Investigating ALK inhibitors alone or in combination as therapeutic options for ALK-positive neuroblastoma

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Cover illustration: The bright future of neuroblastoma patient, who would be cured by targeting ALK and other oncogenic signaling pathways through advance therapeutic medication and research work done to bring the smiles back to the suffering children.
By Komal Kazmi and Sharmeen Kazmi

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Be content in times of trouble and be happy despite difficulties

If you have beautiful eyes you will find the world beautiful but if you have beautiful heart and tongue then the world will find you beautiful

If you cannot write happiness for someone by becoming a pencil then try to be rubber to remove someone's sorrow and tears

***My family is my life, and everything else is secondary. I dedicate this thesis to my parents, my siblings, my wife & my kids for their prayers, love, mammoth support and sacrifice, due to which I completed this PhD journey*.**

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ABSTRACT

Neuroblastoma (NBL) is the third most common pediatric cancer after leukemia and cranial tumours and accounts for around 15% of death in pediatric malignancy. NBL develops due to the poorly differentiated progenitor cells of the sympathetic nervous system. Even after extensive chemo and immune therapeutic options, high-risk and relapse NBLs are still hard to treat and needs better and efficient therapeutic treatment. The most common genetic abnormalities in NBL are chromosome 1p and 11q deletion, 2p and 17q gain, MYCN amplification and mutation in anaplastic lymphoma kinase (ALK) is a tyrosine kinase receptor, part of the insulin receptor (IR) family, which has been involved in several solid and liquid cancers. ALK mutations are found in about 10% of NBL cases but in the relapsed patient population, the ALK-positive percentage increases significantly. Crizotinib was the first ALK tyrosine kinase inhibitor (TKI), which was approved clinically to treat ALK-positive lung cancer. In NBL, crizotinib has had a less striking effect, which urges to discover more efficient and potent ALK TKIs for NBL treatment. The overall aim of this thesis was to investigate ALK inhibitors alone or in combination as therapeutic options for ALK-positive NBL patients.

Alectinib is a second-generation ALK inhibitor and showed a dramatic effect in crizotinib resistant ALK-positive lung cancer patients. In our first project study, we interrogated the alectinib effect in preclinical settings of NBL. *In vitro* kinase assays and cell-based experiments examining ALK mutations show that alectinib is an effective inhibitor of gain-of-function ALK mutants in NBL models. Administration of alectinib showed efficient tumour effect in mouse xenograft model of NBL, in comparison to crizotinib

In the second study project, we interrogated the inhibitory effect of dihydroorotate dehydrogenase (DHODH) in NBL preclinical settings. Pyrimidine nucleotides play a vital role in tumour progression and these pyrimidines can be synthesized through either salvage or the de novo pathway. Tumour cells fulfil their need for nucleotides through the de novo pathway. Dihydroorotate dehydrogenase (DHODH) is an important player of de novo pyrimidine synthesis and by inhibiting DHODH, tumour cells proliferation is inhibited. Low levels of DHODH in NBL tumours is linked to good clinical outcome. BAY2402234, a novel

DHODH inhibitor has shown striking inhibition in acute myeloid leukemia (AML) and we investigated BAY2402234 in NBL settings. In BAY2402234 treated NBL cells and in transgenic mouse models, inhibition of cell proliferation and significant reduction of tumour growth were observed. Biochemical analysis showed that BAY2402234 treatment inhibited MYCN expression and increased p53 and cleaved PARP protein levels. Synergy was observed in ALK-positive NBL cells upon the combination of BAY2402234 and lorlatinib treatment.

This thesis study shows the significance of ALK in NBL. To summarize, alectinib is an efficient inhibitor of ALK kinase activity in ALK addicted NBL and BAY2402234 inhibits NBL cell proliferation *in vitro* and *in vivo*. The combination treatment of BAY2402234 and lorlatinib showed synergy and as a promising future therapeutic option for the NBL patients, this should be considered alone or in combination.

Keywords: NBL, Anaplastic lymphoma kinase, MYCN, DHODH, alectinib, crizotinib, BAY2402234, lorlatinib

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SAMMANFATTNING PÅ SVENSKA

Neuroblastom (NBL) är den tredje vanligaste pediatriska cancern efter leukemi och kraniala tumörer och står för cirka 15 % av dödsfallen i pediatrisk malignitet. NBL utvecklas på grund av de dåligt differentierade progenitorcellerna i det sympatiska nervsystemet. Även efter omfattande kemo- och immunterapialternativ är NBL med hög risk och återfall fortfarande svåra att behandla och behöver bättre och effektiv terapeutisk behandling. De vanligaste genetiska abnormiteterna i NBL är kromosom 1p och 11q deletion, 2p och 17q gain, MYCN amplifiering och mutation i anaplastiskt lymfom kinas (ALK) är en tyrosinkinasreceptor, en del av insulinreceptorfamiljen (IR) som har varit involverad i flera fasta och flytande cancerformer. ALK-mutationer finns i cirka 10 % av neuroblastomfallen, men i den återfallande patientpopulationen ökar den ALK-positiva andelen signifikant. Crizotinib var det första ALK TKI-läkemedlet, som godkändes kliniskt för att behandla ALK-positiv lungcancer. I NBL har crizotinib haft en mindre slående effekt, vilket uppmanar till att upptäcka mer effektiva och potenta ALK TKI för NBL-behandling. Det övergripande syftet med denna avhandling var att undersöka ALK-hämmare ensamma eller i kombination med andra mål som ett potentiellt framtida terapeutiskt alternativ för ALK-positiva neuroblastompatienter.

Alectinib är en andra generationens ALK-hämmare och visade en dramatisk effekt hos crizotinib-resistenta ALK-positiva lungcancerpatienter. I vår första projektstudie förhörde vi alektinibeffekten i prekliniska miljöer av NBL. *In vitro* kinasanalyser och cellbaserade experiment som undersöker ALK-mutationer visar att alectinib är en effektiv hämmare av förstärknings-of-function ALK-mutanter i NBL-modeller. Administrering av alectinib visade effektiv tumöreffekt mus xenograft modell av NBL, i jämförelse med crizotinib.

I det andra studieprojektet undersökte vi den hämmande effekten av dihydroorotatdehydrogenas (DHODH) i NBL prekliniska miljöer. Pyrimidinnukleotider spelar en viktig roll i tumörprogression och dessa pyrimidiner kan syntetiseras genom antingen räddnings- eller de novo-väg. Tumörceller fyller sitt behov av nukleotider genom de novo-vägen. Dihydroorotatdehydrogenas (DHODH) är en viktig aktör för de novo pyrimidinsyntesen och genom att hämma DHODH minskar tumörcellsproliferationen. Låga nivåer av DHODH i NBL-tumörer är kopplat till bra kliniskt resultat. BAY2402234, en ny DHODH-hämmare har visat slående hämning vid akut myeloid leukemi (AML) och vi undersökte BAY2402234 i NBL-miljöer. I BAY2402234-behandlade NBL-celler och i transgena musmodeller observerades hämning av cellproliferation och signifikant minskning av tumörtillväxt. Biokemisk analys visade att BAY2402234-behandling hämmade MYCN-uttryck och ökade p53- och klyvda PARP-proteinnivåer. Synergi observerades i ALK-positiva NBL-celler vid kombinationen av BAY2402234 och behandling med lorlatinib.

Denna avhandling visar betydelsen av ALK i NBL. För att sammanfatta, är alectinib en effektiv hämmare av ALK-kinasaktivitet i ALK-beroende NBL och BAY2402234 hämmar NBL-cellproliferation *in vitro* och *in vivo*. Kombinationsbehandlingen av BAY2402234 och lorlatinib

visade synergi och som ett lovande framtida terapeutiskt alternativ för NBL-patienter bör detta övervägas ensamt eller i kombination.

Nyckelord: NBL, Anaplastiskt lymfomkinas, MYCN, DHODH, alectinib, crizotinib, BAY2402234, lorlatinib

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II. **Muhammad Wasi Alam**., Ganesh Umapathy., Yeshwant Kurhe., Dan E. Lind., Sonia Lain., Palmer, R. H., & Hallberg, B. BAY2402234, a dihydroorotate dehydrogenase (DHODH) inhibitor, abrogates proliferation and induces apoptosis in neuroblastoma cells. Manuscript 2021

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ABBREVIATIONS

ALCL = anaplastic large cell lymphoma

ALK = anaplastic lymphoma kinase

ALKAL1 = ALK and LTK Ligand 1 & 2

ALO17 = ALK lymphoma oligomerization partner on chromosome 17

ALT = alternative lengthening of telomeres

ATIC = 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase

ATM = ataxia telangiectasia mutated

ATR = ataxia telangiectasia and Rad3 related

ATRA = all-transretinoic acid, tretinoin

ATRX = alpha thalassemia/mental retardation syndrome X-linked

BMP = bone morphogenetic protein

BIRC5 = baculoviral inhibitor of apoptosis repeat-containing 5

BARD1 = BRCA1 Associated RING Domain 1

CARS = cysteinyl-tRNA synthetase

CC = chromaffin cells

CDKs = Cyclin-dependent kinases

CHEK2 = checkpoint kinase 2

CLTC = clathrin heavy chain

CAN = copy number alteration

CNS = central nervous system

CT = computerized tomography

CXCR4 = C-X-C motif chemokine receptor

DA = dorsal aorta

DD = DNA damage

DG = dorsal ganglia

DHFR = Dihydrofolate Reductase

DHODH = dihydroorotate dehydrogenase

DLBCL = diffuse large B-cell lymphoma

DLG2 = disc large homolog 2

ECD = extracellular domain

EFS = event-free survival

EGFR = epidermal growth factor receptor

EML4 = echinoderm microtubule associated protein-like 4

ERK = extracellular signal-regulated kinase

FAD = flavin adenine dinucleotide

FDA = Food and Drug Administration

FMN = flavin mononucleotide

G1 = gap 1 phase

G2 = gap 2 phase

GAP = GTPase-activating proteins

GDP = guanosine diphosphate

GD2 = disialoganglioside 2

GEF = guanine nucleotide exchange factors

GOF = gain-of function

GR = glycine-rich region

GTP = guanosine triphosphate

GWAS = genome-wide association studies

IGF-1R = insulin-like growth factor receptor 1

IMT = inflammatory myofibroblastic tumour

InR = insulin receptor

INSS = International Neuroblastoma Staging System

JAK = Janus kinase

KIF5B = kinesin family member 5B

KLC1 = kinesin light chain 1

LDLa = low density lipoprotein class A

LOF = loss-of function

LTK = leukocyte tyrosine kinase

M = mitosis

MAM = meprin A5 protein and receptor protein tyrosine phosphatse mu

MAPK = mitogen-activated protein kinase

MDM2 = Mouse double minute 2 homolog

MHY9 = myosin, heavy chain 9

MNA = MYCN amplified

MIBG = metaiodobenzylguanidine

MRI = magnetic resonance imaging

MSN = moesin

mTOR = mammalian target of rapamycin

MYC = Myelocytomatosis Viral Oncogene Homologue

MYCN = Neuroblastoma MYC oncogene

NBL = neuroblastoma

NC = neural crest

NCA = Numerical chromosome alterations

NCC = neural crest cells

NPM = nucleophosmin

NRG1 = neuregulin 1

NRTK = non-receptor tyrosine kinase

NSCLC = non-small cell lung cancer

NT = neural tube

OS = overall survival

PC12 = pheochromocytoma 12 cells

PFS = Progression-free survival

PHOX2B = Paired-like homeobox 2b

PI3K = phosphoinositide 3-kinase

PINK1 = PTEN-induced kinase 1

PK = protein kinase

PLCγ = phospholipase Cγ

PPFIBP1 = protein-tyrosine phosphatase receptor-type F polypeptide-interacting protein-binding protein 1

PTK = protein tyrosine kinase domain

PTPN3 = protein tyrosine phosphate non-receptor type 3

PTPN11 = Protein Tyrosine Phosphatase Non-Receptor Type 11

RA = retinoic acid

RANBP2 = Ras-related nuclear protein-binding protein 2

RAF = Rapidly Accelerated Fibrosarcoma

RAS = Rat sarcoma virus

RB = retinoblastoma

RS = replication stress

RTK = receptor tyrosine kinase

S = synthesis

SAP = sympathoadrenal precursor cells

SCA = segmental chromosomal alterations

SEC31L1 = SEC31 homologue A

SHANK2 = SH3 and Multiple Ankyrin Repeat Domains 2

SN = sympathetic neurons

SQSTM1 = sequestosome -1

STAT = signal transducer and activator of transcription

STRN = striatin

SV = structural variation

TAT = targeted alpha therapy

TERT = telomerase reverse transcriptase

TFG = TRK fused gene

TKD = tyrosine kinase domain

TMD = transmembrane domain

TPM3/4 = tropomyosin 3 and 4

TRAF1 = TNF receptor-associated factor 1

TSG = tumor suppressor gene

TT = targeted therapy

1. INTRODUCTION

1.1. Cancer

Cancer is classified as one of the most lethal diseases worldwide where major changes occur in the genome aggressively [1, 2]. During 2020, the mortality cases of cancer was around 10 million, and until 2040, statistically the death cases would be increased to 16.4 million [3, 4]. Cancer is a genetic disease where cell proliferation is disturbed and imbalanced, these cancer cells can move to different bodily organs. The emergence of a cancerous cell from a normal cell takes place through several processes, e.g., mutation, deletion, amplification and the translocation of several genes, which leads to an abnormal protein expression and activity as well as modification in DNA repair genes and activation and inactivation of oncogenes and tumour suppressor genes respectively [5-7]. Cancer development can be caused by exposure to carcinogenic substances. Tumour initiation, promotion and progression take place through alterations to a normal cell, which leads to a malignant cell [2, 8] (Figure 1). Although, cancer is termed as a genetic disease but an abnormal cell signaling is an important and ultimate factor in tumour progression that is involved in aberrant activities of enzymes and other important biological process, for instance, apoptosis, cell cycle, differentiation and proliferation. It has been stated that soon cancer would be the top reason for death in the world [9]. There are two gene classes: oncogenes and tumour suppressor genes. Oncogenes cause tumour initiation and progression by acquiring dominant gain-of-function mutations. On the other hand, tumour suppressor genes become non-functional in a recessive aspect [1, 2].

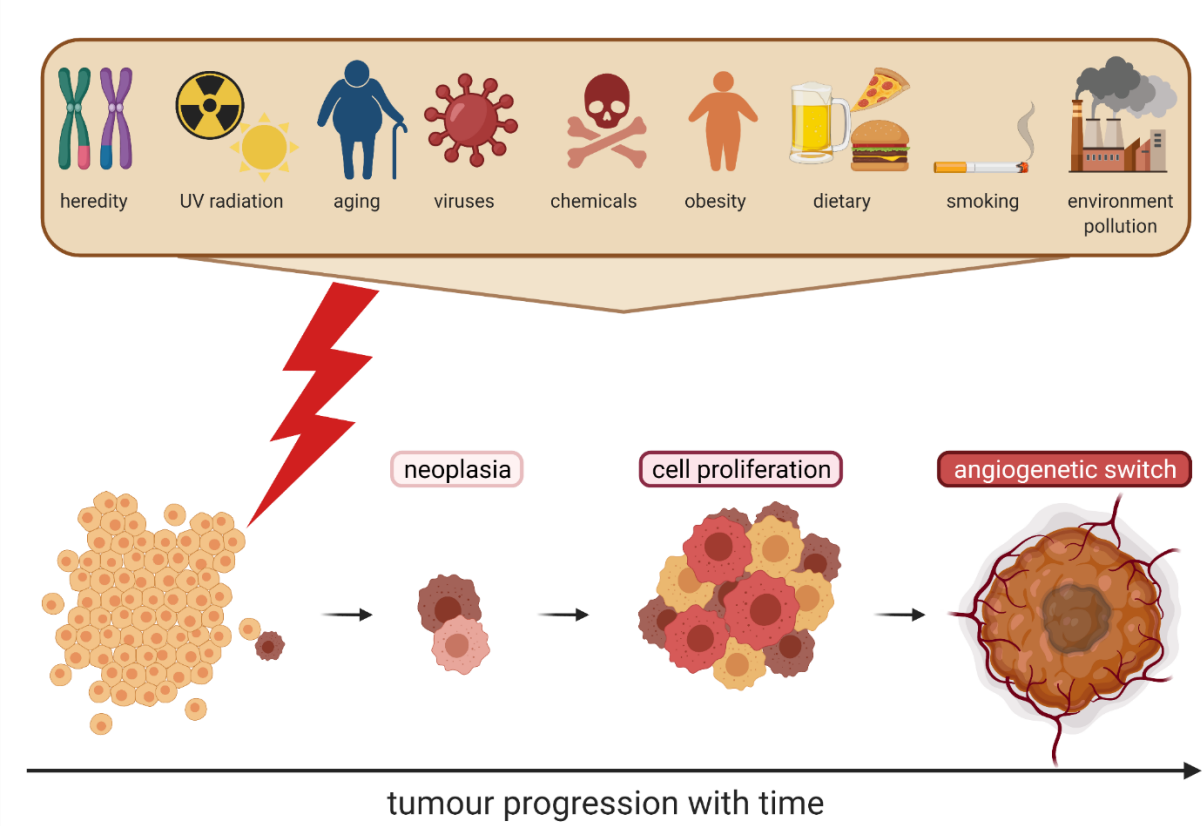

Figure 1. ***Transformation of malignant tumour:*** *The stepwise transformation of tumour from a normal cell with a passage of time by possible different factors involved in this transformation.*

1.1.1. Oncogene and tumour suppressor

Oncogene

The normal cell characteristics are a tightly regulated and well-balanced cell division, differentiation and apoptosis (programmed cell death). Normal cells could undergo programmed cell death once their function has been perturbed [10]. Nevertheless, once the oncogenic signaling is activated this tightly regulated and well-balanced system is disturbed that cause tumour ultimately [11].

Proto-oncogene are responsible for translating numerous different proteins, which are important for cell division, proliferation and differentiation [1, 2, 11-15]. They can be oncogene from proto-oncogene through different ways:

1. Point mutation: Gain-of-function (GoF) point mutation (e.g. in HRAS, KRAS, NRAS, ALK, EGFR).

2. Amplified gene that drives to raise the protein levels (e.g. MYC, MYCN, EGFR, ERBB, DHFR, and RAS).

3. Gene translocation, where one proto-oncogene is fused with other gene and setup a fusion protein that increases the tumorigenic properties (e.g. fusion of BCR with ABL, NPM with ALK, and EML4 with ALK).

Also, epigenetic changes can induce a high protein expression and this can result in the enhanced activity of protein signaling, which disturbs the normal process of cell division, survival and growth, becoming cancer [11, 16-18].

The RAS family (H/K/N-RAS) is one of the most mutated oncogenes in human cancer to date. It can be activated by point mutation and is found in 16% of all cancers [19-25]. The RAS mutation percentage increases and depends on the cancer type [26] e.g., in pancreatic cancer, 95% of mutations are present in the K-RAS. The figure is 50% for colon cancer. K-RAS is the most mutated (85%) among all RAS genes, with mutations for N-RAS (12%) and for H-RAS (3%) [27-29].

All RAS proteins belong to the RAS-related small GTPase superfamily, where they play the role of a molecular switch that regulates intracellular signaling [21, 30, 31]. RAS is activated through the binding of guanosine triphosphate (GTP) and deactivated when it attaches to guanosine diphosphate (GDP). This switch is controlled by GTPase activating proteins (GAP) and guanine nucleotide exchange factors (GEF), which activates downstream signaling cascades e.g., the RAS-MAPK pathway concludes the fate of the cell by triggering and regulating key cellular processes, including cell division, survival and differentiation [28, 31-35] (Figure 2). Receptor tyrosine kinases (RTKs) such as ALK (anaplastic lymphoma kinase) and EGFR (epidermal growth factor receptor) can also activate RAS/MAPK and PI3K/AKT pathways without any mutation in RAS/MAPK through ALK and EGFR [36, 37]. The RAS/MAPK pathway is a potential therapeutic target in many cancers [38-41].

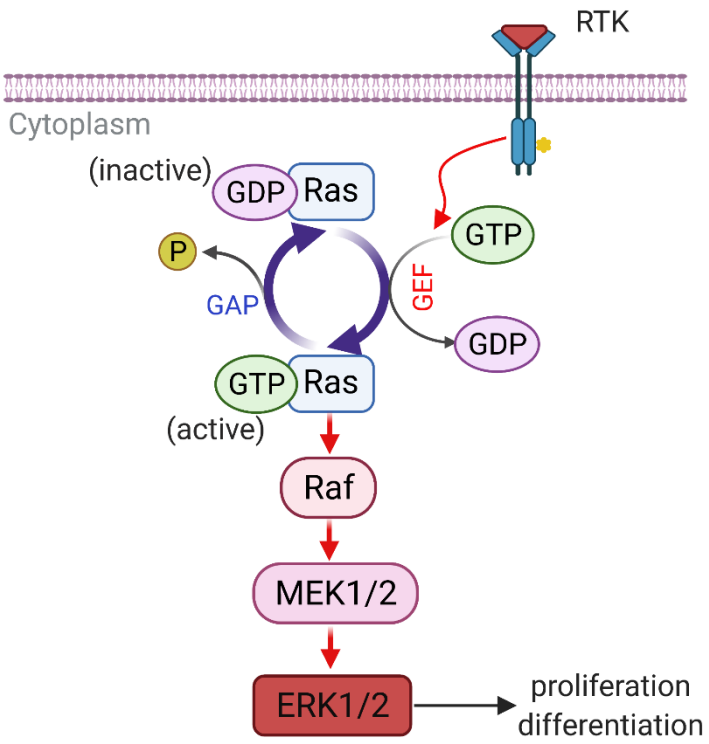

Figure 2. ***RAS activation signaling pathway:*** *The stepwise signaling of RAS to ERK1/2*

Tumour Suppressor Genes

Oncogenesis is not a single step process and conversion to a cancer cell might not only be by the activation of an oncogene but rather by a combination of many other genetic modifications [31]. Tumour suppressor genes (TSGs) play a vital part in preventing the transformation of a normal cell into a cancer cell by checking cell growth, stimulating DNA repair and encoding the proteins that increase apoptosis and cell cycle checkpoints [16, 31, 42-44].

As the activation of an oncogene is important and required to enhance tumorigenesis, deactivation or the loss of tumour suppressor genes also boosts cancer growth and in most tumour suppressor genes, mutations are loss-of-function mutations [31, 45, 46]. The famous 'two hit' tumorigenesis hypothesis was provided by Knudson. This states that a single allele is not enough to induce a tumour. Instead, the loss of both alleles of the retinoblastoma (RB) gene is essential for the development of a rare paediatric eye tumour, the first tumour suppressor gene discovered in retinoblastoma [45, 47, 48]. In many cancers (breast, head & neck, lung and melanoma), RB is deregulated by upstream activators [49, 50].

Many other tumour suppressor genes have been identified. The second tumour suppressor revealed was p53 [50-52]. During the normal process, an E3 ubiquitin ligase, mouse double minute 2 homolog (MDM2), binds and deactivates p53 activity by taking it to proteasomal degradation. Once stress activators like DNA damage, imbalance cell cycle and hypoxic conditions are activated then the complex dissembles and p53 triggers cell cycle arrest or apoptosis [50-52]. p53 is the most common and frequently mutated tumour suppressor gene in human malignancies and becomes a possible remedial target in many tumour types [51]. In colon cancer where p53 is intensely investigated and studied, around 80% of cases pursue the 'two-hit theory' [51], but in some cases, the opposite of Knudson's two-hit hypothesis has been identified where a mutation in one allele is ample to inactivate p53 activity in cells. This is called 'dominant-negative mutation' [53, 54]. The first p53 inhibitor was found by Kamarov and his team in 1999. They showed that mice were protected by serious damage from ionizing radiation by blocking p53-induced transcription with the help of pifithrin-α [55]. It is difficult to revive p53 in tumours when it is missing but this can be attained by employing genetic and pharmacological techniques [56, 57]. The restoration of the transcriptional activity of p53 was shown by Foster's team in 1999 by discovering the small molecule, which showed anti-cancerous activity in a mouse model [58]. Nowadays the technique to restore p53 activity is to target CDKs, MDM2 or RAS-MAPK pathway members [56, 59, 60].

Approximately one mutation occurs in 10^7 cell divisions and the mutation rate is very slow. These mutations cause genetic lesions, which activates gain-of-function in an oncogene or loss of function in a tumour suppressor gene. To induce tumorigenesis, multiple sequential mutations are required. As per the mutation rate, the tumour takes time to grow [61], which is why most tumours develop in the older age groups [62]. Cancer mostly seems to be found in age-related cases and the older population, but incidences of childhood tumours cannot be ignored, and special attention is needed.

1.2. Neuroblastoma

Neuroblastoma (NBL) is a cancer of immature nerve cells and a typical extracranial solid paediatric malignancy. NBL initiates from undifferentiated neural crest derived cells in the developing nervous system. Paediatric cancer-related deaths caused by NLB are around 15% and the tumour is located in the adrenal gland or sympathetic ganglia [63-71]. Worldwide,

NBL is the 3rd most common childhood malignancy and just behind leukemia and cranial tumours [72]. NBL is a heterogeneous and complex disease with around 90% of NBL cases diagnosed under the age of 10 in children (40% of cases are diagnosed in children less than a year old). The median age is around 18 months at the time of diagnosis [67, 73, 74]. NBL can arise sporadically and in a familial form but familial cases are few, with an incidence rate of 1-2% and the rest of cases are sporadic [75]. NBL can be developed along the sympathetic chain with half (around 47%) of the cases arising in the adrenal medulla of the adrenal glands and the other growing areas are the abdomen, thoracic, pelvis, neck and other areas with 24%, 15%, 3%, 2.7% and 7.9% respectively [67, 76-79]. When NBL cases come and investigate in a clinic, it is diagnosed and subject to various factors like the patient's age, the stage of the tumour, histology, the chromosomal aberration, and other markers e.g., MYCN and TrkA status [80-83]. These factors are used to clinically define the stages of NBL according to the International Neuroblastoma Staging System (INSS) [84]. There are five different stages (stage 1 to 4 and 4S) [63, 68, 85]. Stages 1 and 2 of NBL are classified as initial stage tumours, with non-metastatic and localized properties, sensitive to chemo and radiation therapies. Stage 3 tumours cannot be removed fully by surgery and a metastatic tumour cannot be removed remotely. Stage 4 tumours are metastatic and advance tumours with distant metastasis properties. The fifth and last stage is 4S, where the NBL tumour is localized as in stages 1 and 2 and patients are under one year of age. The tumour can specifically spread to the bone marrow, liver and/or skin [63, 68, 85, 86]. This stage of NBL tumours shows spontaneous regression, with very little or no therapy [87-89]. The International Neuroblastoma Risk Group Staging System (INRGSS) divides and describes NBL into three risk groups: low, intermediate and high-risk. These risk groups depending on the INSS stage, the patient's age, chromosomal aberration, histology, tumour differentiation status, MYCN and 11q status [86, 90-92].

1.2.1. Chromosomal and genetic abnormalities in NBL

The precise cause of NBL and its origin (initiation) is not crystal clear but some factors are consistently associated with NBL development. These can either be familial and/or somatic aberrations [75]. Segmental chromosomal alterations, numerical chromosome alterations and mutations are NBL genetic aberrations. Most frequently somatic mutated genes are *ALK, ATRX and PTPN11* with 10%, 2.5% and 2.9% of frequency respectively. Some other mutated genes are *PHOX2B, NRAS, TERT, CHEK2, PINK1 and BARD1* [93-95]. Amplified *MYCN* levels are

found in 25% of all NBL cases and about 50% are found in high-risk patients. The others are chromosomal gain in 17q (65%), loss in 11q (20-45%), loss in 1p and gain in 2p, which are most common and frequent in segmental chromosomal alterations [36, 63, 94-104].

1.2.1.1. Gain of chromosome 2p (ALK, ALKAL2 & MYCN)

Three main players of the ALK signaling cascade are present on the distal arm of chromosome 2, ALK, ALKAL2 (ALK ligand) and MYCN. These players are linked to a poor prognosis in NBL patients [100, 105]. The position of ALK is at 2p23.2-2p23.3, MYCN at 2p24.3 and ALK ligand, ALKAL2 is 2p25.3. All of them are present on chromosome 2p. In NBL patients, the mutation in the ALK gene is found in around 10% of cases [97, 99, 106-108]. In both familial and sporadic NBL cases. ALK point mutation in the kinase domain has been described and is around 10% of all NBL [97, 99, 106-109]. Several mutations in the ALK kinase domain have been revealed but three mutations are known as “hot spot” mutations found in 85% of all ALK kinase domain mutation cases in NBL. These are F1174 (30%), F1245 (12%) and R1275 (45%) [94, 97, 99, 106-108, 110]. Tumour-inducing capabilities have been shown in the combination of ALK-F1174L and MYCN in the transgenic mouse system [111]. Inhibition of ALK activity has been observed in NBL by ALK TKIs treatment that showed striking cell proliferation inhibition and tumour growth reduction in NBL cell lines and mouse models respectively [112-116]. *MYCN* amplification is one of the hallmarks of NBL and is found in 20-25% of NBL cases. The percentage spikes to 50% in high-risk NLB and relates to advanced-stage NBL with a poor prognosis, the worst survival and with an aggressive phenotype [68, 98, 117]. The *MYCN* gene encodes a transcription factor, MYCN, which plays a key role in apoptosis, survival and differentiation [63, 68, 118]. As in NBL, *MYCN* amplification is also reported in other cancers [36, 119-121]. Several genetically engineered mouse models have been generated where *MYCN* overexpression shows tumour initiation of NBL and *MYCN* collaborates with other oncogenes to induce NBL pathogenesis [111, 122]. *ALK*, *MYCN* and *ALKAL2* show evidence of the development, evolution and propagation of NBL [100]. Considering their strong role in NBL development, targeting *ALK*, its downstream targets *MYCN* and *ALKAL2* would be the viable therapeutic option for NBL patients.

1.2.1.2. Chromosome 11q deletion

One of the most common genetic abnormalities in NBL is the deletion of 11q, which accounts for 20-45% of NBL patients. 11q is correlated with a poor prognosis, advanced stage NBL and *MYCN*-independent amplification [98, 123-125]. 4S stage tumours have a better clinical outcome but relapse cases are increased upon 11q deletion in these NBL cases [123, 126]. As it is an effective NBL patient prognosis tool, the International Neuroblastoma Risk Group (INRG) has defined 11q deletion as an important and independent risk factor [90]. Some very important tumour suppressor genes that sit on chromosome 11 are deleted due to the loss of the long arm of chromosome 11. NBL pathogenesis has evolved upon a loss of chromosomes and/or point mutation in tumour suppressor genes [127]. For instance, Disc Large Homolog 2 *(DLG2)* is a newly published tumour suppressor gene that sits on 11q14.1. *DLG2* is revealed as an *ALK* downstream signaling target and its overexpression in NBL cells shows an increased differentiation and a decreased tumour burden in the xenograft mouse model system [128]. Some other examples of a loss of the tumour suppressor gene due to 11q deletions are immunoglobulin superfamily 4 (*IGFSF4*), tumour suppressors in lung cancer 1/cell adhesion molecule 1 (*TSLC1/CADM1*) [86, 129, 130], DNA damage response (DDR) genes (*ATM, CHK1, MRE11 and H2AFX*) [63, 97, 98] and postsynaptic adaptor protein-coding gene, *SHANK2*. The induced expression of *SHANK2* drives differentiation and inhibits proliferation in NBL cells with retinoic acid [131].

1.2.1.3. Chromosome 17q gain

Another abundant abnormality in NBL is the chromosomal 17 gain of the long arm (17q), which refers to a poor patient outcome and found in around 40-50% of cases of NBL [86, 96]. Contrary to 11q loss, 17q gain is directly proportional to *MYCN* amplification or 1p deletion, which drives a poor prognosis in NBL patients under 12months [132, 133]. During chromosomal gain of 17q, an uneven rearrangement happens with other partner chromosomes. 1p is the most common translocation partner, which, in turn, results in a 17q gain and a concurrent 1p deletion [132, 134, 135]. *BIRC5* (survivin), *NM23A* and *PPM1D* are the most important genes, which are found on this chromosome 17. These genes help in tumour cell progression [86, 136-138]. Survivin correlates to a poor patient outcome, overexpressed in NBL tumours and acts as an apoptosis inhibitor [86, 137, 139]. Survivin

inhibition and a *PPM1D* knockdown trigger apoptosis and could be a possible curative clinical target for NBL patients [137, 138, 140, 141].

1.2.1.4. Chromosome 1p deletions

The fragmental deletion of the short arm of chromosome 1 (1p36), occurs in around 35% of NBL cases but the percentage spikes in high-risk NBL where it corresponds to *MYCN* amplification and a poor prognosis in cases of NBL [142-144]. The normal fragment of the short arm of chromosome 1 was transferred into the NBL cell line, resulting in increased neuronal differentiation and abrogated proliferation [145]. *ARID1A*, calmodulin-binding transcription activator 1 (*CAMTA1*), *CASZ1*, chromo-domain helicase DNA-binding domain 5 (*CHD5*), kinesin superfamily protein 1Bβ (*KIF1Bβ*) and microRNA 34a (*mir-34a*) are the important tumour suppressor genes located on the 1p36 site, which correlates to reduced proliferation and induced apoptosis [146-152].

1.2.1.5. Aneuploidy

Ploidy, describes the complete chromosomal sets is also another powerful prognostic factor in NBL. Near diploid or tetraploid forms of DNA are associated with chromosomal abnormalities, for instance, unequal translocation, the gain and loss of fragments and with aggressive primary tumours. On the other hand, the near triploid and hyperploid state of DNA are associated with a better prognosis and low aggression, where whole chromosomal gain and less structural translocation are observed [63, 127].

1.2.2. Diagnostic tools in NBL

When an NBL patient comes to the clinic, they are mostly in the advanced NBL stage with swelling in body parts along with the sympathetic ganglia (such as the abdomen, chest, neck or pelvis), a loss of body weight, increased belly size and with breathing issues. Blood and urine tests are conducted initially to check biochemical levels. In case of elevated levels of catecholamine (adrenaline/epinephrine and nor-epinephrine), the chromaffin cells of the adrenal medulla produce these hormones, and the presence of tumour cells in bone marrow is ample for an NBL diagnosis. Once the patient has been diagnosed based on biochemical analysis, imaging of the tumour has to be performed through computed tomography (CT) scan, magnetic resonance imaging (MRI), a metaiodobenzylguanidine (MIBG) scan,

ultrasound and X-ray. Intravenous injections of MIBG (with a low level of radioactive iodine) are performed to investigate the metastatic NBL cells in skeletal and soft tissues. Further diagnostics are performed by biopsy.

1.2.2.1. Treatment strategies in neuroblastoma

As mentioned before, NBL is divided into 3 risk groups: low, intermediate and high by the Children's Oncology Group (COG). These risk groups and other factors serve to choose the best possible therapeutic option [153]. The most common treatments in NBL patients are surgery, chemotherapy, differentiation therapy, immunotherapy and radiotherapy [154].

1.2.2.2. Surgery

In the low and intermediate-risk groups for non-metastatic NBL tumours, surgery is performed to remove most of the tumour tissue from the primary tumour, with as little tumour tissue left over as possible, showing a therapeutic effect of 97% with a 5-year OS in low-risk groups [155]. The complete removal of the tumour is not possible due to angiogenesis and neighbouring nerves. In intermediate-risk groups, chemotherapy is performed before surgery to reduce the tumour mass and metastatic abrogation [156, 157]. 40-50% OS within 5-years has been shown in high-risk NBL, where surgery and multiple chemotherapy sessions are used in the treatment of the disease. [158, 159]. The 4S group of patients under 18 months of age are not exposed to surgery as the regression of tumours takes place spontaneously with no treatment needed [67, 68].

1.2.2.3. Chemotherapy

Chemotherapy is one of the preferred treatment strategies in NBL and the inclusion of chemotherapy as a treatment is based on the patient’s risk group [69]. The low-risk group needs just a few chemotherapy cycles and the intermediate-risk group requires 2-8 chemotherapy cycles [160]. In chemotherapy, one or more (combination) drugs can be used in the clinics. Chemotherapeutic drugs are highly destructive for dividing cells and trigger growth inhibition and increased apoptosis. At the same time, these drugs are highly toxic to normal cells. Hair loss, vomiting, diarrhoea and anaemia are the most frequent side effects of chemotherapy. Carboplatin, cyclophosphamide, doxorubicin, and etoposide are used for the intermediate-risk group of NBL patients, but for high-risk groups of patients, cisplatin,

cyclophosphamide, topotecan, vincristine and etoposide are employed [160, 161]. For high-risk NBL treatment strategies are: (i) To inhibit metastasis and to reduce the primary tumour size, with 5-8 rigorous chemo cycles employed. (ii) To eradicate the leftover tumour cells, first, an elevated quantity of chemotherapy is used followed by autologous stem cell transplant (ASCT) and radiotherapy. (iii) Immunotherapy and/or differentiation drugs like 13-cis retinoic acid (RA) are employed [69, 162].

1.2.2.4. Retinoid and differentiation therapy

NBL cells are distinguished as poorly differentiated, which gives rise to unsuccessful differentiation of neural crest-derived sympathoadrenal precursor cells with the highest level of spontaneous regression [87, 163, 164]. These indications show a strong need for NBL differentiation-inducing agents, which can induce differentiation and inhibit proliferation. Some agents are used to induce differentiation, for instance, fenretinide, nerve growth factors (NGF) and derivatives of vitamin A (13-cis-RA & all-trans-retinoic acid (ATRA)) are used and have been described to activate differentiation of neurons and proliferation inhibition *in vitro* [165-168]. In high-risk NBLs, a striking improvement with event-free survival has been reported with 13-cis RA treatment [169]. In high-risk NBL, studies have shown the clinical potency of 13-cis RA as maintenance therapy for minimal residual disease [162, 170]. In clinics, 13-cis-retinoic acid has a better effect than ATRA [165]. Combination treatments with RA give a better survival rate [171]. In high-risk NBL, RA and anti-GD2 monoclonal antibodies are used in combination as maintenance therapy [162, 172]. Further clinical investigations are ongoing for the combo treatment between RA and anti-GD2 antibodies in patients with high-risk NBL (clinicaltrials.gov).

1.2.2.5. Immunotherapy

Four decades ago, increased levels of sialic acid and gangliosides on NBL cell surfaces have been described but this was not associated with a prognosis [173]. They play their role in adhesion, migration and metastasis [174]. GD2 stands for disialoganglioside, mostly expressed on neural crest surface or neuroectodermal derived cells and tissues like skin melanocytes, mature neurons, astrocytes and peripheral pain fibres [172]. These findings made a strong case for targeting GD2 for immunotherapy. Initially, for NBL surface glycolipid antigen, four monoclonal antibodies were introduced, with three IgM and one IgG [175]. In

NBL, chimeric anti-GD2 antibodies (ch14.18) known as dinutuximab and mouse 3F8 are two frequently examined anti-GD2 monoclonal antibodies [176]. The combination of dinutuximab with granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2 (IL-2) and 13-cis-RA show better EFS. After intense chemotherapy, these combinations are used in treatment as maintenance therapy for the minimal residual disease [171, 177]. In 2015, the FDA permitted dinutuximab (Unituxin) to be used as a first and frontline therapy treatment in high-risk NBL patients [178-180]. At present, anti-GD2 monoclonal antibodies like 3F8 (NCT00072358) clinical trials is completed and Hu3F8 (NCT02650648) is under clinical investigations for NBL (clinicaltrials.gov).

1.2.2.6. Radiation therapy

Radiotherapy came into the NBL therapeutic protocol when cells of NBL origin, the adrenergic neuroblast, had shown radiation sensitivity, especially in high-risk patients [181]. Improvements in both the PFS and OS of intermediate and high-risk NBL patients has been seen after radiation therapy was included along with chemotherapy [182]. The use of radionuclide (radiolabel iodine) and MIBG (131I-MIBG) in combination with radiotherapy has shown better results in NBL [154, 183]. NBL cells are keen to uptake MIBG due to the norepinephrine transporter (NET) and the later pile up in neurosecretory granules in the cancer cells where these cells are significantly destroyed by 131I-MIBG radiation [184, 185]. Even with a 66% striking response rate with 131I-MIBG targeted therapy, long term dose-limiting toxicities are reported [183, 186, 187].

1.2.2.7. Novel targeted treatment in neuroblastoma

Surgery, chemo and radiation therapies have displayed significant potential and effects in low and intermediate-risk NBL cases, but for high-risk cases, the prognosis is still the worst [140]. Conventional therapies are unsuccessful in refractory/relapsed NBLs cases and within five years, almost 90% of patients are dead [140, 188, 189]. These investigations reflect that in high-risk NBL, especially in refractory NBL, clinical therapeutic resources are restricted or deficient and can also trigger severe toxicity, leading to the need for better, efficient, less-toxic, novel, potent, specific and targeted therapeutic agents [190]. The main strategy to identify a targeted therapy compounds or a combination of compounds is to kill the tumour cells without disturbing the normal cell [191, 192]. Intensive preclinical research is ongoing to

identify novel agents, but as NBL is a rare disease, there have been only a few phase III clinical trials (https://clinicaltrials.gov/). Currently, many novel targets have been discovered by investigating the mechanistic insight of NBL tumorigenesis and relapse. For instance, these factors are ALK, BIRC5, CDK4/6, CHEK1, MEK, MYCN etc., which can be targeted through targeted therapies in NBL [140, 193].

NBL is a well-known disease of an abnormal signaling protein in cells. Due to this aberrant activity in NBL, the Neuroblastoma New Drug Development Strategy (NDDS) has been endorsed so that protein products that trigger the increase of tumour propagation and growth should be given more consideration for targeted therapy [140, 193]. ALK, ALT (alternative lengthening of telomere lengthening), ATRX, Aurora kinase, BCL2 BET, BIRC5, BRIP1, CHEK1, CDK4/6, CDK2/9, CDK7, MDM2, MEK, mTORC1/2, PARP, TERT telomerase and WEE1 are the updated targets of NDDS [140].

In preclinical studies, ALK tyrosine kinase was targeted and inhibited by a small molecule that displayed growth hindrance of ALK-positive NBL cells in cell lines and in the mouse model system [112, 113, 194-198].

At present, the MAPK signaling pathway MEK inhibitors, the PI3K and CDK4/6 inhibitors are being tested in clinical trials [193]. Bromodomain (BET), CHEK1 and MDM2 inhibitors are also in the initial stages of clinical trials [140].

In normal cells, with each DNA replication, telomere length decreases gradually, which later triggers senescence and apoptosis [199]. These telomeres are present at both chromosome terminals, which blocks the degradation and fusion of chromosomes [200]. Tumour cells avoid the normal telomere shortening process and disturb the normal balance of the cell by inducing increased telomerase activity that leads to unlimited cell replications [2, 201-203]. It has been reported that telomerase is activated in about 90% of cancers [201, 204]. Telomerase has a catalytic unit known as telomere reverse transcriptase (TERT) [203]. Correlation of TERT with alternative lengthening of telomeres (ALT), a mechanism does not depend on telomerase, another method, which cells use to keep their telomeres elongated via homologous recombination [205-207]. TERT rearrangement and MYCN amplification activate telomere maintenance, which is a marker for poor prognosis in NBL and associate with high-risk NBL. It would be beneficial if novel therapeutical agents for telomerase activity

and ALT could be explored to treat high-risk NBL [69, 208-213]. One inhibitor that could inhibit telomerase enzymatic activity was part of the clinical trial named Imetelstat (GRN163L), but this was discontinued to its highly toxic properties [214, 215]. There is another agent, 6- thio-2'-deoxyguanosine that shows great potential to inhibit telomerase activity. To treat high-risk NBL, targeting the telomerase and ALT pathway therapeutically might be a beneficial approach [140].

1.2.2.8. Apoptosis activation

Tumour cells escape from apoptosis (programmed cell death) and keep proliferating. One interesting way to handle these cells is to enhance apoptosis, which would inhibit the progression of the tumour and induce cell death. Dihydroorotate dehydrogenase (DHODH) inhibitors have shown a significant induction in apoptosis and a reduction in NBL cell proliferation both in cell lines and in mouse models [216]. Recently, the highly potent and novel DHODH inhibitor, BAY2402234, has shown striking proliferation inhibition and has induced apoptosis in acute myeloid leukemia (AML) [217]. In the year 2000, a group published a retinoid called Fenretinide, which triggers apoptosis in NBL cells [218]. Synergy has been seen in apoptotic enhancement upon a combination of fenretinide with chemotherapy agents in NBL [219]. TrkB is a neurotrophic receptor associated with high MYCN levels and shows a poor prognosis in NBL patients [86, 220]. Entrectinib is a Trk inhibitor approved by the FDA as an orphan drug to treat NBL patients [221]. Survivin (*BIRC5*) belongs to the apoptosis inhibitor family and targets BIRC5, which correlates to poor patient outcomes but is another very interesting way to induce apoptosis and to treat NBL patients. Sepantronium bromide (YM155) is a small potent molecule that degrades the promoter activity of *BIRC5* and induces apoptosis in retinal progenitor cell lines [222]. YM155 inhibits *BIRC5* expression and induces apoptosis in NSCLC [223].

1.2.2.9. Inhibition of MYCN

MYCN expression is one of the most important prognostic markers that correlate to poor patient outcomes in NBL and targeting MYCN could be valuable [63, 68, 127]. As it is impossible to target MYCN directly due to the absence of a proper motif for a therapeutic agent to bind to its DNA binding site, indirect targets to block MYCN are required and are currently necessary [224]. NVP-BEZ235 is a PI3K/mTOR inhibitor that abrogates MYCN activity

indirectly through mTORC inhibition but not PI3K activity and induces apoptosis [225]. Some other investigations conclude that MYCN is transcriptionally regulated by *ALK* through *AKT/ERK5* [111, 226, 227]. Some examples of indirect MYCN inhibition are Aurora kinase A/B inhibitors, BET, MYCN and the MAX interaction inhibitor, ornithine decarboxylase (ODC1) inhibitors, [171, 226, 227]. Additional targeted therapies could be beneficial in NBL like DNA methylation, suppression of the heat shock protein 90 (Hsp90), cell cycle checkpoint inhibitors, targeting noncoding RNAs and protein glycosylation [171, 228].

1.2.3. Receptor Tyrosine Kinase superfamily

There is a huge protein kinase family, which transfers high energy phosphate (PO_4) to serine (S), threonine (T) or tyrosine (Y) in substrate proteins [229, 230]. The superfamily of tyrosine kinase consists of 90 and is further divided into two different groups; (i) Receptor tyrosine kinases (RTKs) 58 and (ii) Non-receptor tyrosine kinases (NTRKs) 32 [231, 232]. The 58 RTKs are additionally classified into 20 subclasses [37, 231, 232]. All RTKs have a similar domain structure comprising an extracellular domain containing a ligand-binding domain (ECD), a transmembrane domain (TMD) and an intracellular protein tyrosine kinase domain (TK) [37, 233-236] (Figure 3). The receptor can be activated by four steps: biding to the ligands, ligand activates receptor dimerization, autophosphorylation of tyrosine and activated protein signaling [37, 234, 237]. The ligand binds to ECD and enhances the dimerization of receptors triggering the trans-auto-phosphorylation of tyrosine and downstream signal activation [238, 239]. Various important cell processes e.g., growth, differentiation, proliferation, survival, migration, metabolism etc. are controlled by RTKs [240, 241]. The constitutive activation of RTKs can be carried out by point mutation (gain-of-function), overexpression and chromosomal translocation, which activates tumorigenesis. The constitutive expression activation of RTKs induces numerous key cellular processes [241, 242]. Since tumorigenic kinase signaling activity is associated with a poor prognosis, a beneficial option would be to inhibit tyrosine kinase activity by tyrosine kinase inhibitors (TKIs). The journey of TKIs started two decades ago, when the FDA approved Gleevec/Imatinib, the first TKI to cure chronic myelogenous leukemia (CML) by inhibiting the activity of Abl, and after seven years, Gleevec received FDA approval for KIT receptor-positive gastrointestinal stromal tumours (GISTs) [243, 244]. The successful targeted therapeutical entry of TKI made this an important target and several pharma drugs came to the market [245-253]. TKIs work in four different ways,

competing with:

(i) ATP

(ii) Kinase substrate

(iii) Both

(iv) Act in an allosteric manner [254].

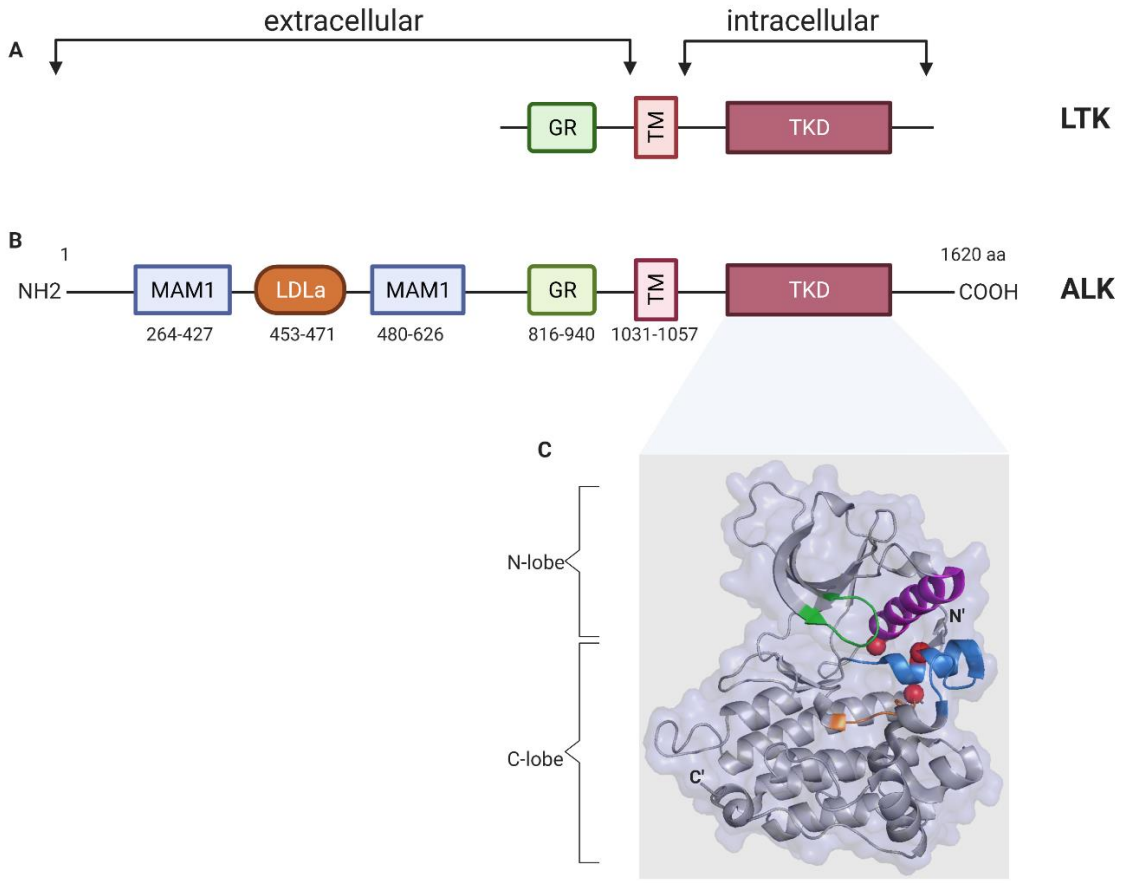

Figure 3. ***The structure of human ALK and LTK:*** *ALK has 1620 aa, (A and B) Both ALK and LTK have an extracellular domain (ECD), a transmembrane domain(TM) and a tyrosine kinase domain (TKD). The difference between ALK and LTK is that in ALK ECD, there are two meprins, A-5 protein and receptor protein tyrosine phosphatase mu (MAM) domains and one of each low-density lipoprotein class A (LDLa) and glycine-rich (GR) domain, but LTK contains only one GR. C. The crystal structure of an inactive form of ALK kinase domain (PDB: 3LCT), which contains: a GR loop (green), an α-C helix (magenta), a catalytic loop (orange) and an activation loop (blue). The ALK "hotspot" mutations: F1174, R1275, F1245 (red balls). Adaption was done with permission [36].*

1.2.3.1. Anaplastic lymphoma kinase (ALK)

ALK was initially revealed and named in 1994 after it was found to be a fusion partner with nucleophosmin (NPM), by the chromosomal translocation of the kinase domain of ALK (2p23) with the N-terminal site of NPM (5q35) [255-257]. Three years later, two independent research groups discovered full-length ALK [258, 259] and its expression was found in the

development of the central and peripheral nervous system. The human ALK protein consists of 1620 amino acids (aa), which is translated from the ALK gene and localized at chromosome 2p. The 1620 aa encodes a 177 KDa protein and due to the modification of full-length ALK through the N-linked glycosylation mature form of ALK, it is detected at 200 KDa [258, 259]. The shortened form of ALK is detected at 140 kDa due to the proteolytic cleavage [36, 260]. Due to ALK's 47% sequence similarity with the insulin receptor (InR), ALK is a member of the IR superfamily [257]. ALK and leucocyte tyrosine kinase (LTK) has around 50% similar sequences and belong to an Insulin receptor (IR) superfamily [258, 259]. ALK expression was found in a specific region of the growing brain with more expression in the peripheral ganglia and other regions of mice brains [258, 259, 261]. Significantly, abrogated sympathetic neuron proliferation is observed once ALK is inhibited [262]. No lethal or big phenotype difference has been detected in ALK loss-of-function mice, but neurogenesis, testosterone and a behavioural response are defected [263-266]. On the other hand, mice with ALK gain-of-function triggers neurogenesis and leading to propagation along with MYCN [111, 267, 268]. These investigations show and highlight how important ALK expression is in brain and testis growth. Several fusion partners of ALK have been discovered and published up to now. These activated ALK and played a key role in several tumours [269-271]. The role of ALK as a fusion gene like EML4-ALK in non-small cell lung cancer (NSCLC) [272, 273] and gain-of-function mutations ALK in NBL [97, 99, 106, 108, 274] have made ALK an important and beneficial therapeutic target [109, 275, 276].

Structure of ALK

The 1620 aa of full length ALK is divided into 3 parts; an extracellular ligand-binding domain with 1020 aa, a transmembrane spanning domain with 21 aa and an intracellular tyrosine kinase domain with 561 aa [36, 258, 259] (Figure 3).

Extracellular domain

The extracellular domain carries two Meprin, the A-5 protein and the receptor protein tyrosine phosphatase Mu (MAM) region, a low-density lipoprotein class A (LDLa) region, and a glycine-rich (GR) region [258, 259, 277, 278] (Figure 3). The extracellular part of ALK seems to be for ligand binding, co-receptor interaction and dimerization, which could trigger

intracellular kinase domain activation due to conformational changes [271, 279]. Two ALK ligands, ALKAL1 and ALKAL2, have been discovered recently [280, 281].

ALK intracellular kinase domain

Just as for other RTKs, the intracellular kinase region of ALK comprises a small conserved N-terminal lobe and a large C-terminal lobe [282-284] (Figure 3). N and C terminal lobes are merged by a 'hinge' domain, which creates a binding pocket of ATP or a substrate [282, 284]. There are five stranded antiparallel β-sheets and a regulatory αC-helix, responsible for catalytic activity. The activation loop sits in the helical large C-terminal lobe and this A loop has an inhibitory property. Upon inactive conformational change, it inhibits the substrate-binding domain of the kinase domain [36, 282, 284]. It has been shown that protein kinase consists of regulatory (R-spine) and catalytic (C-spine) spines [283, 285]. These spines are similar in all kinases, where the R-spine plays an important role in defining the active and inactive conformation of the ALK kinase. In ALK I1171, C1182, H1247, F1271 and D1311 are hydrophobic in the regulatory spine. The C-spine contains residues like V1130, A1148, L1256, C1255, L1257, L1204, L1318, I1322 and plays a catalytic controller role through ATP binding [271, 283, 285, 286]. Interestingly, just as for the IR superfamily, the A-loop of the C-terminal has an auto-phosphorylation motif Y'XXX'YY. A small difference is observed in IR and ALK tyrosine phosphorylation. In NPM-ALK fusion, ALK phosphorylation goes in order from the first to the third (like Y1278, Y1282 and Y1283) but in IR the second tyrosine is phosphorylated first, then the first and the third are phosphorylated [282, 284, 287, 288]. This observation states that upon ALK inactivation, Y1278 cannot be accessed and its phosphorylation is critical [282, 284, 285]. Contrary to previous reports, Guan et al investigated the phosphorylation preferred sequence in wild type ALK and found that the third tyrosine (Y1283) is critical instead of the first tyrosine (Y1278). The authors made their statement stronger by inducing the single mutation of phenylalanine to Y1278F, Y1282F and Y1283F. Only Y1283F showed efficiently inhibited ALK phosphorylation (ligand mediated) of and its downstream signaling ERK1 and ERK2 [289].

The biological role of ALK in model organisms

The specific and accurate role of ALK in mammalians is still uncertain, even though ALK participates in several tumour types, both in childhood and adult cancers. ALK seems to be a key player of neurogenesis in several animal models and homo sapiens [36, 258, 261].

Drosophila melanogaster, an experimental model system, has DAlk similar to mammalian ALK, which consists of an extracellular ligand-binding domain, a transmembrane domain and an intracellular kinase domain, where the kinase domain shows a high sequence similarity with the IR superfamily [277]. It has been shown that visceral mesoderm development needs ALK in embryogenesis and embryos without ALK do not survive since the fonder cells are missing [290-292]. Once the *Drosophila* ALK (DAlk) binds to its ligand jelly belly (Jeb), ALK signaling is activated via the MAPK-ERK1/2 pathway to intervene in the specification of founder cells [290-293]. It has been claimed that Jeb/DAlk signaling is critical for the synaptic connectivity in motor circuits development and the visual system of the fruit fly [294, 295]. Also, ALK mRNA is found in *Drosophila* CNS and visceral muscles [277]. The gut-less phenotype has been found in loss-of-function DAlk mutants [293]. In early embryogenesis, DAlk plays a critical role in the development of the gut visceral musculature [277, 293]. Strikingly, tiredness and vision defects are the main side effects in cancer patients treated with ALK inhibitors [296, 297]. Another study shows that by controlling the decapentaplegic (Dpp) transcription, which is a homolog of mammalian TGF- β, DAlk helps in embryonic endoderm development [298]. Neuroblast growth has been protected by DAlk via the PI3K/AKT signaling pathway in starvation conditions [299]. Some other studies have discovered that DAlk signaling is involved in body weight and associative learning in the fruit fly [300].

Another animal model widely used in cancer research is Caenorhabditis elegans (C.elegans). HEN1 and SCD-2 (suppressor of constitutive dauer formation) in C.elegans is the same as jeb and ALK respectively. SCD-2 is required for dauer development, which was suppressed at the 3rd larval stage in C.elegans [301-303]. Initially, it was found as a suppressor in TGF-β mutant screening and was given this name [302]. C.elegans ligand, Hen-1, is the same as Jeb, which has an LDL receptor repeat and participates in sensory integration [304]. The function of SCD-2 and Hen-1 belong to a similar pathway and to confirm the double mutant has been performed no difference was found between the single or double mutant [305].

In the kinase signaling investigation in zebrafish, Danio rerio has been performed in both Alk (DrAlk) and Ltk (DrLtk) [306-308]. DrAlk shows a strong expression in the developing CNS. Serious problems in neuronal differentiation of the CNS have been observed when DrAlk is inhibited, with no effect on neuron progenitor development [308]. However, another investigation showed ligands for zebrafish Alkal (Alkal1, Alkal2a and Alkal2b), which are important for iridophores (NC-derived pigment cells) formation [306, 309, 310]. There are more similarities in human ALK and DrLtk in the extracellular ligand-binding domain. Both DrLtk and hALK have two MAM domains but no MAM domain exists in human LTK [310].

Intense investigations have been carried out on dAlk and DrAlk ligands, but the human ALK receptor was known as an orphan receptor for a very long time [304, 311]. Two closely similar proteins have been discovered to be LTK ligands and are named FAM150A and FAM150B, now have new names as ALKAL1 and ALKAL2 [312]. ALKAL1 and ALKAL2 activity have been shown in the cell line [280, 281].

Neurogenesis and testosterone levels are disturbed in loss-of-function ALK double mutant mice but this is not life-threatening [264-266]. The expression of ALK mRNA found in specific areas of the CNS and PNS in the sympathetic chain, which highlights the role of ALK in the nervous system development [258, 259, 261]. Mouse NCCs show ALK expression, which shows the effect of ALK on NCC's migration [313]. The increment in ganglia and neurogenesis has been detected in gain-of-function knock-in mice, where ALK mutant induction promotes the proliferation of neuroblasts in ganglia of the sympathetic chain [264, 268, 314]. To get a full insight into NBL tumorigenesis, understanding the role of ALK in neural crest cell development is required.

1.2.3.2. ALK in cancer and signaling

Many different fusion partners of ALK have been revealed and discovered to date in several cancer types, which shows ALK is a translocation hotspot target [36] (Figure 4). In ALCL, around 80% of cases depend on NPM-ALK fusion [315, 316] and the other ALK fusion cancerous protein is EML4-ALK, which is around 9% in NSCLC [272, 273, 315, 317]. The activation of transcription, subcellular localization is carried out by a fusion partner in ALK fusion, with an outcome of constitutive activation [36]. Point mutation is not described in primary ALK fusion but it normally spikes in relapsed cases as a resistance mutation [36, 318].

The fusion of ALK is reported in adult tumours but in childhood tumour abnormality in full-length ALK has been described [36]. CRKL-C3G-RAP1, MEK5/ERK5, RAS/MAPK, PI3K/AKT, PLCγ and STAT3/STAT5 pathways have been activated through the constitutive activation of fusion and full length ALK [36, 315, 319] (Figure 4). Several adaptor proteins have been activated through ALK activation [36, 271, 315, 319]. Different cancer types have shown that constitutive activation of ALK leads to tumorigenesis, which makes ALK a potential therapeutic target for drug development [97, 99, 106, 108, 272-274, 320] (Figure 4).

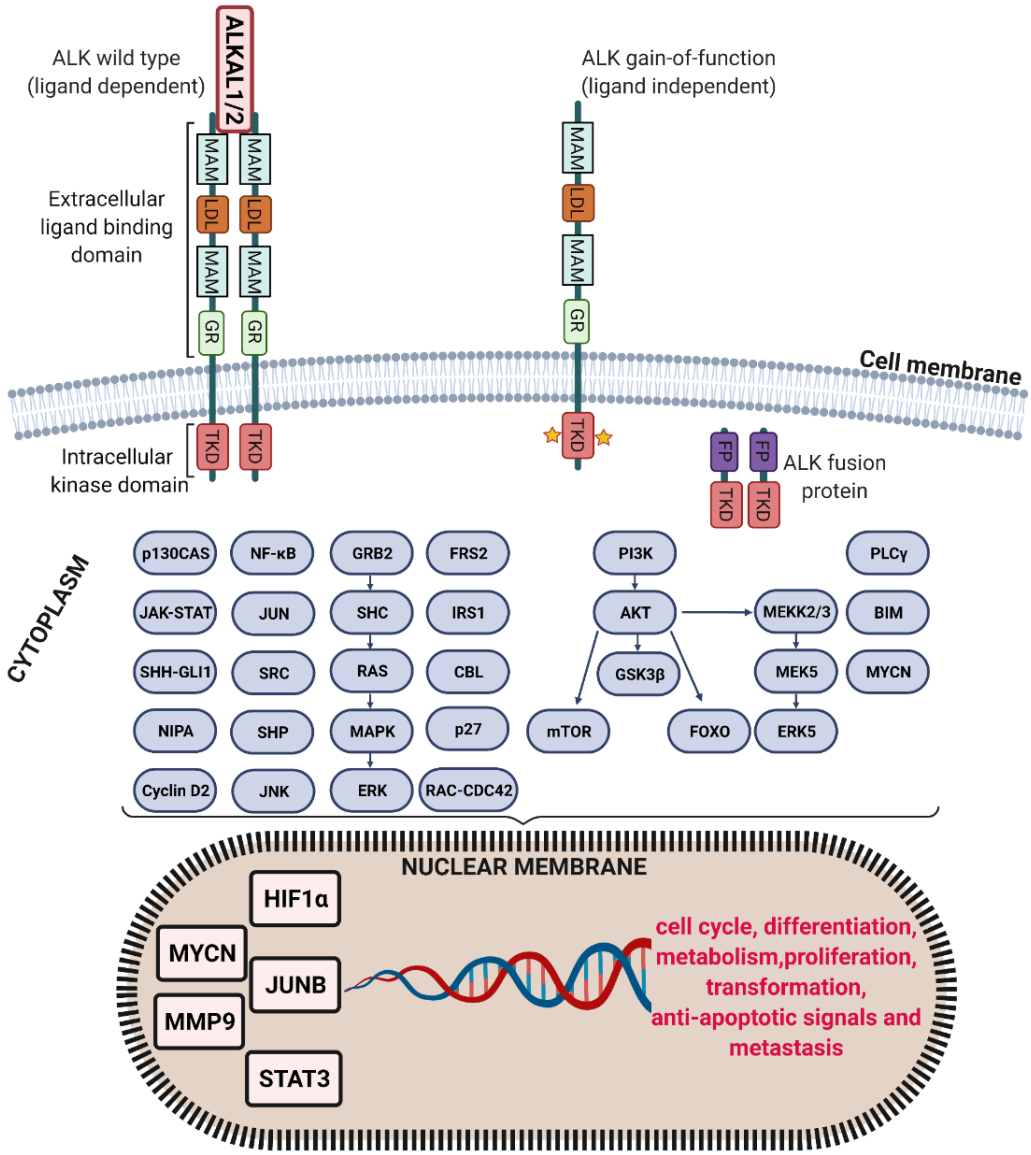

Figure 4. ***ALK signaling cascades and downstream targets:*** *Modes of activation is shown in this figure, where ALK signaling can be activated through three ways. (i) ALK wild type activates through ligands binding (ligand dependent manner) (ii) ALK GoF mutant (ligand-independent manner) (iii) ALK binds with fusion protein (ligand-independent manner). ALK activation activates numerous signaling pathways, for instance, Janus kinase (JAK)–signal transducer and activator of transcription (STAT), PI3K-AKT, RAS-MAPK etc., through numerous transcription factors such as MYCN, JUNB, HIF1α etc., that leads to distinct cell responses involve in survival, anti-apoptotic signals, differentiation, proliferation, transformation and metastasis. Adaption was done with permission [36].*

1.2.3.3. ALK positive cancers

In NBL, normally the ALK activation is done through tyrosine kinase domain mutation and ALK overexpression. There are some other cancers, which are related to oncogenic ALK abnormalities. As mentioned, ALK was first identified as a fusion partner to NPM in ALCL [257], since then around 30 fusion partners has been described, which makes locus of ALK as a hot-spot target for translocation [315]. Some examples of ALK fusion partners are ALCL, DLBCL, IMT and NSCLC

1.2.3.4. Anaplastic large cell lymphoma (ALCL)

ALCL was first identified as a neoplasm, having Ki-1 antigen (CD-30) [321]. *ALK* got its name and was first identified as a fusion gene along with NPM in ALCL in 1994, where initial 117 amino acids of NPM fused with last 563 amino acids of ALK, which includes an ALK kinase domain. This dimerization facilitates the auto-phosphorylation of the kinase domain of ALK leads to oncogenic signaling of ALK [255-257]. The NPM-ALK fusion happens in about 80% of ALCL cases (Amin and Lai, 2007). Other than NPM-ALK, several more ALK fusion partners found in ALCL are MSN, ALO17, TFG, TPM3, TPM4, MHY9, ATIC, CLTC, TRAF1 [322].

1.2.3.5. Diffuse large B-cell lymphoma (DLBCL)

The DLBCL is a disease where neoplasia occurs in B-cells. DLBCLs are the most common lymphomas, represent about 40% of all lymphomas. The location of primary tumour is lymph nodes, but rarely present in nasopharynx, stomach and tongue [323, 324]. Although, in DLBCL, *ALK* aberration represents >1%, but *ALK* rearrangements are correlated to poor outcome and not effective to chemotherapy treatment [323, 325]. ALK targeted therapy seems to be a good option to treat ALK positive DLBCL [326]. The ALK fusion partners found in DLBCL are *CLTC* [327], NPM, SEC31A [328], SQSTM1 [329-331].

1.2.3.6. Inflammatory myofibroblastic tumours (IMT)

The IMT is a rare disease, where neoplasia occurs in mesenchymal cells that mostly initiates in the abdomen, lungs soft tissues, retroperitoneal region and pelvis mostly present in young adults and kids [332, 333]. In approximately 50% cases of IMT, ALK fusion have been involved with TPM3, present on chromosome-1 [334]. Numerous other ALK fusion partners have been

described, for instance, ATIC [335], CARS [336], CLTC1 [337, 338], PPFIBP1 [339], RANBP2 [338, 340], SEC31L1 [341] and TPM4 [334]

1.2.3.7. Non-small cell lung cancer (NSCLC)

Lung cancer has the highest rate of death among all cancer types in the world. In 2020, 1.80 million death cases were registered [3]. Lung cancer is divided into two types, non-small cell lung cancer (NSCLC), which accounts for 85% cases and small cell lung cancer, occurs in 15%. Smoking of cigarette is one of the most important risk factor associated to NSCLC development [342, 343]. ALK rearrangement has been found in NSCLC and around forty thousand cases register each year with ALK-dependent NSCLC worldwide [344, 345]. The cases register with ALK-dependent NSCLC are mostly young, with a casual or non-smoking background [346, 347]. The fusion of EML4 with ALK is the most common and both present on the same chromosome-2, where N-terminal region of EML4 gene fuses with tyrosine kinase domain of ALK gene [272]. Until date approximately fifteen different EML4-ALK variants have been identified [272, 344]. Several different partners of ALK translocation in NSCLC are KIF5B [348, 349], KLC1 [350], PTPN3,STRN [351] and TFG [273].

1.2.3.8. ALK in neuroblastoma

ALK gain-of-function mutations have been found in familial and sporadic NBL cases, making this an interesting target to investigate the mechanism of action of ALK and its downstream signaling partners [97, 99, 106, 108] (Figure 4). Heritable mutations are quite common in familial NBL, but most cases are sporadic [45]. Mendelian-based familial cases are just 1-2% and most have ALK mutations. Point mutation has been found in around 10% of sporadic NBL cases [94, 95, 110] and this percentage is increased to 26% in refractory NBL [352, 353]. The point mutation of ALK happens and sits in the intracellular kinase domain of ALK [36]. In NBL, the mutation in ALK has been divided into (i) gain-of-function ligand-independent, (ii) ligand-dependent, (iii) kinase-dead mutations [354]. Gain-of-function mutation in the tyrosine kinase domain of ALK induces constitutive activation of ALK, which leads to the oncogenic signaling activation of ALK and its downstream pathways [36, 354]. This constitutive activation correlates with poor patient outcomes in high-risk NBL patients [110]. The most common and hot spot ALK mutations found in NBL are Phe1174 (30%), Phe1245 (12%), and Arg1275

(45%), approximately 85% among all ALK kinase domain mutations [94, 110]. ALK activation in these mutations is known to be ligand-independent [115, 274, 354] (Figure 4).

The low copy number of an unbalanced gain of the short arm of chromosome 2, the location of *ALK* and *MYCN*, is described in around 23% of NBL, which induces *ALK* mRNA leading to a high ALK protein expression and showing a poor prognosis [94, 355]. Interestingly, it has also been shown that the *ALK* copy number gain and point mutation in the ALK kinase domain rarely happens [94].

ALKAL2, the ALK ligand, is located at chromosome 2p.25 in proximity to ALK and MYCN and is found in the gain of an unbalanced chromosome 2p abnormality along with ALK and MYCN [100]. Deletion of exons has also been reported to activate ALK signaling in NBL, leading to ALK extracellular domain truncation [356-358] (Figure 4).

ALK kinase domain mutation has been found in about 10% of primary NBL patients during diagnosis and this number spikes to 20% in refractory patient samples [94, 95, 352]. Mutations in the RAS/MAPK signaling pathway have been found in patients who receive chemotherapeutic treatment [359, 360]. These results describe how important ALK may be as a driver in primary as well as relapse NBL cases [353, 360, 361]. ALK as a therapeutic target for primary and refractory ALK-positive NBL would be beneficial. Interestingly, in adult animals, the mRNA and protein levels of ALK go down in every tissue and further drop to a lower level until the age of 3 weeks where it is maintained at the minimum level [258]. Mice are viable in ALK loss of function [264, 266]. All these results and investigations are a strong indication to employ an ALK inhibitor as a targeted therapeutic agent in adult and childhood ALK-driven NBL.

1.2.3.9. Synergistic collaboration between ALK and MYCN in neuroblastoma

Chromosomes 2p.23 & 2p.24 have two important oncogenes, *ALK* and *MYCN* respectively. Amplification of this combination occurs in 2-3 % of NBL cases [94, 108, 110, 362]. Most ALK gain-of-function mutants and especially F1174 (one of the hotspot mutations) are correlated to MYCN amplified cases which relates to a poor prognosis in NBL patients [94]. These investigations have shown the link and combinatory effect of these two abnormalities. This mechanistic link was initially revealed where the group showed that both wild type and mutated ALK induces MYCN transcription in neuronal cell lines and NBL [226].

Synergistic cooperation of ALK and MYCN incudes transformation in mouse fibroblastic cells (NIH3T3) [226]. This synergistic tumorigenic correlation was observed in mouse and zebra fish models of NBL [111, 314, 363] (Figure 5). Combined expression of ALK kinase domain mutant and MYCN showed complete and higher tumour penetrance, induced development and earlier onset of tumour [111, 314, 364]. MYCN overexpression abrogates the differentiation of the chromaffin cell and induces the proliferation of sympathetic neuroblasts, but ultimately arose the apoptosis response appears in the NC-derived progenitor, which gives low penetrance tumorigenesis in NBL [111, 364]. On the other hand, no tumorigenesis was seen in constitutive ALK gain-of-function neuroblasts alone [111, 364]. Neuroblast proliferation is increased in gain-of-function ALK knock in mouse, with elongated ganglia and enhanced neurogenesis [268]. These studies showed that transformed cell of MYCN are dependent on ALK signaling to induce tumorigenesis, which strengthen the therapeutic advantage to target ALK in ALK-addicted and MYCN-amplified NBLs (Figure 5).

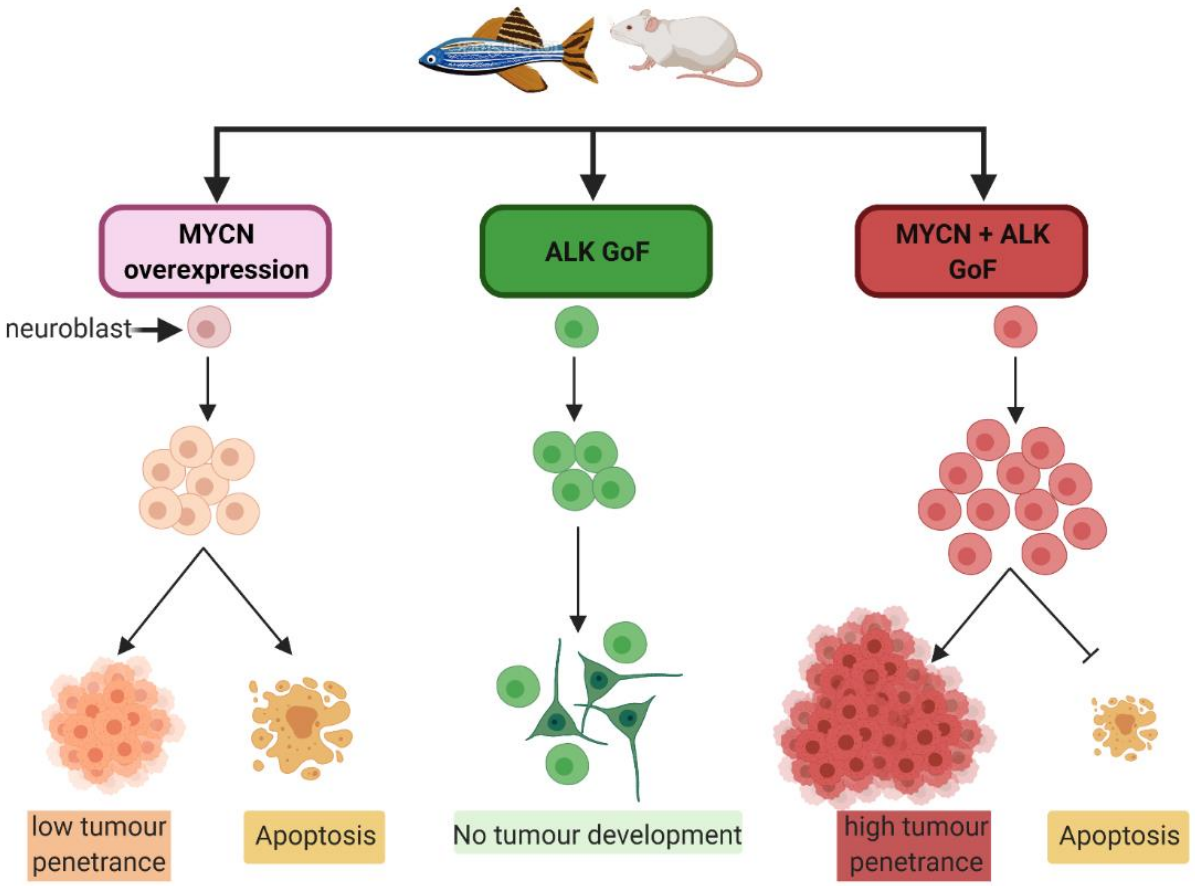

Figure 5. ***Mechanistic illustration of NBL progression and development in synergistic cooperation between gain-of-function ALK mutants with MYCN:*** *MYCN overexpression in neuroblast induces cell proliferation and drives apoptosis in few cells that leads to low tumour penetrance. The overexpression of ALK gain of function alone does not induce tumour, rather increases neuronal differentiation. Cooperation of activated ALK and MYCN leads to high tumour penetrance [111, 268, 364].*

1.2.3.10. Role of ALK in neuroblastoma differentiation

In chicken, sympathetic neuroblast-induced neuronal differentiation has been observed when ALK is activated [365]. Another investigation in mice showed a proliferation induction and differentiation inhibition when ALK-F1174 activation is driven via the SOX10 promoter [366, 367]. Early sympathetic progenitor cells of mice have shown a blocked differentiation and induced proliferation when ALK-F1174 is expressed ectopically in migrating NCCs, but no tumorigenic activity has been observed [366]. In NBL tumours, the induced protein expression of ALK (41%) and MYCN (39%) has been found, with around 86% of NBL tumours with high protein expression of ALK and MYCN, displayed poorly or undifferentiated histology [368].

These investigations showed the importance of ALK and MYCN activity in neural crest cells differentiation and its role in NBL initiation and propagation

1.2.3.11. Targeting ALK in cancer

The Pathogenic activity of ALK signaling was revealed in ALCL [255-257] and several other cancer types showed the involvement of oncogenic ALK signaling [271, 369-371]. ALK chromosomal translocation leads to the formation of a constitutively active oncogenic ALK fusion protein, which is the most frequent form of ALK aberration in human cancer. This ALK translocation in ALCL is about 55%, about 50% in IMT and as much as 9% in NSCLC [316, 317, 372].

Mostly point mutations happen in the ALK kinase domain, which is around 10% in NBL [36, 94, 99, 108]. To treat ALK-driven cancers, tyrosine kinase inhibitors (TKIs) are generated, which bind to the ATP binding pocket and inhibit the activity of ALK signaling. Some of these are crizotinib, alectinib, brigatinib and lorlatinib [274, 373]. Below is a summary of some of the TKIs used in the experiments.

Crizotinib

Crizotinib (PF-2342066/XalkoriTM) is the first approved ALK TKI, known as a first-generation ALK inhibitor. It was initially discovered as an efficient c-MET kinase inhibitor, which is potent for other RTKs such as ALK and ROS-1 [247]. In 2011, an exceptional clinical result hastened its approval by the FDA, during the first and second clinical trial of NSCLC patients for advance ALK-positive NSCLC and it has been suggested for use in NBL patient treatments [275, 317, 374]. It is an ATP competitive TKI and is orally bioavailable [320]. In phase 3 of the clinical trial, crizotinib displayed a better effect than chemotherapy [370, 375]. Even after the initial response, the development of secondary resistance mutation needs the next generation of ALK TKIs [375]. In the first clinical trial of eleven NBL patients with a known ALK status, response was observed only in 1 patient [374] but the other paediatric cancers with ALK-fusion like ALCL and IMT showed a remarkable response, with 80% and 86% respectively [376]. Informal evidence of crizotinib treatment for chemotherapy-relapsed NBL patients observed a complete initial response where high levels of ALK protein expression were reported [377]. These investigations draw important attention to discover new and more potent ALK TKIs to treat NBL patients.

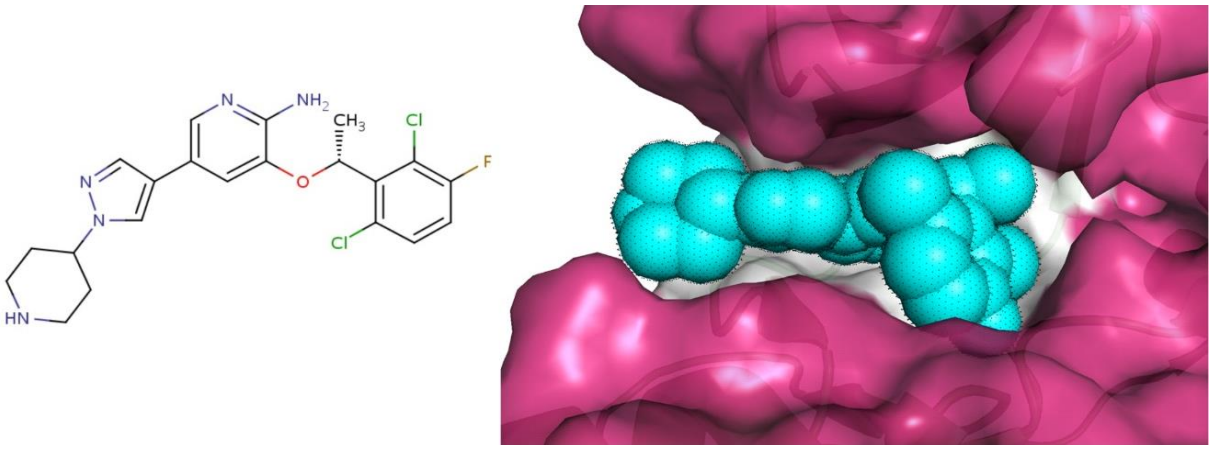

Figure 6. ***ALK tyrosine kinase inhibitor (TKI)-Crizotinib:*** *Chemical structure [378] is shown to the left side and on the right side, crizotinib (light blue) binding into the ATP binding pocket of the ALK kinase domain (dark pink).*

Alectinib

Alectinib is an ATP-competitive, second-generation, selective and orally available ALK TKI [379-382], initially approved in Japan in July 2014 and later in December 2015 in the USA. Alectinib was approved to treat recurrent ALK-positive NSCLC especially for those patients who were intolerant to crizotinib [380]. Better ALK inhibition and blood-brain barrier transport in alectinib comparing to crizotinib and longer median progression-free survival were observed [383-385], with an augmented safety profile has observed when compare with crizotinib in NSCLC [384, 386]. Additionally, alectinib displayed robust activity towards L1196M gatekeeper mutation and the F1174L and R1275Q, known as hot spot mutations [380]. Even V1180L and 1171T showed resistance to alectinib [387] but hotspot mutations are the most frequent in all NBL mutated cases and the use of alectinib as a therapeutic drug could be beneficial with these mutations. About crizotinib, while the naïve NSLCLC median survival for the patient treated with alectinib is 28 months, in crizotinib resistance the PFS is around nine months [383]. Alam et al. showed abrogation of ALK activation in NBL cell lines and exogenously expressed ALK activity in xenografts mouse model when treated with alectinib [112].

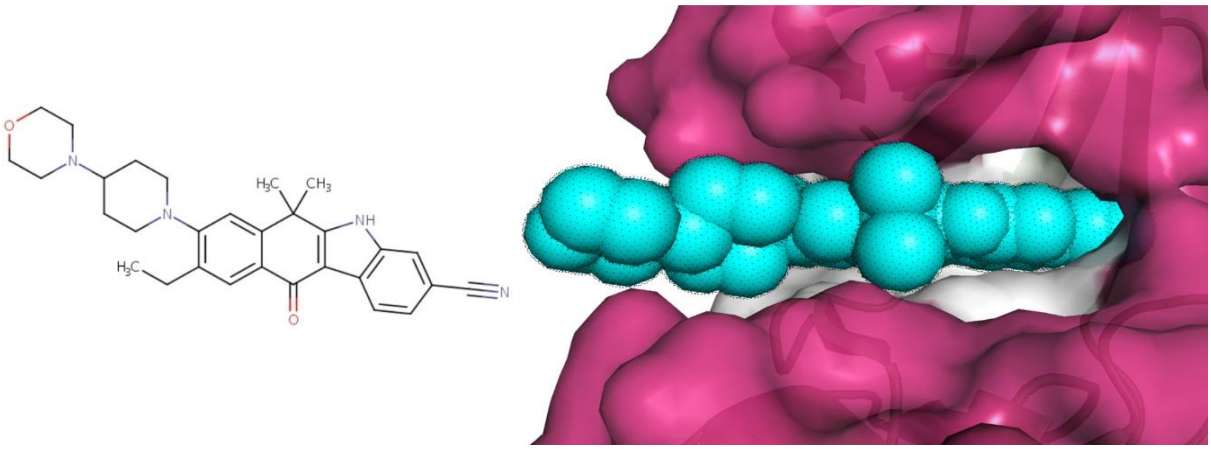

Figure 7. ***ALK tyrosine kinase inhibitor (TKI)-Alectinib:*** *Chemical structure [378] is shown to the left side and on the right side, alectinib (light blue) binding into the ATP binding pocket of the ALK kinase domain (dark pink).*

Ceritinib

Ceritinib, an ALK competitive second-generation ALK TKI, is in capsule form and taken through the mouth [388]. FDA approval was given in 2014 to treat crizotinib resistant ALK-positive NSCLC patients [389] and has been shown remarkable tumour reduction in both resistant and sensitive crizotinib ALK-mutations in ALK-arranged NSCLC mouse models [388, 390]. Ceritinib's clinical trials have been shown much higher efficacy compared to the crizotinib and showed median PFS of 7-8 months in NSCLC [391]. Ceritinib inhibits not only ALK but also IGF-1R, INSR and STKK22D [390, 392]. Several drug-related side effects of ceritinib are found e.g. tiredness, diarrhoea, vomiting, stomachache, high aminotransferase levels and low serum phosphate levels [389, 391]. Alectinib and crizotinib resistant ALK mutations have shown sensitivity to ceritinib [387, 390]. During phase 3 clinical trial, ceritinib shows twice better PFS than chemotherapy as first-line therapy in ALK-rearranged NSCLC [393]. Another study shows the synergistic effect of ceritinib and ribocilib (CDK 4/6 inhibitor) on reduced tumour growth in NBL xenograft [394]. The phase 1 trials are done for IMT, NBL and ALCL (NCT01742286). For high-risk NBL cases, combination treatment of ceritinib with several drugs is going on (NCT02559778).

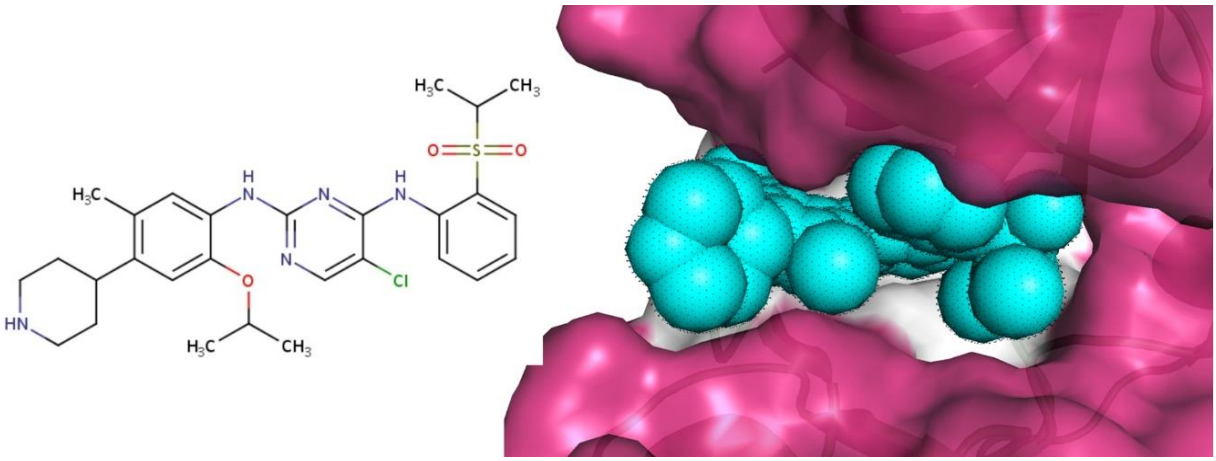

Figure 8. ***ALK tyrosine kinase inhibitor (TKI)-Ceritinib:*** *Chemical structure [378] is shown to the left side and on the right side, ceritinib (light blue) binding into the ATP binding pocket of the ALK kinase domain (dark pink).*

Brigatinib

Brigatinib is another ALK competitive ALK TKI that inhibits ALK kinase activity and other targets, for instance, EGFR, IGFR-1 and ROS1 [395]. During brigatinib preclinical trials on ALK-addicted NSCLC has been shown more potent on all ALK resistant mutants of alectinib, crizotinib and ceritinib [395]. FDA approval was given to brigatinib in 2017 to treat those patients who are crizotinib resistant [396]. In NSCLC patients which were resistant to crizotinib, ALK mutants showed around 13 months PFS during phase I and II trials [389]. Several drug-related side effects of brigatinib are found e.g. nausea, fatigue, dyspnoea, vomiting, pyrexia, arthralgia and diarrhoea [389]. Siaw et al investigated and found the effect of brigatinib in NBL, which shows ALK activity abrogates strikingly and better than crizotinib both *in vitro* and *in vivo* [116]. Another study has shown an interesting result of brigatinib induced PFS in CNS metastatic and CNS non-metastatic cases, as normally ALK TKIs displays bad CNS penetration [397].

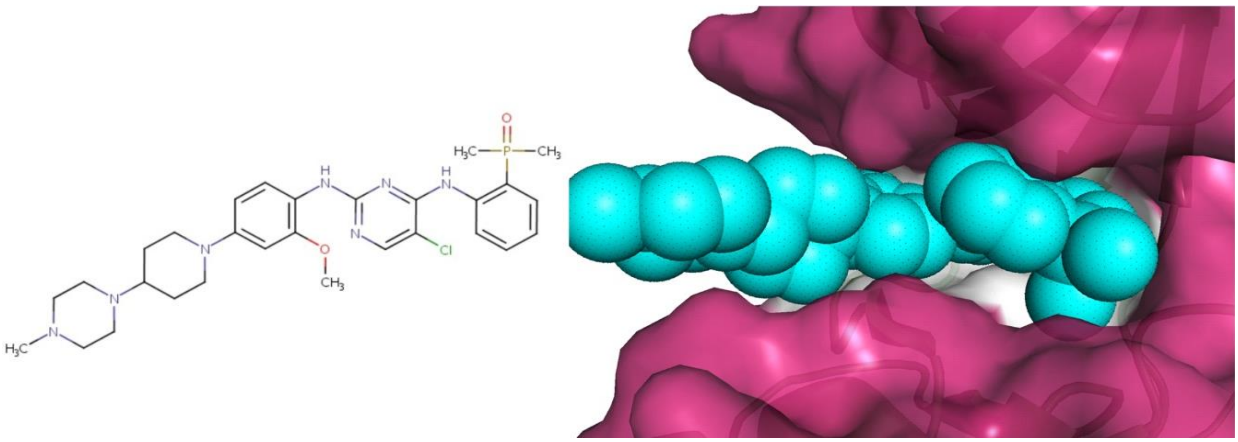

Figure 9. ***ALK tyrosine kinase inhibitor (TKI)-Brigatinib:*** *Chemical structure [378] is shown to the left side and on the right side, brigatinib (light blue) binding into the ATP binding pocket of the ALK kinase domain (dark pink).*

Lorlatinib

Lorlatinib (PF-06463922) is an ATP-competitive molecule that is an effective and potent ALK/ROS-1 with an ability to cross the blood-brain barrier and gives better efficacy to all AK resistant mutations compared to all other generations of ALK TKIs both in the cell lines and also in animal models [197, 198, 398, 399]. Due to its unique macrocyclic structure, it conquers ALK-TKI resistant mutations with enhanced and better CNS activity and can inhibit [400]. In preclinical data, it abrogates to those mutations where resistance was observed with all other ALK TKIs. It is also shown in biochemical assays that when including the ALK wild type, almost all other ALK mutations were inhibited at very low concentrations [398]. Phase I/II clinical trials for NBL is ongoing for lorlatinib (NANT, NCT03107988) [193]. Since lorlatinib efficacy is remarkable, lorlatinib could be the best therapeutic target to treat ALK-driven NBL in a mono treatment, as well as in combination with other signaling targets to escape acquired long-term treatment resistance.

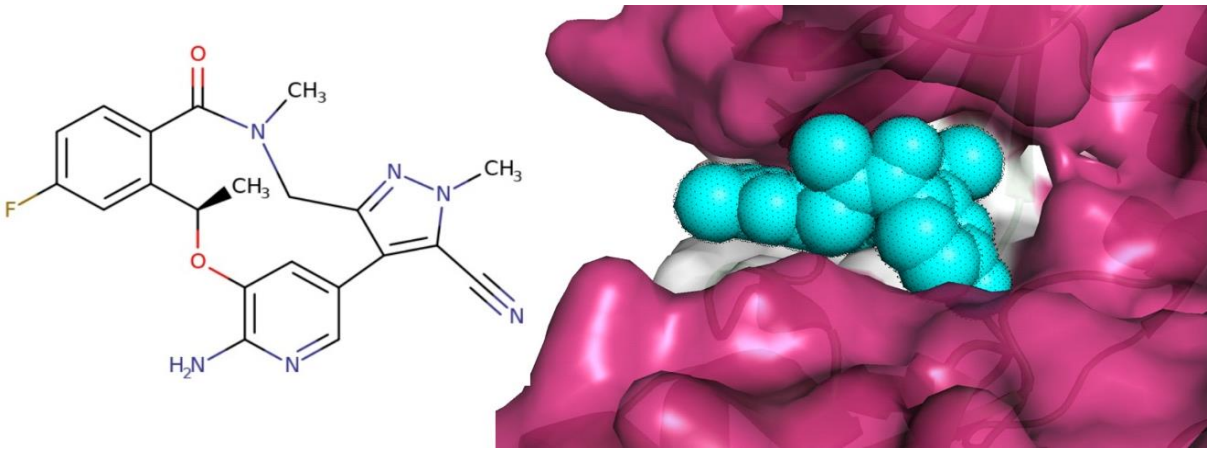

Figure 10. ***ALK tyrosine kinase inhibitor (TKI)-Lorlatinib:*** *Chemical structure [378] is shown to the left side and on the right side, lorlatinib (light blue) binding into the ATP binding pocket of the ALK kinase domain (dark pink).*

1.2.4. Dihydroorotate dehydrogenase (DHODH)

1.2.4.1. Characterization of DHODH enzymes

DHODH gene sits on an ORF region of human chromosome 16q22 with 1191 bp length, which translates the DHODH protein of 397 amino acids [401]. The crystal structure of the DHODH enzyme has flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and iron [402].

Several organisms express dihydroorotate dehydrogenase (DHODH) and this enzyme is divided into two classes (i) Class 1 and (ii) Class 2 based on many factors like co-factor, localization, sequence similarity and substrate. Class 1 is classified into class 1A, class 1B and class 1S. These three classes are localized in the cytoplasm [403, 404]. Class 1A enzymes are homodimers and FAD and FMN are their co-factors, present in gram-positive bacteria [405, 406]. While Class 1B enzymes are heterodimers and NAD+ acts as an electron acceptor, usually present in gram-negative bacteria [407, 408]. Class 1S utilizes serine as a catalytic base as it is not able to use a natural electron acceptor [403, 404]. Eukaryotes and prokaryotes (some) both express class 2 enzymes, where DHODH in eukaryotes is attached to an inner mitochondrial membrane while attached with the cytosolic membrane in prokaryotes [404, 409]. Humans relate to Class 2 monomeric group and DHODH is present in the inner membrane of mitochondria [410]. DHODH is an iron-containing flavin-dependent enzyme, participates in the de novo synthesis of pyrimidine [401]. There is around 30% sequence similarity between class 1A and class1B, and 20% similarity between soluble class 1 and class 2 DHODH [404].

1.2.4.2. Structure and function of human DHODH

The first DHODH structure was discovered from Lactococcus lactis in 1997 [411], and in 2000 DHODH structure of human was identified. In the structure of humans DHODH, there are two domains (i) A large C-terminal catalytic domain (CAT), which has an enzyme active site and (ii) the little N-terminal membrane-bound domain, both are attached by the loop [404, 412]. The redox site is at the C-terminus while the mitochondrial localization signal is at the protein N-terminal. Then there is a hydrophobic region that keeps holding it with membrane [413]. The α-helical domain is the binding domain for many inhibitors and the binding domain for

ubiquinone Q10, which goes down during catalysis [412, 414]. R136 and Y356 residues interact with ubiquinone are found an interactive partner to DHODH inhibitors [412, 415].

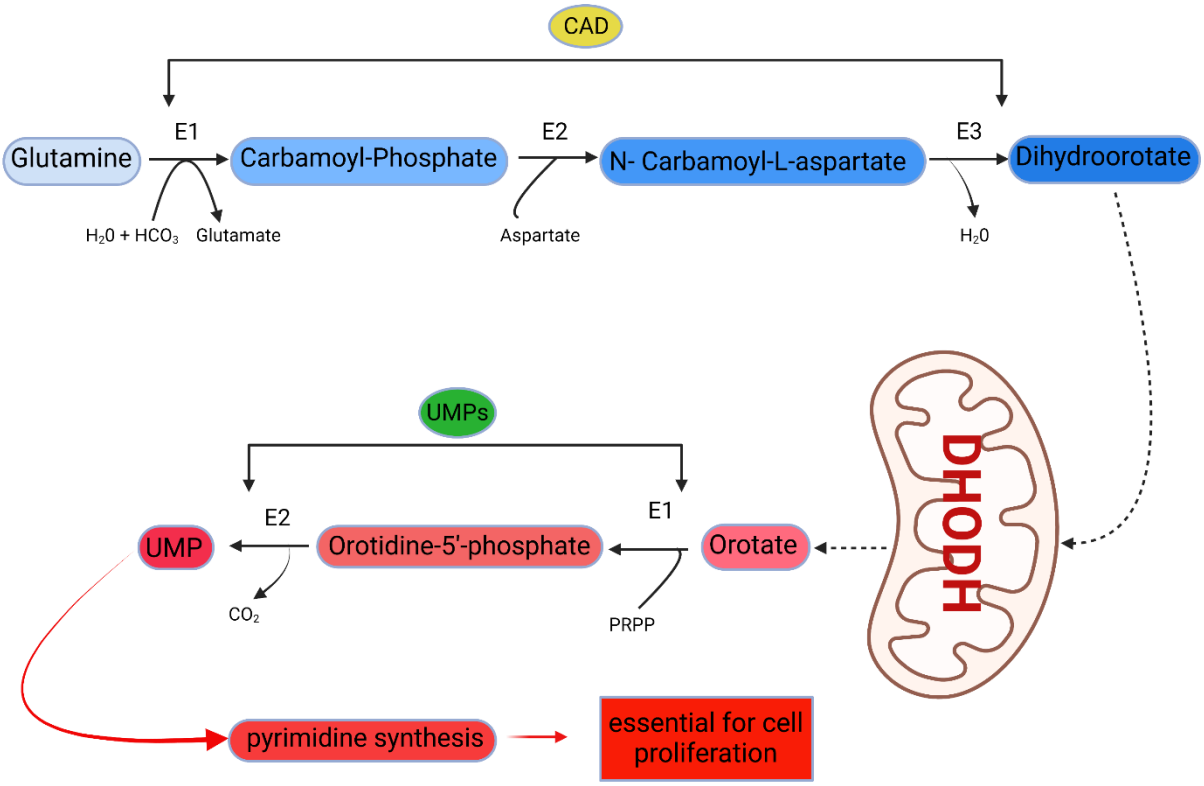

Figure 11. ***The schematic illustration of the six enzymatic reactions of the de novo pyrimidine synthesis pathways:*** *In this figure, CAD is abbreviated for Carbamoyl-Phosphate Synthetase 2, Aspartate Transcarbamylase, And Dihydroorotase, DHODH is abbreviated for Dihydroorotate Dehydrogenase and UMPs is abbreviated for Uridine Monophosphate Synthetase. This illustration is adapted and modified [416], copyrights reserved for MDPI 2019.*

1.2.4.3. Role of DHODH in cancer and as a drug target

In total there are six universally conserved enzymatic reactions in the pyrimidine de novo synthetic pathway and DHODH catalyses the fourth enzymatic reaction step and for tumour treatment, it would be a potential drug target [403, 417] (Figure 11). The function of DHODH in humans is to catalyse the fourth step which oxidises the dihydroorotate to orotate, by a redox reaction where ubiquinone (CoQ) converting to ubiquinol (CoQH2), a substrate of respiratory complex III, in the mitochondria [404, 418, 419] (Figure 11). The cofactor of this redox reaction is FMN that works as an electron acceptor [419, 420].

DHODH is a key player in the synthesis of pyrimidine through the de novo synthesis pathway. In idle or complete differentiated cells salvage pathway fulfils the nucleotide while to complete the nucleotide requirement of highly proliferating cells de novo synthesis pathway

is used [419]. In contrast with the proliferation of normal cells, cancerous cells have disturbed metabolism of pyrimidine which is correlated to tumour development and transformation [421]. In addition, DHODH is a crucial player in tumour progression by supplying rapidly proliferating cancer cells with nucleic acids [422] thereby making DHODH a potential drug target in cancer. Several cancers have shown anti-proliferative and apoptotic effect by inhibiting DHODH e.g. acute myeloid leukemia AML [217, 423], breast cancer [424], cervical cancer [425], colorectal cancer (CRC) [426], glioblastoma stem cells (GSCs) [427], lung cancer [428], multiple myeloma cells [429], NBL cells [216, 430], pancreatic cancer [431], renal cell carcinoma (RCC) [432]. Another group has recently been described that MYCN, which is associated to poor clinical outcome in NBL, induces the pyrimidine nucleotide production via transcriptional upregulation of DHODH, and DHODH suppression reduces the proliferation of MYCN-amplified NBL cells [430].

Inhibition of DHODH displayed proliferation inhibition due to pyrimidine depreciation. Studies have shown that DHODH inhibition is more potent to treat cancers that are addicted to pyrimidine through the de novo pathway rather than the salvage pathway. For example, inhibition of DHODH activates ATR, which increases DNA damage and cell death [433]. Hypoxic and low nutrient environments make DHODH inhibitors more sensitive to the tumour cells [434, 435]. Several important transcription factors regulate DHODH for instance, GATA2, MYC, NF-kB1, p300 and POU2F2 [436].

In another study, where DHODH activity has measured through fluorescence in cancer cells and neighbouring normal cells of the same patient, striking induction of DHODH activity has observed in cancerous tissue then compare with nearby normal tissue [425].

DHODH play an important role in tumour development and metastasis, which makes it an important therapeutic target for the development of the drug. Cancer cells have shown an inhibitory effect upon treatment with DHODH inhibitor. Several inhibiting compounds of DHODH have been published, for instance, brequinar (BRQ) [437], which was initially used as an immunosuppressant, and later it displayed anti-tumorigenic properties in different cancers *in vivo* and *in vitro* [438]. Another DHODH inhibitor is leflunomide [419]. Leflunomide inhibits proliferation and enhanced apoptosis in multiple myeloma cells, NBL cells and mouse models [216, 429]. Leflunomide inhibition has also shown an anti-proliferative effect on renal carcinoma cells [432]. Leflunomide decreases cell movement and shows a metastasis

inhibitory effect in breast cancer cells [439]. Leflunomide inhibits cell movement in breast cancer cells, which shows its anti-metastatic effect [439]. An active metabolite of leflunomide is teriflunomide [440] initially used for rheumatoid arthritis (RA) and multiple sclerosis (MS), also involved in antitumour activity through DHODH inhibition [427]. There are other known DHODH inhibitors for instance ALASN003 [441], till date FDA only approved ASLAN003 as an orphan drug designation.

BAY2402234 is a novel and potent DHODH inhibitor, which has shown prolific results in acute myeloid leukemia (AML) cell lines and mouse models, where proliferation inhibition and induced differentiation have been observed in a monotherapy treatment [217]. Additionally, omics data has shown increase expression of differentiation, apoptotic and p53 pathway levels and inhibition of many kinases phosphorylation of MAPK pathway [217]. Currently, there is a planned clinical trial (NCT05061251) of BAY2402234 for recurrent glioma, which will be starting soon (https://clinicaltrials.gov/ct2/home).

2. GOALS/AIMS

Thesis main goal is to understand the novel and different therapeutic strategies to overcome NBL and to target ALK and other oncogenic signaling pathways.

Specific aims of paper I

- To interrogate the therapeutic potential of alectinib in the cell lines and the pre-clinical models of NBL.

- To establish better profile of inhibition of alectinib in comparison to previous TKIs for ALK gain-of-function mutant alleles found in NBL.

Specific aims of paper II

- To investigate the effect of DHODH inhibitor (BAY2402234) on NBL cell lines and transgenic mouse models.

- To analyse the synergy between BAY2402234 and lorlatinib in ALK-addicted NBL.

3. MATERIALS AND METHODS:

Below is a short introduction of the common materials and methods used to produce all the data. Detailed materials and methods are written in a published paper and manuscript.

3.1. Cell culture

Collagen pre-coated dishes are used to culture NBL cells. RPMI-1640 medium in addition to 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (PE/ST) are used to grow NBL cell lines at 37°C with 5% CO_2 and 95% humidity. A MEM/EBSS medium is used to grow PC-12 cells with 7% horse serum (HS), 3% FBS and 1% PE/ST.

3.2. Immunoblotting

Whole cells were harvested in a RIPA buffer, kept on ice and 10 minutes centrifugation at 4°C. Protein concentrations were calculated, run on bis-acryl-tris gels and subjected to SDS polyacrylamide gel electrophoresis (PAGE). Samples were then transferred to polyvinylidene difluoride (PVDF) membrane., 5% bovine serum albumin (BSA) or skimmed milk were used for blocking and primary antibodies were added to the membrane overnight at 4°C. They were then washed with PBS-T and then secondary antibodies were added at room temperature for 1 hr. ECL prime was used to detect western blot. Western blot was quantified by Image Studio™ Lite software (LI-COR Biotechnology - UK).

3.3. Apoptosis assay

NBL cells were plated and incubated with indicated concentrations of inhibitor for 24 hrs or 48 hrs. Lysates were collected and the protein concentration was calculated. Western blotting was performed, the membrane was blocked in milk and then PARP antibody was used to immunoblot the membrane. PARP antibody detects full length PARP and cleaved PARP1 both. Respective actin/GAPDH concentration was used to normalize cleaved PARP1 levels for quantification.

3.4. Proliferation assay

NBL cells were plated on 48 or 96-well plates and left to grow overnight. The next day, the specified concentrations of drugs were used to treat cells, either in mono drug treatment or

in combinations (for synergy experiments). The analysis of the experiment was carried out either in Incucyte S3 (Essen BioScience, USA) or by a resazurin assay.

3.5. Neurite outgrowth assay

Wild type ALk or mutant ALK were transfected in 100 µl of Ingenio electroporation solution (Mirrus Bio LCC) with 2 x10^6 PC-12 cells. After electroporation, a fresh medium was added to the cells and seeded into 24-well plates. Specified concentrations of inhibitors were used on transfected cells for 48 h. Neurite outgrowth was quantified/scored 48 h post-transfection. The scoring is carried out by counting neurite-carrying cells (double the length compared to normal cells) GFP-positive cells vs GFP-positive cells (all) by using a Zeiss Axiovert 40 CFL microscope.

3.6. Cell cycle analysis

1 x 10^6 NBL cells were seeded in a 6-well plate. Type I Bovine collagen solution (0.4%) (Advance BioMatrix, LOT#7434) was used to pre-coat the plates. The next day, cells were treated with the desired concentration and for the desired time. After treatment, the cells were harvested, fixed and followed the instruction protocol of "fixed cell cycle-DAPI assay protocol" (NucleoCounter NC-3000, Chemometec, Denmark). Analysis of the cell cycle was performed by using NucleoCounter NC-3000 (Chemometec, Denmark) and following the manufacturer's protocol. Cell cycle data were determined by the plot manager in NucleoView NC-3000 software.

3.7. Mouse xenografts model

4-6 weeks old BALB/cAnNRj-Foxn1nu (Janvier Labs, France) or Balbc/nude female mice (Charles River, Germany) were hypodermically injected 1 x 10^6 in the left flank. The tumour size was measured continuously, once the tumour size reached 150 mm^3, the mice were divided into a drug or vehicle-treated group, which were orally administrated. Xenograft tumours were excised and weighed at the end of the experiment and fixed in 4% paraformaldehyde for 3days. All experimental protocols and procedures were conducted by following the Regional Animal Ethics Committee approval, Jordbruksverket (230-2014, 01890-2018).

3.8. Transgenic mouse model

Alk-F1178S$^{KI/0}$; TH-MYCN$^{Tg/0}$ on a 129X1/SvJ background (JAX stock #000691) were screened by ultrasound, 2–3 times a week from approximately 35 days old. Tumours were monitored until they reach a size of 3–6 mm in average diameter when mice were randomized into treatment groups. Mice were treated P.O. once daily with BAY2402234 at 2.5 mg/kg of body weight (n=6), or vehicle control treatment (2% DMSO/30% PEG300). Tumour imaging was performed on days 0, 7 and 14 by ultrasound. On day 14, the animals were sacrificed and the tumour was harvested except for two mice in the treated group that were released and imaged after an additional 7 days (day 21). Screening and 3D image acquisition were performed using the Vevo 3100 imaging systems (FUJIFILM VisualSonics, Toronto, Canada). The VisualSonics MX550D (25-55 MHz, 40 µm axial resolution) linear array transducer was used for all image acquisition. For 3D scanning the probe was attached to a step motor, animals were anaesthetized with isoflurane and their respiration rate, ECG and body temperature were monitored during the procedure. Images were analysed and the tumour volume was measured in VevoLab (Fujifilm VisualSonics, Toronto, Canada). ‘

3.9. Immunohistochemistry

Tumour samples fixation was done in 10% neutral buffered formalin, dehydrated, cleared and embedded in paraffin, then sectioned at 5 µm. After deparaffinization and rehydration, Heat-Induced-Epitope-Retrieval was performed with 10mM citric acid, 0.05%Tween 20. Blocking was performed with 5% of normal goat serum before sections were incubated with primary antibody and diluted in Signalstain® Antibody Diluent (CST, #8112) overnight at 4°. Sections were incubated with Signalstain® Boost IHC Detection Reagent (HRP, Rabbit; CST, Cat. #8114). The signal was developed with Signalstain® DAB Substrate Kit (CST, Cat. #8059). Slides were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich, Cat. #MHS1-100ML), before dehydration and mounting. Digital images of sections were obtained with a Hamamatsu NanoZoomer-SQ Digital slide scanner.

4. RESULTS AND DISCUSSION

4.1. Paper I

"Alectinib, an Anaplastic Lymphoma Kinase Inhibitor, Abolishes ALK Activity and Growth in ALK-Positive NBL Cells"

Alectinib has been used in the clinic for relapsed ALK-positive NSCLC and for those patients who are intolerant to crizotinib. We have shown that ALK activity is inhibited by alectinib and the growth of ALK-addicted NBL cell lines in a dose-dependent manner. Alectinib showed a nearly two-fold inhibition over the first generation ALK inhibitor, crizotinib. Mutated ALK variants found in NBL cases were examined and the capability of alectinib to block phosphorylation, on Y1604, in PC12 cells. Interestingly, the activation of ALK gain-of-function alleles were inhibited by alectinib, with IC_{50} values of 2.2 to 22 fold less than those observed with crizotinib. In a mouse xenograft model of NBL, alectinib has shown potent anti-tumour growth. Tumour volume showed a relatively significant reduction in a treatment group. No effect on mice weight and no observable side effects were observed after treatment with alectinib. ALK dependent NBL cell lines have shown increased PARP cleavage and cell cycle G1 arrest after 24 h of treatment. Cell cycle progression is delayed in ALK-addicted cells and induced apoptosis has been shown after alectinib treatment

To summarize: Alectinib suppresses the activation of ALK in NBL cell lines, in biochemical assays and ALK activity in mice xenografts, which was exogenously expressed. In conclusion, these results suggest that alectinib is a potent inhibitor for ALK and highlights further investigation of alectinib in the ALK-positive NBL setting.

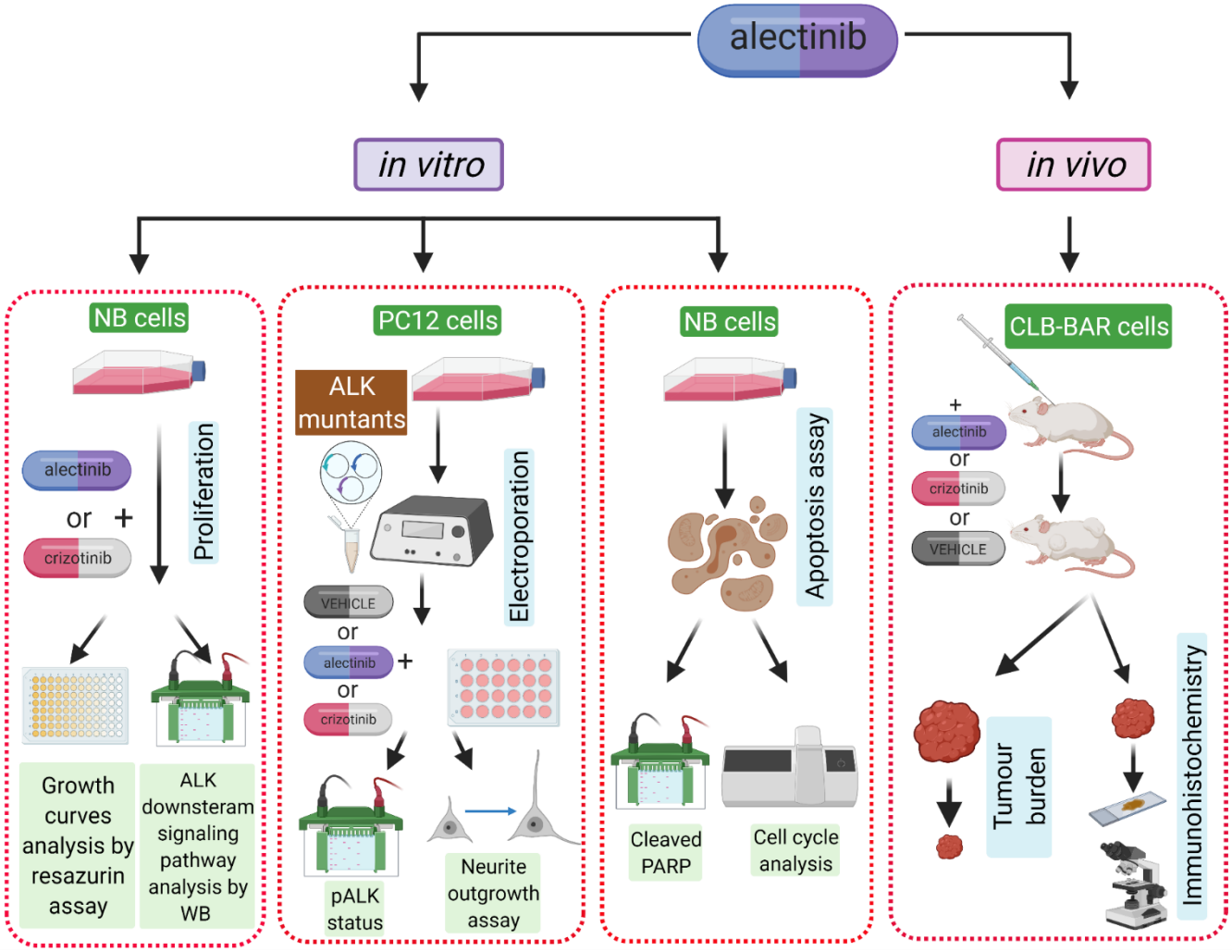

Figure 12. ***Project I workflow illustration:*** *The preclinical analysis of alectinib in NBL setting.*

4.2. Paper II

“BAY2402234, a Dihydroorotate Dehydrogenase (DHODH) Inhibitor, Mediates Abrogation of The Proliferation and Apoptosis of NBL Cells”.

Pyrimidine nucleotides play a vital role in tumour progression and these pyrimidines can be synthesized through either salvage or de novo pathway. Tumour cells fulfil their need for nucleotides through the de novo pathway. Dihydroorotate dehydrogenase (DHODH) is an important player of de novo pyrimidine synthesis and by inhibiting DHODH tumour cells proliferation is decreased. BAY2402234, a novel DHODH inhibitor has shown striking inhibition in acute myeloid leukemia (AML) and we investigated BAY2402234 in NBL preclinical settings. In BAY2402234 treated NBL cells and transgenic mouse models, inhibition of proliferation and tumour growth are observed respectively. Once the low levels of DHODH confirm better prognosis in NBL on an online tool, R2 database, we employed the panel of NBL cell lines. BAY2402234 showed proliferation inhibition in nM concentrations. The combination of BAY2402234 and ALK inhibitor (lorlatinib) showed synergy in the proliferation of ALK addicted NBL cell lines, showing the better therapeutic effect of the drug combination. BAY2402234 treatment induced p53 and cleaved PARP and reduction of MYCN proteins levels. In *Alk-F1178S; Th-MYCN* mouse model, a significant reduction of tumour growth has been observed once treated with BAY2402234 and the immunohistochemistry result of Ki-67 on tumour samples displayed a significant anti-proliferative effect.

Summary: BAY2402234 is an efficient inhibitor of DHODH, which inhibits NBL proliferation and growth in NBL cell lines and transgenic mice respectively. The combination treatment of BAY2402234 and lorlatinib showed synergy and as a promising future therapeutic option for the NBL patients, this should be considered alone or in combination.

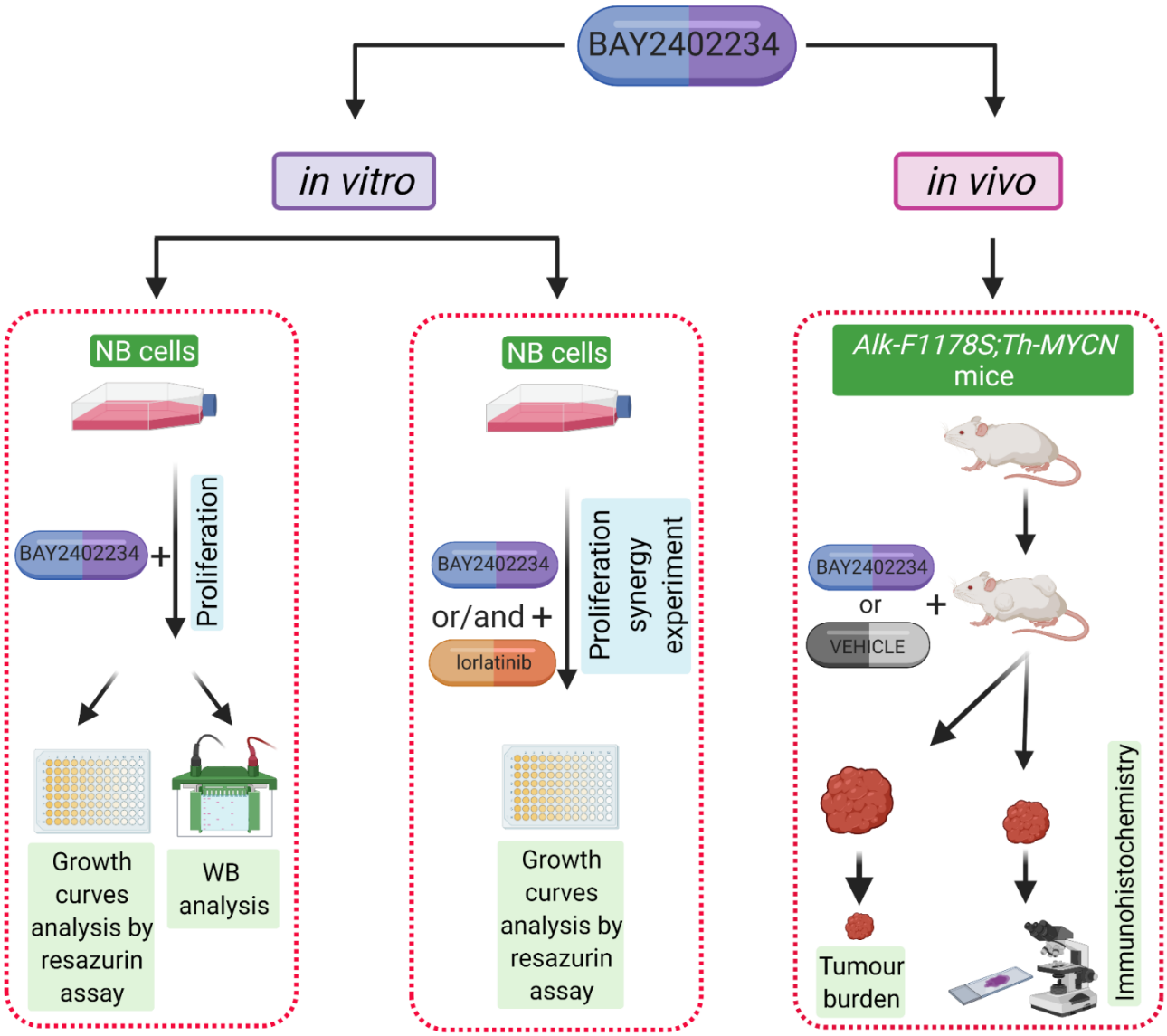

Figure 13. ***Project II workflow illustration:*** *The preclinical analysis of BAY2402234 in NBL setting.*

5. CONCLUSIONS

5.1. Paper I

- Alectinib inhibits ALK activity in NBL cell lines.
- Alectinib efficiently abrogated NBL cell proliferation and reduced tumour burden in NBL mice model.
- Alectinib abrogates the ALK downstream signaling with better efficiency than crizotinib
- Alectinib showed better potency in inhibition of ALK-wt and ALK mutants compare with crizotinib
- Alectinib induces apoptosis and inhibits the cell cycle progression

5.2. Paper II

- Low DHODH expression is associated with good overall survival in NBL tumours.
- BAY2402234 inhibits the proliferation of NBL cells in nM concentrations comparing with leflunomide where μM concentrations were used.
- BAY2402234 and lorlatinib showed synergy in ALK-addicted NBL cell lines.
- BAY2402234 treatment showed a significant reduction of tumours in the transgenic mouse model.
- BAY2402234 should be used alone or in combination with ALK TKIs, as a prospective future therapeutic strategy for ALK positive NBL patients.

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APPENDIX