhibition of AKT was associated with reduced cancer cell survival and activation of the intrinsic apoptotic pathway. In addition, Strictinin also prevents TNBC cell migration and invasion by reducing β-catenin activity [105].

The role of ROR1 in tumor growth, survival, cell proliferation, migration and metastasis in various cancers has been repeatedly demonstrated. ROR1 expression pattern has been associated with disease progression in various malignancies. Overexpression of ROR1 has been associated with aggressive disease. Wnt5a is thought to be a ROR1 ligand that stimulates signaling via activation of PI3K/AKT/mTOR (non-canonical Wnt) and canonical Wnt signaling. Thus, ROR1 appears to be a promising target for precision cancer medicine.

Further research and development on ROR1 inhibitors and molecular properties of this RTK are needed to optimize ROR1-targeted therapeutics in malignancies, especially in combination with other anti-cancer drugs. However, preclinical small molecule data

are promising, particularly with regards to the treatment of patients with antibodies, BiTE and ROR1-CAR.

3 RESEARCH AIMS

The overall goal of this thesis was to characterize the role of tyrosine-protein kinase ROR1 in tumor cells and to evaluate the potential of ROR1 inhibitors in combination with non-ROR1 targeting drugs on both hematological and non-hematological malignancies.

The specific aims of the papers were:

I. To investigate the apoptotic effects of a first-in-class ROR1 small molecule, KAN0439834 of ROR1 inhibitor in human pancreatic cell lines alone and combination with other targeted therapy.

II. To elucidate the association of ROR1 expression in DLBCL patients outcome and the anti-tumor effect of the second generation of ROR1 SMI, KAN0441571C alone and in combination with targeted therapeutics.

III. To evaluate the induction of apoptosis of a ROR1 inhibitor, KAN0441571C alone and in combination with venetoclax, in ibrutinib-resistant ROR1+ CLL cells.

IV. To characterize the apoptotic effects of ROR1 inhibitor, KAN0441571C, alone and in combination with other targeted therapeutics in MCL cell lines and primary cells.

V. To investigate the clinical and functional characteristics of ROR1 expression in NSCLC patients and the effects of a ROR1 inhibitor (KAN0441571C) alone or in combination with EGFR small molecule inhibitors in five NSCLC cell lines.

4 MATERIALS AND METHODS

Detailed descriptions of all the methods and materials in this thesis can be found in publications and manuscripts. Therefore, the selected methods are explained here.

4.1 Flow cytometry

Flow cytometry was used to stain the surface markers of the cells and determine the induction of apoptosis. Cells were then stained with appropriate surface antibodies. Cells were used fresh or stored in liquid nitrogen until use. After drug treatment, cells were incubated for 24 or 48 hours. Flow cytometry analysis of PBMCs was performed for CD45, CD19, CD3, CD5, ROR1 and the corresponding isotype controls. Results were analyzed using Novocyte 3000 and Novoexpress software (ACEA Biosciences, San Diego, CA, USA).

For apoptosis assay, cells were collected, washed with PBS, and resuspended in 100 µl of binding buffer (BD Biosciences) containing FITC-conjugated Annexin-V and PI (BD Biosciences) and incubated at room temperature for 20 minutes [103] and run by flow cytometry. Viable cells were determined as the double negative Annexin V/PI population. Apoptosis was analysed by flow cytometry (FACS Canto II, BD Biosciences).

4.2 Drug synergy studies

Cytotoxicity studies to evaluate drug synergies in this thesis were performed using cytotoxicity assay (MTT) and Annexin V/PI apoptosis assay. MTT assay is used to measure cell viability, proliferation and cytotoxicity. The MTT assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells and is quantified by measuring absorbance at the 595 nanometers using a multi-well spectrophotometer [106] (Figure 6). The darker the solution, the greater the number of viable, metabolically active cells. To calculate the drug combination interactions, we used two methods. The zero interaction potency model (ZIP) was used to calculate delta scores (synergy scores) to quantify the degree of drug synergy with (https://synergyfinder.org/). The ZIP model calculates drug-drug interaction by comparing the change in potency (effect at a given dose) of dose-response curves between individual drugs and their combinations. The second method, Chou-Talalay, is based on the median effect equation derived from the law of mass action principle. This unified theory is the common link between single and multiple entities and first and higher-order dynamics. This general equation includes the Michaelis-Menten, Hill, Henderson-Hasselbalch, and Scatchard equations in biochemistry and biophysics. In addition, the theoretical description for the combination index (CI) isobologram equation provides a quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations. The computer software CompySyn

automatically calculates synergism and antagonism at all assumed doses or potencies based on this equation [107].

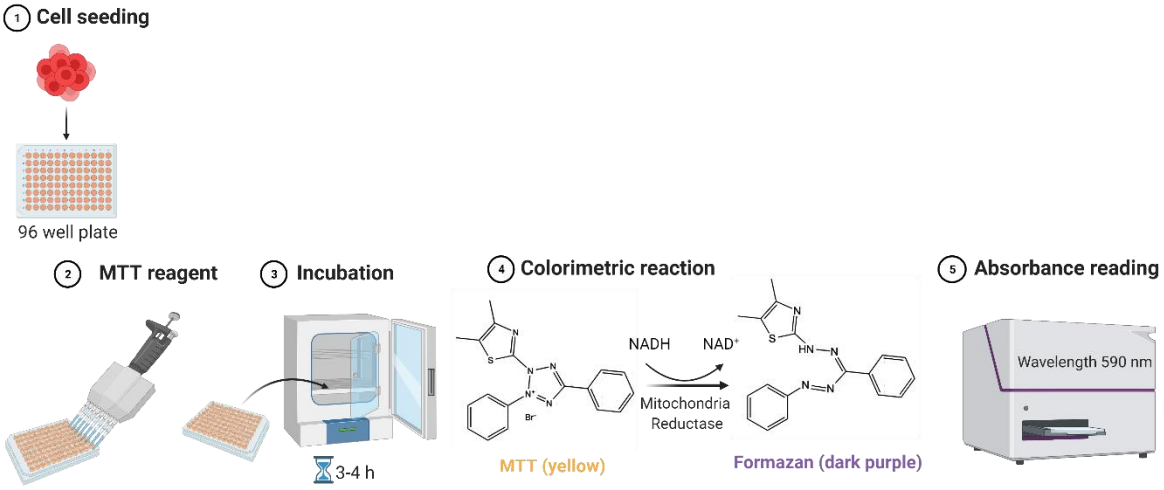

Figure 6. The workflow of MTT Cell viability assay. *Created with BioRender.com*

4.3 Zebrafish model

The zebrafish model can reduce, refine and, to some extent, replace other model system for higher vertebrates. The advantages of zebrafish include low-cost, rapid development, transparent embryo, exceptional imaging properties, gene knockdown tools, and high-throughput screens, that make zebrafish a clinically relevant animal model for basic and translational research. Therefore, we decided to use zebrafish (embryos < 5 days) to test our therapeutic in an *in vivo* model. Zebrafish embryos younger than five days are unlikely to experience stress, pain or suffering. Therefore, the experiments do not require ethical approval under EU Directive 2010/63/EU.

Zebrafish embryos (48 h post-fertilization) were injected with approximately 500 stained tumor cells in five nL medium into the perivitelline space to confirm whether the cell line forms tumors. KAN0441571C was added to the medium at four different concentrations: 25, 100, 250, 1000 nM. One group (n=21) was untreated. Tumor size was determined by normalizing the area of the tumors at each given time point to that of the same tumor at day 0. Tumors were photographed under fluorescence microscope at 0, 24 and 72h post-implantation (Figure 7).

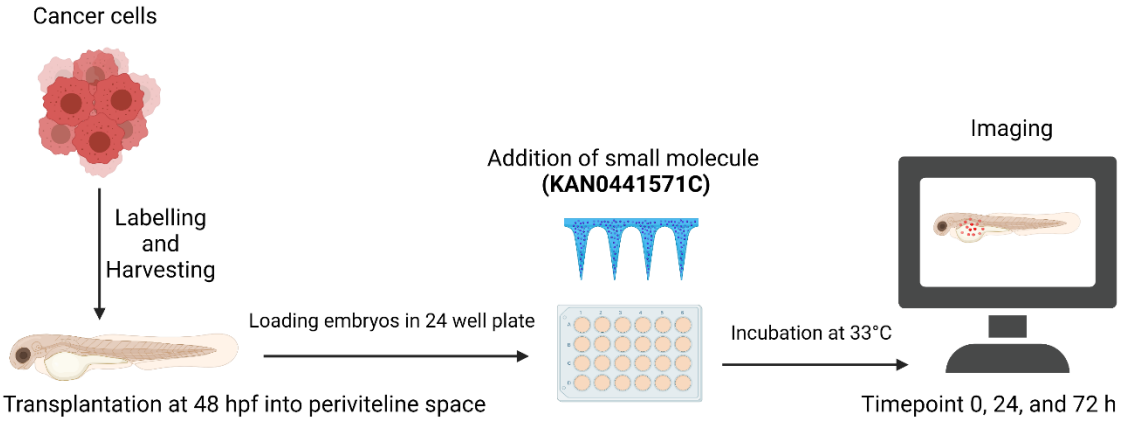

Figure 7. The workflow of zebrafish tumor cells and drug treatment. *Created with BioRender.com*

4.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Student's t-test and Mann–Whitney U-test were used to comparing the data. EC_{50} values were calculated from the dose-response curve by non-linear curve fitting (HillSlope). The Kruskal-Wallis test was applied to compare ROR1 expression in different clinical patient subgroups. Overall survival (OS) was measured from the time of diagnosis to death or last follow-up, and time to progression was illustrated graphically by the Kaplan-Meier method. Statistical significance was estimated using the log-rank test. P-values < 0.05 were considered significant.

4.5 Ethical aspects

All clinical material was collected after informed consent from patients. All studies were performed under the ethical principles of the Declaration of Helsinki and in compliance with national laws. Approval was obtained by the Regional Ethics Committee (www.etikprovningsmyndigheten.se).

5 RESULTS, DISCUSSION AND CONCLUSIONS

This section presents the main results and discussions of the studies. For more details, see the corresponding articles or manuscripts at the end of this thesis.

5.1 PAPER I

A receptor tyrosine kinase ROR1 inhibitor (KAN0439834) induced significant apoptosis of pancreatic cells which was enhanced by erlotinib and ibrutinib
Daneshmanesh et al. PLoS One. 2018;13(6): e0198038

This paper assessed the effect of a first-in-class ROR1 small molecule, KAN0439834, on the apoptosis of human pancreatic cancer cell lines. The cytotoxicity of KAN0439834 alone and in combination was compared with ROR1 murine mAb as well as other drug inhibitors including gemcitabine, erlotinib, and ibrutinib.

The result shows that all eight pancreatic cell lines express the ROR1 protein either on the surface or intracellular part of ROR1, confirmed by flow cytometry and Western blot. The surface expression of ROR1 was heterogeneous from 40% (Capan-1) to71% (AsPC-1). Moreover, pancreatic tumor cells phosphorylated ROR1 at a 130 kDa protein band as a fully glycosylated ROR1.

In addition, KAN0439834 induced cancer cell death with EC_{50} values of 250±650 nM depending on the cell line. Thus, compared with ROR1 mAb, KAN0439834 is much more effective in the cytotoxicity of pancreatic cancer cells. Among these pancreatic cell lines, PaCa-2 is most sensitive to KAN0439834 with 82% apoptosis compared to the 35% for anti-ROR1 mAb. On the other hand, Capan-1 was the most resistant cancer cell to KAN0439834 but still more effective than the anti-ROR1 mAb. We also tested KAN0439834 on healthy primary cells (B and T cells), and no significant killing effect was observed [25].

The cytotoxicity of erlotinib and ibrutinib alone was EC_{50} values of 5000±15000 nM depending on the cell line. Significant additive effects on tumor cell death were observed when these inhibitors were combined with KAN0439834. The combination of KAN0439834 and anti-ROR1 mAb induces apoptosis through cleavage of caspase-3 and PARP and downregulation of MCL-1 and Bcl-xL proteins.

Pancreatic cancer is one of the most invasive malignancies with a low surgical resection rate [108], having the lowest survival rate of all cancers in Europe [109]. Unfortunately, due to the high heterogeneity, metabolic reprogramming and early local invasion and metastasis of pancreatic cancer, patients benefit little from current conventional therapy [110]. Therefore, optimizing early diagnosis and developing targeted therapy for pancreatic cancer is key to increasing patient survival [111]. The oncogenic signaling pathways of pancreatic cancer include RTK signaling pathways such as EFGR, VEGFR, IGF-1R, RON, Wnt, and PI3K/AKT/mTOR signaling pathway [110, 112]. These signaling pathways play important roles in a variety of cancer-related cellular processes, including those in pancreatic cancer, including cell proliferation, apoptosis, differentiation, migration, metabolism, angiogenesis, and immune regulation [113, 114].

The main conclusion of this paper is that KAN0439834 as a single agent has a higher killing effect than gemcitabine, erlotinib, ibrutinib, and an anti-ROR1 mAb. On the other hand, an additive cytotoxic effect of erlotinib, gemcitabine, and ibrutinib is obtained. Moreover, erlotinib, gemcitabine, and ibrutinib do not dephosphorylate ROR1 in pancreatic cells [25]. However, cell lines with a wild-type *KRAS* are more sensitive to erlotinib (EGFR inhibitor) than those with mutated *KRAS* [115]. Still, KAN0439834 was equally effective in inducing apoptosis of pancreatic cancer cell lines regardless of the *KRAS* status.

Tumor cells treated with both KAN0439834 and anti-ROR1 mAb were found to have 25% increased cytotoxicity. This may be due to targeting both the intracellular and extracellular portions of the ROR1 molecules. This is consistent with reports that the combination of anti-HER2 mAb and lapatinib can enhance the apoptotic effect of HER2-positive breast cancer cells compared to either drug alone [116, 117].

Both KAN0439834 and anti-ROR1 mAb dephosphorylated low-density lipoprotein receptor-related protein 6 (LRP6) (a co-receptor for ROR1), which may be due to the dimerization of ROR1 and LRP6 upon ligand activation, which transmits downstream signals via SRC and the canonical and non-canonical Wnt signaling pathways [118]. Phosphorylation of the PI3K/AKT/mTOR pathway and the transcription factor CREB was inhibited, which is important in tumorigenesis [119].

While ibrutinib and erlotinib dephosphorylate EGFR [35, 36], KAN0439834 does not dephosphorylate EGFR and BTK, suggesting different mechanisms of action. The additive effects of KAN0439834, erlotinib, and ibrutinib may be because these drugs cross-talk with the EGFR pathway [120, 121], and besides, both KAN0439834 and ibrutinib are inhibitors of Wnt signaling pathways [121, 122]. In addition, EGFR can heterodimerize with HER2, activating the tyrosine kinase domain of EGFR [123].

In summary, this paper introduces the *in vitro* killing effect of the first-in-class ROR1 small molecule inhibitor of ROR1-RTK as a novel targeted therapy and suggests a potential combination approach for pancreatic tumor cells.

5.2 PAPER II

ROR1 is expressed in diffuse large B-cell lymphoma (DLBCL) and a small molecule inhibitor of ROR1 (KAN0441571C) induced apoptosis of lymphoma cells
Ghaderi et al. Biomedicines. 2020; 8(6):170

This paper evaluated ROR1 overexpression and anti-tumor effects of KAN0441571C, in preclinical DLBCL cells. In addition, a combination of ROR1 small molecule inhibitor with ibrutinib and venetoclax was studied. Finally, we analyzed the correlation between ROR1 expression and overall survival in R/R *de novo* DLBCL patients. This analysis was retrospective and had a limited number of patients, and should be considered a hypothesis-generating approach.

DLBCL is the most common form of non-Hodgkin lymphoma (NHL), and the most common lymphoid malignancy in the Western world, accounting for 25-40% of all NHL [124]. Despite major efforts to improve treatment options for advanced-stage DLBCL, this

remains a challenge due to the heterogeneity of DLBCL and poor prognosis [124, 125]. Expression of ROR1 was observed more frequently in primary refractory DLBCL, Richter´s syndrome, and transformed follicular lymphoma and less frequently in relapsed and non-relapsed DLBCL patients. Primary refractory de novo DLBCL patients were observed to express ROR1+ compared to non-refractory and relapsed patients ($p < 0.05$). There was no significant correlation between ROR1 expression and the origin of the cancer cells in the non-relapsed and relapsed/primary refractory subgroups.

ROR1+ DLBCL patients (n = 17) had a five-year rate OS of 42% compared with 7% of patients with ROR1+ tumors (n = 16) ($p < 0.05$). This multivariable analysis was independent of sex and Ann Arbor stage in patients with overexpressed ROR1 but dependent on age and International Prognostic Index (IPI). In the future, ROR1 expression as a prognostic marker in DLBCL needs to be thoroughly assessed using cohorts of newly diagnosed *de novo* DLBCL patients. Interestingly, overexpression of ROR1 in R/R *de novo* DLBCL cases has been shown to correlate with shorter survival of these patients [126, 127], which supports the association of ROR1 with poor DLBCL prognosis and more aggressive disease along with previous publications in various other cancers [128].

KAN0441571C was found to be cytotoxic in all DLBCL cell lines expressing ROR1, while no cytotoxicity occurred in the ROR1 negative U2932 cell line. The apoptotic effect of KAN0441571C is similar to venetoclax but better than ibrutinib in DLBCL cell lines, while the combination of KAN0441571C and venetoclax had an additive apoptotic effect. As these drugs have different mechanisms of action, it might be interesting to explore this combination further. Furthermore, we showed that KAN0441571C inhibits the phosphorylation of ROR1 in DLBCL cells and induces apoptosis by inhibiting BCL-2 and MCL-1, which are pro-survival molecules acting on the intrinsic mitochondrial pathway. However, in the presence of high concentrations of KAN0441571C, caspase 8 was also cleaved, suggesting activation of the extrinsic pathway. In addition, KAN0441571C inactivated both the canonical and non-canonical Wnt pathways and the transcription factor CREB. Apoptosis of DLBCL tumor cells was characterized by BCL-2, BCL-xL, and MCL-1 downregulation and cleavage of PARP, caspases 3, 8, and 9, and BAX protein upregulation in the OCI-LY3 cells. Moreover, the cell cycle control proteins p21, p27, and p53 were upregulated, indicating induction of cell cycle arrest.

Studies show that tumor cell growth and drug resistance can be promoted by stromal cells [129]. To test whether ROR1 can suppress these stromal cell-mediated effects, we co-cultured the DLBCL cell line OCI-Ly3 with and without stromal cells that were ROR negative. When we incubated these cells with KAN0441571C for 24 h, we saw that in the absence of stromal cells, the apoptosis of DLBCL cells was elevated, compared to the setting where stromal cells were present. This suggests that ROR1 inhibition cannot completely overcome the tumor-promoting effects of stromal cells.

Finally, zebrafish embryos transplanted with tumor cells were treated with KAN0441571C and observed for three days, and the ROR1 inhibitor produced a significant and consistent reduction in cells.

In conclusion, patients with relapsed/refractory DLBCL and those not eligible for high-dose chemotherapy with stem cell support have an urgent medical need for new therapeutic alternatives. Our data shows promise for the second generation ROR1 inhibitor, KAN0441571C, as a new drug candidate that should be further investigated in clinical trials in high-risk patients DLBCL.

5.3 PAPER III

A ROR1 small molecule inhibitor (KAN0441571C) induced significant apoptosis of ibrutinib-resistant ROR1$^+$ CLL cells
Ghaderi et al. eJHaem. 2021; 2: 498– 502

In this paper, we collected six CLL patients before and after developing clinical resistance to ibrutinib. In addition, one of these CLL samples had developed dual resistance to both ibrutinib and venetoclax. Therefore, the study aimed to evaluate the effect of ROR1 small molecule inhibitor (KAN0441571C) in an *ex vivo* model before and after the acquisition of resistance to ibrutinib. In addition, we evaluated venetoclax as a control to see if it was effective in ibrutinib-resistant cases. Finally, we were interested in investigating the combined effect of KAN0441571C and venetoclax for induction of apoptosis.

The main finding of the paper is that KAN0441571C induced apoptosis in both ibrutinib-sensitive and -resistant ROR1+ CLL cells from the same patient. Furthermore, in the case of venetoclax, both ibrutinib-sensitive and -resistant cells responded equally well. However, in double-refractory cells are required 10–15 times higher concentrations of venetoclax are required to achieve the same apoptotic effect as in ibrutinib-sensitive and -resistant cells.

KAN0441571C dephosphorylated ROR1 in both ibrutinib-sensitive and -resistant cells, while the effects on BTK phosphorylation were different. The combination of KAN0441571C and venetoclax had a synergistic apoptosis effect in ibrutinib-resistant CLL cells in all six patients. Yet, the mechanism underlying the synergism between KAN0441571C and venetoclax is unknown. However, it is interesting to note that in MCL, ibrutinib in combination with venetoclax or monoclonal ROR1 antibodies overcame drug resistance to ibrutinib. Inhibition of ROR1 by siRNA or monoclonal antibodies resulted in downregulation of the intracellular protein NF-kB p65. Activation of the NF-kB pathway could antagonize the apoptotic response mediated by ROR1 [46]. These results are consistent with our findings of a synergistic apoptotic effect in CLL cells by targeting ROR1 and Bcl-2 in ibrutinib-resistant cells.

Expression of ROR1 in malignancies was first discovered in CLL cells [60], and high expression of ROR1 is correlated to progressive disease [63]. ROR1 Overexpression might be due to increased activation of AKT and related signaling molecules compared to cells with low ROR1 expression [45]. Our previous studies show that the ROR1 inhibitors KAN0439834 and KAN0441571C induce tumor cell death in ROR1+ CLL and DLBCL cells [25, 103].

Ibrutinib is an irreversible inhibitor of the protein Bruton's tyrosine kinase (BTK), which inhibits the proliferation and survival of malignant B-cells [130]. Ibrutinib is clinically effective in CLL, but patients may develop resistance which in 80% of cases is associated with a mutation of the BTK gene at the ibrutinib binding site as C481S, as well as mutations in the domain of the immediate downstream effector phospholipase Cγ2 (PLCγ2) [131, 132]. However, venetoclax (a Bcl-2 inhibitor) is effective in ibrutinib-resistant patients [133]. BTK is a crucial tyrosine kinase that regulates the B cell antigen receptor (BCR) signaling cascade, resulting in activation of downstream NF-κB and PI3K and promoting CLL clone survival [134].

Ibrutinib has limited direct pro-apoptotic activity *in vitro* and requires interfering with cross-talk between CLL cells and the lymph node microenvironment [135]. Therefore, the apoptotic effect of ibrutinib cannot be assessed in the present *ex vivo* setting. Overlap between BCR complex and ROR1 signaling through activation of BTK has been reported in CLL, MCL, ALL, and Burkitt's lymphoma [45, 46, 48].

In conclusion, our results suggest that a ROR1 inhibitor can induce apoptosis in ibrutinib-resistant CLL cells and preliminarily in venetoclax-resistant cells. Our data support a potential combination strategy of ROR1-targeted drugs with ibrutinib or venetoclax to target CLL tumor cells.

5.4 PAPER IV

A ROR1 small molecule inhibitor (KAN0441571C) induced significant apoptosis of mantle cell lymphoma (MCL)
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In this manuscript, we characterized the expression of ROR1 in MCL primary cells and cell lines. The anticancer activity of the small molecule ROR1 inhibitor, KAN0441571C, was analyzed in preclinical *in vitro* and *ex vivo* models. Previous studies show that ROR1 is highly expressed in MCL cells, a rare but aggressive and incurable form of non-Hodgkin's lymphoma [47, 136].

Furthermore, KAN0441571C dephosphorylated the TK domain of ROR1 and the combination of the ROR1 inhibitor with other therapeutic drugs, including ibrutinib, venetoclax, idelalisib, everolimus, and bendamustine, resulted in a synergistic effect in primary MCL cells (n=11). Besides, low doses of the drugs in combination appeared to be as effective as high doses. For example, in primary MCL cells, the two best synergistic effects of KAN0441571C with other inhibitors were observed between a low dose (50nM) of KAN0441571C and a low dose (5000nM) of idelalisib, and a low dose (50nM) of KAN0441571C and a low dose (5nM) of venetoclax.

KAN0441571C alone dephosphorylated not only ROR1 but also PI3K, AKT, BTK, and mTOR in primary MCL cells. However, ROR1, PI3K, and mTOR phosphorylation inhibition were observed when KAN0441571C was combined with venetoclax, ibrutinib, and idelalisib. In addition, the pro-survival (anti-apoptotic) proteins BCL-2 and MCL-1 were also down-regulated by drug combinations than by the drugs alone.

Furthermore, the anti-apoptotic proteins BCL-2 and MCL-1 were also down-regulated by drug combinations than by drugs alone in primary MCL cells.

Regarding MCL cell lines, a combination of KAN0441571C with ibrutinib, venetoclax, or bendamustine results in an additive or mostly synergistic apoptotic effect. KAN0441571C, venetoclax, and ibrutinib alone had similar killing effects in different MCL cell lines, whereas bendamustine appeared to be less effective in two (Mino and Granta-519) of the five cell lines. In addition, the combination of KAN0441571C with venetoclax or ibrutinib had a synergistic apoptotic effect in all cell lines compared to each drug alone. However, KAN0441571C with bendamustine showed a synergistic effect in all MCL cell lines except Granta-519.

Different MCL cell lines appeared to utilize one or the other signaling pathway. For example, KAN0441571C caused dephosphorylation of Src in the Z138 cell lines as well as PI3Kδ, mTOR, and transcription factor c-JUN. The extracellular signal-regulated kinases (ERK) pathway, usually dysregulated in malignancies and induces tumor cell proliferation [137], was also inactivated in MCL cells by ROR1 inhibition. The levels of cleaved caspases 3 and PARP increased significantly, at least in the Z138 cell line.

In the Granta-519 cell line, ERK and AKT were dephosphorylated by KAN0441571C, which might be mainly because of ERK inhibition in the AKT/mTOR pathway. Also, a dose-dependent increase of cleaved caspase 9 in Granta-519 cells indicates activation of the intrinsic apoptotic pathway.

MCL cells exhibit high expression of MCL-1 and BCL-2 [138]. BCL-2 and MCL-1 promote cell survival in lymphoma by inhibiting the activation of pro-apoptotic proteins [139]. Members of the BCL-2 family are critical regulators of the mitochondrial apoptotic pathway [140], and genetic mutations in BCL-2 have been associated with lymphomagenesis and chemotherapy resistance [141]. Our result showed that the anti-apoptotic proteins of BCL-2 and MCL-1 are slightly downregulated in primary MCL cells, while the pro-apoptotic Bax protein remains unchanged.

In summary, our data show that the combination of ROR1 inhibitors with current drugs could effectively dysregulate signaling pathways in MCL tumor cells, which contributes to efficient therapeutic strategies for both untreated and refractory MCL.

5.5 PAPER V

ROR1 targeting tyrosine kinase inhibitor induced significant apoptosis of non-small cell lung cancer (NSCLC) cells in combination with erlotinib and ibrutinib
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This study investigated the clinical and functional characteristics of ROR1 expression in 287 lung tissues from surgically resected NSCLC patients. In addition, the anti-tumor effect of the ROR1 inhibitor KAN0441571C was evaluated *in vitro*, alone or in combination with erlotinib (EGFR inhibitor) and ibrutinib (BTK inhibitor), which have a different mechanism of action other than ROR1 inhibitors.

Our data show that ROR1 expression in tumor cells was more common in non-squamous and squamous (87 and 57%) NSCLC cases than in patients with

neuroendocrine (21%) features (p=0.0001). KAN0441571C dephosphorylated ROR1 and induced apoptosis of NSCLC cell lines in a time- and dose-dependent manner. Overall survival of NSCLC patients tends to be shorter in ROR1+ than in ROR1- patients. ROR1 was more highly expressed in women compared to men with squamous NSCLC (p=0.002). KAN0441571C induced greater apoptosis in NSCLC cells than erlotinib, and a combination of KAN0441571C and erlotinib showed synergistic or additive effects on lung cancer cells. KAN0441571C also inhibited proliferation and metastatic function of cell lines, inactivated AKT/PI3K/mTOR signaling pathway, and induced apoptosis of tumor cells by down-regulating MCL-1 and BCL-2 and PARP and caspase 3 cleavage.

As lung cancer remains the first cause of cancer-related mortality, the promise of effective targeted therapies remains unfulfilled. [142]. Small cell lung cancer (SCLC) NSCLC is two types of lung cancer, with NSCLC being the most common, accounting for 80-85% of cases. Unfortunately, conventional treatments for lung cancer are not effective, and there is an urgent medical need to develop new targeted therapies for lung cancer [143].

KAN0441571C alone appeared to be superior to both ibrutinib and erlotinib. However, a combination of KAN0441571C with ibrutinib and erlotinib increased the cytotoxicity of KAN0441571C in 3 of 5 cell lines except for NCI-H23 and A549. In addition, the combination of KAN0441571C and erlotinib/ibrutinib showed a synergistic or additive apoptotic effect in all lung cancer cells tested.

KAN0441571C inhibited ROR1 phosphorylation and induced apoptosis via the extrinsic and intrinsic pathways by blocking pro-survival BCL-2 and MCL-1 molecules and upregulating the BAX protein. KAN0441571C inactivated the non-canonical Wnt signaling pathway (PI3K/AKT/mTOR) and the transcription factor CREB. The ROR1 inhibitor was more effective than the EGFR inhibitor in inducing apoptosis of NSCLC cell lines.

Our data showed that inhibition of ROR1 by KAN0441571C prevented phosphorylation of ROR1 and EGFR. Dephosphorylation of EGFR may be due to dephosphorylation of ROR1, which led to inactivation of EGFR-ROR1 dimers, followed by suppression of survival signaling by these key dimers in NSCLC cells. Thus, inhibition of ROR1 and a combination of ROR1 and EGFR inhibitors could be effective in treating TKI-resistant lung cancer cells with different mechanisms of action and prevent further mutations in EGFR molecules of NSCLC cells.

In conclusion, ROR1 is expressed in NSCLC. Therefore, ROR1 may play an important role in the biology of the disease. The 2nd generation ROR1 inhibitor (KAN0441571C) was highly effective in inducing apoptosis of NSCLC cells and superior to erlotinib and ibrutinib, but with different MOA. Therefore, the development of new drugs with different MOA than those clinically available is approved to improve prognosis in NSCLC. Currently, several new drugs for lung cancer treatment are in clinical development, including EGFR inhibitors such as erlotinib [144]. Consequently, targeting NSCLC cells by combining ROR1 and EGFR inhibitors may be a promising approach for treating NSCLC. Thus, the combination of the two drugs resulted in potent

tumor cell killing and could be an interesting approach to be investigated in further preclinical models.

6 FUTURE PERSPECTIVES

As the complex molecular mechanisms upon which cancer cells rely become better defined, more effective therapeutic strategies may emerge that simultaneously target disease-specific molecules or different signaling pathways. In addition, the combination of targeted therapies with cytostatic drugs results in better outcomes in cancer treatment and overcoming drug resistance.

Studies confirm the critical roles of ROR1 in the cellular characteristics of human cancer. Wnt5a, as a ligand for ROR1, stimulates ROR1 signaling by activating PI3K/AKT/mTOR (Wnt non-canonical) and Wnt5a-canonical signaling pathways. The overexpression of ROR1 was correlated with disease progression in various cancers. ROR1 targeting small molecules inhibited ROR1 activity and caused significant apoptosis of tumor cells and their functions in different pre-clinical cancer models. Thus, ROR1 appears to be a promising RTK for precision cancer medicine. Additional improvement of ROR1 inhibitors, such as those that target other parts of the ROR1 molecule with more specificity, is essential. ROR1 function in several tumors is still unknown. A better understanding of ROR1 partners involved in tumor cell function for each specific tumor is of great importance. A few of these partners, such as EGFR in lung cancer and B-cell receptor complex in some B-cell malignancies, have shown the vital role of these partners in tumor cells survival. Therefore, advanced knowledge of ROR1 functions and characteristics as a druggable target in carcinogenesis is required to optimize ROR1 inhibitors, specifically in combination with other anticancer-targeted drugs.

The main goal of this thesis was to uncover the novel role of small molecule ROR1 inhibitors in tumor cell death. Furthermore, this study confirms the benefit of treatment strategies combination by using KAN0441571C with other inhibitors, as it targets different tumor-derived proteins, as shown in Figures 8 and 9.

In summary, ROR1 has proven to be an attractive biological target for cancer therapy. However, there is an urgent need to better understand the biology of ROR1 in order to optimize therapeutic approaches. Additionally, the role of ROR1 in tumorigenesis is not fully understood and requires further research. Targeted small molecule anti-cancer drugs can accelerate the progression of next-generation inhibitors to improve the treatment outcomes, overcome drug resistance, and develop combination therapy strategies.

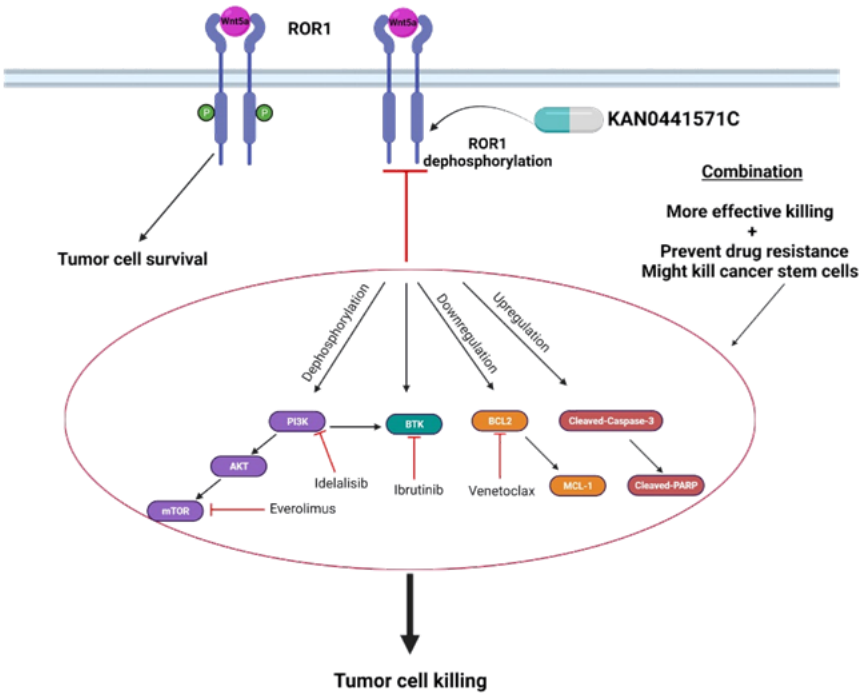

Figure 8. Schematic representation of the signaling pathways potentially involved in the mechanism of action of KAN0441571C in combination with other target inhibitors in B-cell malignancies. *Created with BioRender.com*

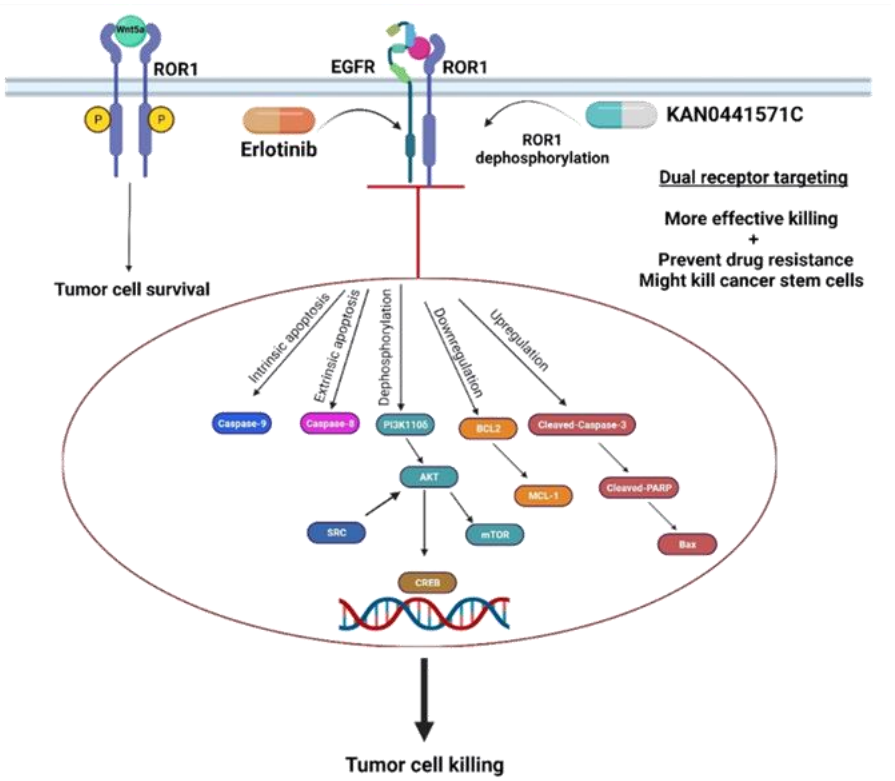

Figure 9. Schematic proposed model of the signaling pathways potentially involved in the mechanism of action of KAN0441571C in combination with erlotinib in EGFR+ solid tumors. *Created with BioRender.com*

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