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Neuroblastoma and its Target Therapies: A Medicinal Chemistry Review

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This review is dedicated to the memory of Isabella Mary Gerges (1998–2005).

Neuroblastoma (NB) is a childhood malignant tumour belonging to a group of embryonic tumours originating from progenitor cells of the sympathoadrenal lineage. The heterogeneity of NB is reflected in the survival rates of those with low and intermediate risk diseases who have survival rates ranging from 85 to 90%. However, for those identified with high-risk Stage 4 NB, the treatment options are much more limited. For this group, current treatment consists of immunotherapy (monoclonal antibodies) in combination with anti-cancer drugs and has a 40 to 50% survival rate. The purpose of this review is to summarise NB research from a medicinal chemistry perspective and to highlight advances in targeted drug therapy in the field. The review examines the medicinal chemistry of a number of drugs tested in research, some of which are currently under clinical trial. It concludes by proposing that future medicinal chemistry research into NB should consider other possible target therapies and adopt a multi-target drug approach rather than a one-drug-one-target approach for improved efficacy and less drug-drug interaction for the treatment of NB Stage 4 (NBS4) patients.

1. Introduction

Neuroblastoma (NB) is the third most common solid tumour in infancy and childhood[1] and is responsible for 15% of all paediatric cancer deaths worldwide:[2] it is extremely rare in adults. 90% of NB occurs in children under the age of 10 with the majority of cases diagnosed between birth and five years of age.[3] A patient's age at diagnosis is one of the prognostic indicators for the disease with survival rates at younger ages being better than at older ages. Currently the US Children's Oncology Group (COG) has advocated for an age cut-off greater than 18 months for risk stratification.[4] In the UK approximately 100 children are diagnosed with the disease each year with the majority being less than five years old. It is the most common cancer in the first year of life and said to account for approximately one fifth, to one quarter of all cancers in this age group.[5] NB is often present at birth but not usually detected until later and in rare cases of adrenal NB, can be detected before birth by foetal ultrasound.[6] According to Fisher and Tweddle in 2012 NB may be detected through obstetric ultrasound scanning in the third trimester usually seen in the adrenal glands, with incidental findings of the disease visualised as early as 23 weeks with careful sonography.[7] In 2015, the UK National Screening Committee (UKNSC) recommended not reversing its current policy of universal screening for NB in children in the first year of life because only a small number of deaths are prevented by screening, and the harms from overdiagnosis that include psychological and physical harms, are great.[5]

Called the "great masquerader" NB is described as a heterogeneous developmental tumour, as the disease can be caused by varying or different genes or alleles that arise from the embryonic sympathoadrenal cells of the neural crest.[8] Unlike cancers that are associated with environmental factors such as asbestos and malignant mesothelioma, or lifestyle behaviour such as smoking and lung cancer, NB is closely associated with its genetic/molecular characteristics, although some research has suggested that certain exposures may be more common in children with NB.[4][5] Tumours of the NB type have been called '"enigmatic"[5] because of their unique and often unpredictable clinical behaviours, such as spontaneous regression, tumour maturation to benign ganglioneuroma, rapid progression to life-threatening disease and aggressive progression refractory to therapy'.[4] It can occur at any point along the migratory pathway of the sympathetic nervous system.[9] The neural crest is the temporary structure composed of multipotent progenitor cells presented during embryogenesis and emerging from the dorsal part of the body.[10] The progenitor cells are proliferative and strictly regulated by the interaction of different signalling pathways.[11] After migration, these pluripotent sympathogonia form the sympathetic ganglia, adrenal medulla chromaffin cells, and paraganglia,[12] reflecting the classic localisation of NB (Figure 1).[13]

Most cases of NB develop in the adrenal glands above the kidneys but it can also grow in the neck, chest, abdomen, pelvis, or areas adjacent to the spinal cord.[14] The most common symptoms of NB are the result of pressure by the tumour or bone pain from cancer that has spread to the bone. Protruding eyes and dark circles around the eyes are common

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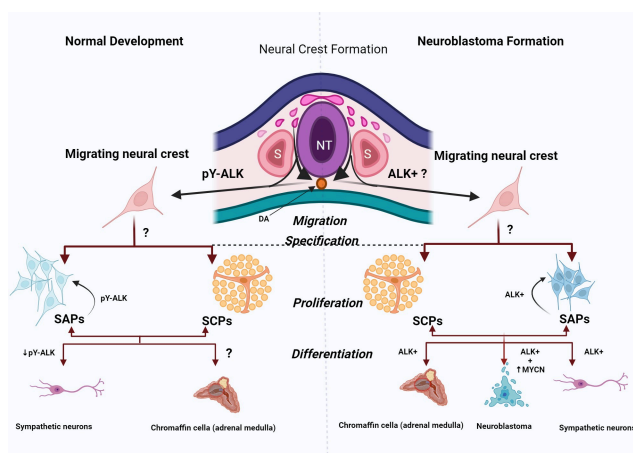European Chemical
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Figure 1. Neuroblastoma formation[12] Reproduced from Ref. [12], Copyright 2021 and recreated with BioRender.com.

and caused by the cancer spreading to the area behind the eye.[14]

Because of its varied pathology, it can have significantly mixed clinical behaviour.[15] A characteristic feature of NB is its clinical heterogeneity from localised tumours to widespread and early haematogenous metastasizing. This clinical heterogeneity reflects the complexity of genomic abnormalities characterised in NB tumours[16] leading to significant differences in outcome across tumour subtypes. In studies of infants the disease has been known to spontaneously regress without therapy, whereas older children with unfavourable histology tend to have poor outcomes. Inomistova et al.,[16] explain that there are multiple mechanisms by which cells can progress into malignancy that involves the concerted accumulation and functional cooperation between genetic and epigenetic changes, and not their order of occurrence that results in carcinogenesis.[16] The occurrence of many cancers is the result of the accumulation of genetic and epigenetic changes: epigenetics is concerned with heritable changes in the functioning of genes without changes in the DNA sequence. These epigenetic modifications consist mainly of DNA methyl-

ation, histone modification, chromatin reorganization, and expression of non-coding RNA. In the case of cancers, it is nearly impossible to reverse genetic alterations, whereas epigenetic changes "can dynamically respond to signals from the physical, biological and social environment".[16]

Inomistova, et al. in 2019, reported that the role of MYCN gene amplification in NB pathogenesis was established in the 1980s, due to its association with high-risk tumour and low patient survival. Since then, several other genetic abnormalities have been associated with NB, including gains of whole chromosomes and large-scale chromosomal imbalances, such as loss of heterozygosity (LOH) at chromosomal arm 1p, 3p, 14q, and 11q, unbalanced gain at 1q, 11p, and 17q and numerous mutations in key genes such as *ALK*, *PHOX2B*, and *PTPRD*.[16] In the case of MYCN it was found to be amplified in 20 to 25% of NB; ALK represents 1–2% of familial NB; PHOX2B germline mutations are found in a subset of familial NB representing 4% of sporadic cases; LOH 40–45% with the gain of chromosome 17q occurring in 80% of NB.[16] With chromosome 17q gain found to be the parameter for poor outcome according to Inomistova et al., understanding the gene abnormalities caused by 17q "will be crucial for fully understanding the NB progression." Thus, elucidating the genetics and epigenetic origins of NB has been vital to the search for actionable therapeutic targets.

Recovery from NB and choice of treatment depends on age and what stage the disease has progressed to. For patients with low, and intermediate risk disease, survival rates can range from 85 to 90%.[17] However, about half of NB patients are diagnosed with high-risk disease, and despite aggressive treatment, have a survival rate of between 40 to 50%.[2] The clinical NB staging system is categorised as Stages 1, 2 A, 2B, 3, 4 and 4S.[1] Stages 1 and 2 (2 A and 2B) are limited to a small disease area and have a good overall prognosis. Stage 3 disease appears to represent a heterogeneous group with the appearance of non-metastatic (Stages 1 and 2) and metastatic disease (Stage 4). Stage 4S NB is called "special" NB because it is treated differently. The cancer is localised with the spread of disease limited to liver, skin, and/or to a very limited extent, bone marrow. The finale stage is metastatic NB (Stage 4) and is often

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fatal despite aggressive therapy. As stages 1, 2, 3 and 4S of NB are linked with favourable histology that records a survival rate ranging from 85 to 90%[17] the focus of this review will be on Stage 4 NB (NBS4) due to its unfavourable histology (metastatic disease)[1] that has a survival rate of between 40 to 50%.[2]

1.1. Screening and Diagnosis of NB

Initial NB screening programmes have been devised using urinary metabolites VMA and HVA, for children under the age of one in Japan, Germany and Canada. For those who test positive, further diagnostic testing of screen-detected cases are performed (e.g. imaging, serology, histology).[5] Despite the screening test being highly specific with less than 0.1% of infants having false positive results requiring investigation, a number of missed (interval) cancers have been diagnosed in children who screened negative both in the Japanese programme and in Canada, indicating there is a problem with the sensitivity of screening in detecting tumours that will progress to life-threatening disease.[18]

Following a review of the evidence for NB screening programmes, the UK National Screening Committee (NSC) in 2015 failed to recommend universal screening for NB because of the inability of markers to identify disease that will regress or progress.[5] There was also no evidence to compare the accuracy of makers predicting prognosis concerning event free survival (EFS) or overall survival (OS). This included markers used during initial screening such as urinary metabolites for children under one, and markers used during subsequent diagnostic testing of screen-detected cases (e.g. imaging, serology, histology).[5] The external review commissioned by the UK NSC relied upon three retrospective cohort studies from the International Neuroblastoma Risk Group (INRG) project, that included the study by Cohn et al. who examined data for 8,800 patients from multiple trials who were diagnosed with NB, Ganglioneuroblastoma (GNB), or ganglioneuroma (GN) maturing between 1990 and 2002.[5] Despite 17q chromosome gain being a significant parameter for poor outcome, Cohn et al. did not examine the prognostic significance of 17q gain (segmental gain on the long arm of chromosome 17) because data on this was available for less than 5% of the cohort.[5]

According to Fisher and Tweddle, in 2012, careful sonography has the ability to detect NB tumours as early as 23 weeks. The identification of genetic abnormalities associated with NB including gains of whole chromosomes and large-scale chromosomal imbalances is not dissimilar to that of Down's Syndrome (DS) that results from an extra chromosome. In the case of DS, the ultrasound measurements for estimating the chances of a having DS child include nuchal translucency (NT) and crown rump length (CRL) along with a maternal blood sample. NT is the ultrasound appearance of a collection of fluid under the skin at the back of the baby's neck. The thickness of the NT is measured and used as part of the combined test to calculate the chance of having a DS baby. Having obtained a result for increased thickness of the NT in the first trimester that was later discounted at the second trimester, Isabella, whose memory this review is dedicated to, was diagnosed with NBS4, six months before her fifth birthday: there was no MYCN amplification but 17q gain was present. At birth, there was pronounced discolouring around her eyes and nose that was dismissed as a "port wine" birthmark, that faded with time.

1.2. Receptors as Targets for Drug Treatment

Treatment for NB is generally divided into 3 phases; induction, consolidation and maintenance. Children in the high-risk category are initially treated with multi-agent chemotherapy, surgery and radiotherapy, followed by consolidation therapy with high-dose chemotherapy (which may cause severe or complete depletion of bone marrow cells; also known as myeloablative therapy) and autologous stem cell transplant.[19] Radiotherapy may also be given after stem cell transplant. In recent years the treatment of NB has included the combination of monoclonal antibodies with therapeutic agents, with the survival rate for NBS4 remaining at 40 to 50%[20] indicating other molecular targets are urgently needed.

Receptors are proteins that represent the most important molecular drug targets in medicine.[21] It is essential in drug design to know the different types of receptors, structures, locations, and how they are initiated.[21–22] Table 1 shows the different types of receptors and how they vary from location, effect, and time scales with an example of each kind.[22] Figure 2 lists four different types of receptors: three are membrane-bound and include tyrosine kinase receptors (TKR), G-protein coupled receptors (GPCR), and ion channel receptors (ICR); the fourth type is an internal receptor known as nuclear receptors (NR).[22]

Table 1. Comparison between the four types of receptors.[22]

	ICR	GPCR	TKR	NR
Location	PM	PM	PM	Nucleus
Effector	Ion channel	Enzyme or ion channel	Enzymes	Regulation of gene action
Time scale	Milliseconds-seconds	Seconds-minutes	Minutes-hours	Hours-days
Example	Nicotinic receptors	Adrenoreceptors	Insulin receptors	Steroid receptors

Note: Ion Channel Receptors (ICR), G-Protein-Coupled Receptors (GBCR), Nuclear Receptors (NR), Tyrosine Kinase Receptors (TKR) and Plasma membrane (PM).
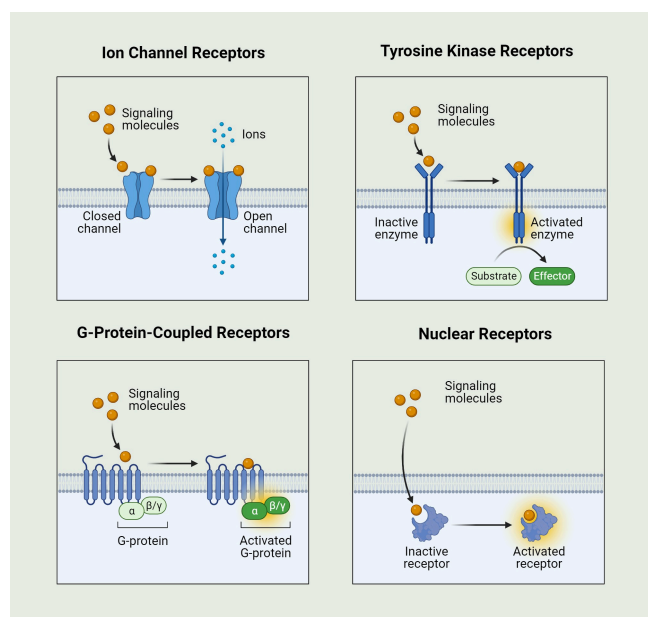
Figure 2. An overview of the activation process of the four types of receptors. Created with BioRender.com.

Also, the activation (initiation) process is different for each type, as shown in Figure 2.

2. Clinical Trials on NB (All Stages) and the Search for a Cure

Drug design and development involves 12 stages: identifying target disease; identifying drug target; establishing testing procedures; finding a lead compound; Structure Activity Relationships (SAR); identifying pharmacophore; optimising target interactions; optimising pharmacokinetics properties; preclinical trials; chemical development and process development; patenting and regulatory affairs, and finally, clinical trials.[21] There are four phases in clinical trials:[21]

1) Phase 1: carried out on healthy volunteers. This phase is useful in establishing dose level, and for studying pharmacokinetics, including drug metabolism;

2) Phase 2: carried out on patients and as double blinded studies to demonstrate whether a drug is therapeutically useful. This stage also attempts to establish a dosing regime and identify side effects;

3) Phase 3: is carried out on a larger number of patients to establish statistical proof for efficacy and safety,

4) Phase 4: Continues after the drug has reached the market to study the long-term effects when used chronically and to identify unusual side effects.

The results from clinical trials can be found at https://classic.clinicaltrials.gov/

Up to the 12th of August 2023, 502 clinical studies (birth-17) have investigated NB and other paediatric cancers, of which 230 have been completed, and 63 currently active; a further 74 have been terminated/suspended/withdrawn. Of the remaining studies, eight are not yet recruiting, 87 are recruiting, and 40 are of unknown status. See Table 2 for clinical trials covering all stage NB.

2.1. Clinical Trials on NB Stage 4 (NBS4)

Up to the 12th of August 2023, a total of 306 clinical trials have been recorded as investigating NBS4, of which 154 are completed, 37 active, 37 terminated/suspended/withdrawn, 53 are currently recruiting, six are not yet recruiting, and 19 unknown status (Table 3).

Table 3 shows that of the 306 clinical trials on NBS4 only four are in phase four clinical trials. Of these four, only one, Dinutuximab (a monoclonal antibody), is concerned with treating NBS4. The four studies include: 1) a completed trial on the *Efficacy of Prophylactic Itraconazole in High-Dose Chemotherapy and Autologous Hematopoietic Stem Cell Transplantation*; 2) one recruiting on *Paediatric Long-Term Follow-up and Rollover Study*; 3) one of unknown status on *G-CSF Alone or Combination With GM-CSF* on the prevention and treatment of infection in children with malignant tumour, and 4) one not yet recruiting on *Dinutuximab*, indicating the need for more research in the treatment of NBS4.

Table 2. Clinical trials on all stage NB (n = 502).						
Phase 1 to 4 clinical trials	Number of studies (n = 502)	Completed (n = 230)	Active (n = 63)	Recruiting (n = 87)	Unknown status (n = 40)	Not yet recruiting (n = 8)	Terminated/ suspended/ withdrawn (74)
4	4	1	0	1	1	1	0
3	44	22	4	8	6	0	4
2	218	95	25	38	23	5	32
1	226	112	33	35	7	2	37
Early phase 1	10	0	1	5	3	0	1

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Table 3. Clinical trials on NB Stage 4 only (n = 306).

Phase 1 to 4 clinical trials	Number of studies (n = 306)	Completed (n = 154)	Active (n = 37)	Recruiting (n = 53)	Unknown status (n = 19)	Not yet recruiting (n = 6)	Terminate/ suspended/ withdrawn (n = 37)
4	4	1	0	1	1	1	0
3	31	19	2	4	3	0	3
2	137	62	16	25	12	3	19
1	132	72	18	23	2	2	15
Early phase 1	2	0	1	0	1	0	0

2.2. Examples of Drugs currently in Clinical Trial for NBS4

Table 4 lists the drugs currently in clinical trial for NBS4 (excluding monoclonal antibody).

Having looked at the drugs currently in clinical trial for the treatment of NBS4 the next step is to look at current targets. Zafar et al. in their work, (Figure 3), give a good summary of current target therapies for NBS4.[23]

From Table 5, it can be seen there are at least three different target groups for NBS4 including: A-MYC driven, B-Telomere maintenance/elongation aberrations, and C-ALK Mutation/ Amplification.[4]

3. Therapeutic Possibilities for Treating NBS4

According to Nguyen et al.[24] although clinical trials have a highly effective infrastructure, conducting clinical studies in paediatric oncology poses challenges due to the low occurrence of the disease, inadequate preclinical models, and testing programs. The complexity in conducting clinical trials in paediatric oncology is further compounded in the case of NBS4 because of the genetic heterogeneity of the disease.[24] Figure 3[23] summarises current targeted therapy in NB, and includes designing inhibitors for MEK, PI3 K, AKT, mTor, Bcl2, MDM2, Topoisomerase, DNMT, HMTs, HAT, HDAC, Bromodo-

main, and most recently monoclonal antibodies as immunotherapy. The aim of targeted treatment is to stop cell proliferation. Despite patients sharing the same diagnosis, patients can respond differently to targeted therapeutic approaches.[24]

In the following section the medicinal chemistry approach to seven drugs tested in clinical trials for NBS4 will be discussed (Table 5). Looking at the drug development approach should give us a greater understanding of how the drugs work and potentially direct further research towards developing a drug that can increase the survival rate of patients with NBS4. The seven drugs selected showed initial promising results for treating NBS4 with four out of the seven reaching phase two clinical trials. The four drugs reaching phase two for NBS4 include: Alisertib (Aurora Kinase inhibitors); Crizotinib (ALK inhibitors); Gefitinib (EGFR tyrosine kinase inhibitors) and Sorafenib (P21 inhibitors). The remaining three drugs reached phase one clinical trial and include: GSK525762/I-BET762 (bromodomain inhibitors); Roscovitine/Seliciclib (CDK inhibitors); and Trametinib (MEK1/2 inhibitors). The seven drugs are discussed according to their date of submission for clinical trial beginning with Gefitinib that entered clinical trial in 2003 (see Table 4 for the date when the seven drugs were submitted for clinical trial and phase). The section will conclude with an example of one of the most recent immunotherapies, monoclonal antibody therapy, Dinutuximab, recommended in July 2018 in the UK by the National Institute for Health and Care Excellence (NICE) as a target therapy for treating NBS4.[25]
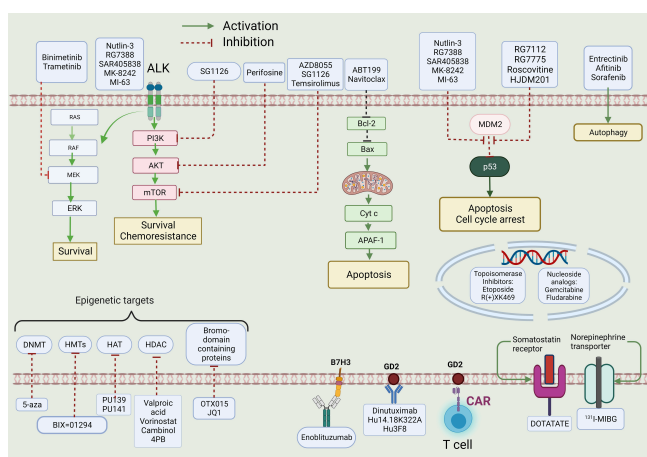
4. Medicinal Chemistry Approaches to Drug Discovery

4.1. Gefitinib (ZD1839)

The isolation of murine epidermal growth factor (EGF) by Cohen and Carpenter, in 1975 heralded the era of targeted cancer treatment.[26] According to Gill et al. in 1984 the possibility of designing a compound to inhibit the EGFR signalling with the hope that it would be used as a treatment for cancer, was discovered by Mendelsohn and his team in 1980.[27] The team managed to produce two monoclonal antibodies, 225 and 528, that could block the downstream signalling by competing with

Figure 3. Targeted therapy in NB.[23] Reproduced from Ref. [25], copyright 2021, and recreated with BioRender.com.

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Table 4. Drugs currently in clinical trials for NBS4 (excluding monoclonal antibody).				
Drug	Phase	Status	Location	Date Started
Crizotinib	NCT00939770- Phase 2	Completed	United States	2009
Lorlatinib	NCT03107988-Phase 1	Recruiting	United States	2017
Ceritinib (LDK378)	NCT01742286-Phase 1	Completed	United States	2013
Entrectinib (RXDX-101)	NCT02650401-Phase 1	Recruiting	United States	2016
DMFO (Eflornithine)	NCT02097810-Phase 1	Completed	United States	2014
	NCT01059071-Phase 1	Completed	United States	2010
Also called α-difluoro-methyl ornithine, "Mostly in combination with other drugs."	NCT02559778-Phase 2 NCT02679144-Phase 2 NCT02395666-Phase 2 NCT02139397-Phase 2 NCT02030964-Phase 1	Recruiting Recruiting - Active, not recruiting a single drug after remission) - Active, not recruiting a single drug after remission) - Active, not recruiting a single drug after remission)	United States United States United States United States United States	2015 2016 2015 2014 2014
Gefitinib	NCT00132158-Phase 1 NCT00068497-None applicable Phase	Completed Completed	United States United States	2005 2003
Erlotinib	NCT00030498-Phase 1	Completed	United States	2003
Ensartinib	NCT03155620-Phase 2 NCT03213652-Phase 2	Recruiting Recruiting	United States United States	2017 2017
Prexasertib	NCT02808650-Phase 1	Completed	United States	2016
Ribociclib (LEE001)	NCT01747876-Phase 1	Terminated	United States, Spain, Germany, Italy, Australia, Singapore, France	2012
Alisertib	NCT02444884-Phase 1 NCT01601535-Phase 1 and 2 NCT01154816-Phase 2	Completed Completed Completed	United States United States United States	2015, 2012, 2010
GSK525762 or Molibresib	NCT01587703-Phase 1	Completed	United Kingdom	2012
SF1126	NCT02337309-Phase 1	Terminated	United States	2015
Copanlisib	NCT03458728-Phase 2	Terminated	Germany	2018

Table 4. continued

Drug	Phase	Status	Location	Date Started
Trametinib	NCT02124772-Phase 2 NCT02780128-Phase 1	Completed Recruiting	United Kingdom United States	2015 2016
Larotrectinib	NCT03213704-Phase 2 NCT02637687-Phase 2	Recruiting Recruiting	United States Germany	2017 2015
Venetoclax	NCT03236857-Phase 1	Completed	United Kingdom	2017
Sorafenib	NCT02298348-Phase 1	Active, not recruiting	United States	2012
Roscovitine	NCT00999401-Phase 1	Completed	United States	2009

Table 5. Current targets for treatment of NBS4.[24]

NBS4 groups	Biomarkers	Mechanism of Action with an example
A-MYC driven	MYC Family Protein (MYCN/MYC) Overexpression	BET Bromodomain inhibitors: (GSK525762, RO6870810, CPI-0610) CDK inhibitors: (Roscovitine, THZ1) Aurora Kinase Inhibitors: (CD532, Alisertib/MLN8237)
	Nuclear Hypertrophy	rRNA synthesis inhibitors: (CX-5461) Protein Translation Inhibitors: (Halofuginone)
B-Telomere maintenance/elongation aberrations	TERT Overexpression	Telomerase Inhibitors: (Imetelstat) P21 Inhibitors: (Sorafenib, UC2288)
	Loss For ATRX Expression	ATR Inhibitors: (AZD6738)
C-ALK Mutation/Amplification	ALK protein Overexpression	ALK Inhibitors: (Crizotinib, Gefitinib, Trametinib Lorlatinib, Ceritinib (LDK378), Ensartinib, Entrectinib)

EGF or TGFα.[27] A murine chimeric version of murine mAb 225, known as Cetuximab (IMC—C225, Erbitux) was identified resulting in the development of Gefitinib by AstraZeneca in 1994. The drug was later approved by the Federal Drug Agency (FDA). In the US in 2015 as a first-line cancer therapy for metastatic non-small-cell lung cancer (NSCLC) with EGFR mutations.[28] Work by AstraZeneca started with identifying an inhibitor for EGFR and was followed by SAR studies, where they identified a 4-anilinoquinazo line. From this work, compound I (Figure 4A) was chosen to be the lead compound.[21]

In vitro studies (laboratory testing on cancer cells) on compound 1 were promising and, *in vivo* studies (animal testing on rats and mice) showed it was quickly metabolised to two compounds by P450 enzymes.[21] Working on the oxidation of the aromatic methyl group and aromatic-para position produced compounds II (Figure 4B) and III (Figure 4C) while replacing the methyl group with a chloro substitution, produced compound IV (Figure 4D). Compound IV showed better *in vivo* activity since it has proven to be resistant to metabolism. Further alterations and various alkoxy substituents

at the 6-position were studied, leading to the Gefitinib discovery (Figure 4E). This includes a morpholine ring, often introduced to enhance water solubility.[21]

Synthesis of Gefitinib[29]

The AstraZeneca synthesis of Gefitinib is one of the syntheses mentioned by Maskrey et al. in 2018[29] (Figure 5) and is used as an example in this review.

Three clinical trials of Gefitinib have been identified:
– NCT00068497 started September 2003 and completed January 2013 without an applicable phase.
– NCT00132158 phase 1 started August 2005, completed April 2012.
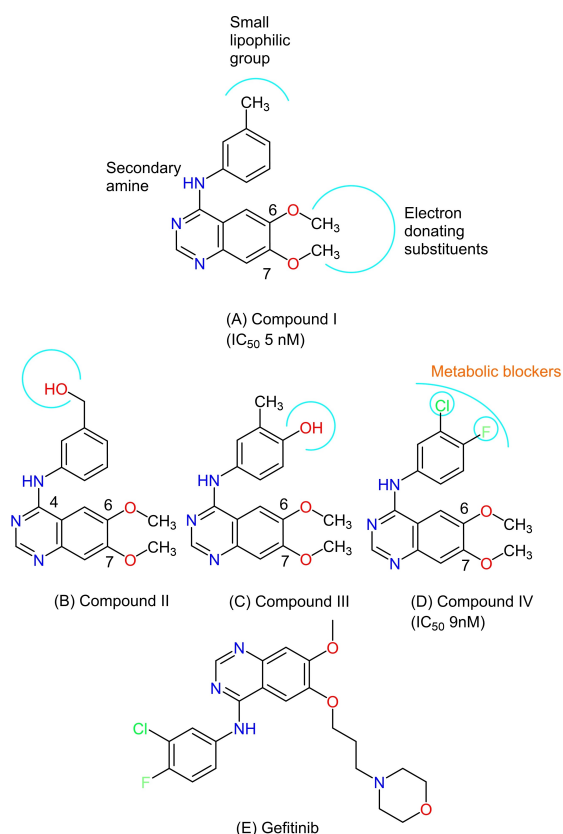– NCT00135135 phase 2 started August 2005, completed June 2008.

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Review

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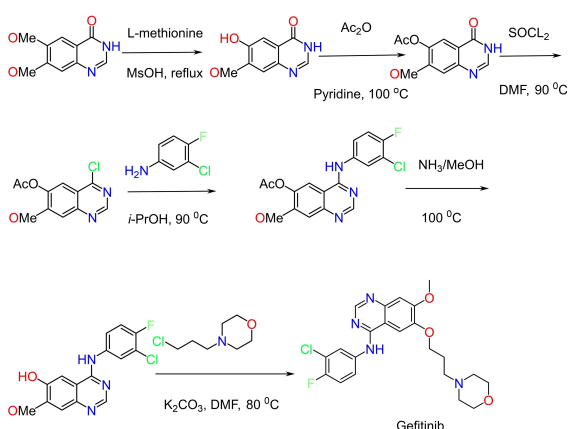**Figure 4.** The development of Gefitinib.

Figure 5. Synthesis of Gefitinib.[29]

4.2. Crizotinib (PF 2341066)

The role of receptor tyrosine kinase c-MET in normal cell development, and its overexpression in cancer, particularly in all types of solid tumours and other metastatic processes, was investigated. Having noted that its overexpression was correlated with poor prognosis or metastatic progression, it became a leading candidate for molecular targeted cancer therapies.[30] The discovery of Crizotinib originated from structure-based drug design (SBDD) studies in the search for a lead compound. Sunitinib (Figure 6A) was one of three known candidates to be
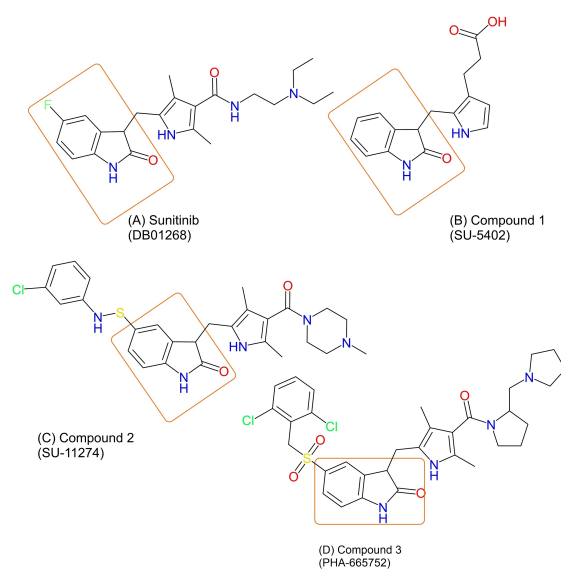
Figure 6. Chemical structures of (A) Sunitinib, (B) Compound 1, (C) Compound 2, and (D) Compound 3.

used as a lead compound for C–MET inhibition.[30] Structure of 1 (SU5402) (Figure 6B) known from a previous study was used in a comparable interaction study with compound 2 (Figure 6C). Further optimisation work on Sunitinib started by improving the lipophilic efficiency index (LipE = pIC_{50}-cLogD) of binding effectiveness,[31] along with testing the drug's safety and Absorption Distribution Metabolism Excretion (ADME).[32]

Optimisation of compound 2 (SU-11274, IC_{50} of 10 nM)[33] led to compound 3 (PHA-665752, IC_{50} = 9 nM) (Figure 6D) with selectivity 50 times greater for c-MET, when compared to a variety of serine-threonine kinases, and tyrosine kinases.[34]

In vivo studies on compound 3 (PHA-665752), showed phosphorylation inhibition in tumour growth and xenograft.[35] According to Cui et al. compound 3 showed poor pharmaceutical properties, and its development had to be stopped.[30] However having done the *in vivo* studies and despite the lack of success, the binding of compound 3 (PHA-665752) to 2WKM (protein data bank code PDB) revealed an unusual activation loop conformation created by residues 1228–1245, which interfered with substrate and ATP binding. These findings led to the design of an inhibitor for this specific binding pocket,[30] a novel 5-Aryl-3-benzyloxy-2-aminopyridine c-MET Inhibitors (Figure 7).

Further optimisation using SAR led to the production of 63 compounds and compound 61 was the best inhibitor. The two pure enantiomeric forms of 61 were prepared to evaluate the relative c-MET activity (Figure 8).

The R enantiomer 63 was found to be the most potent. By comparing the cocrystal structure of compound 3 (Figure 6D) with Crizotinib (Figure 8), it was observed that Crizotinib showed a better alignment with Tyrosine 1230 and less conformational strain. Further SAR studies on Crizotinib revealed that the R-methyl and 2,6-dichloro moieties on the 3-benzyloxy group are crucial for determining the low potency against c-MET.[30]
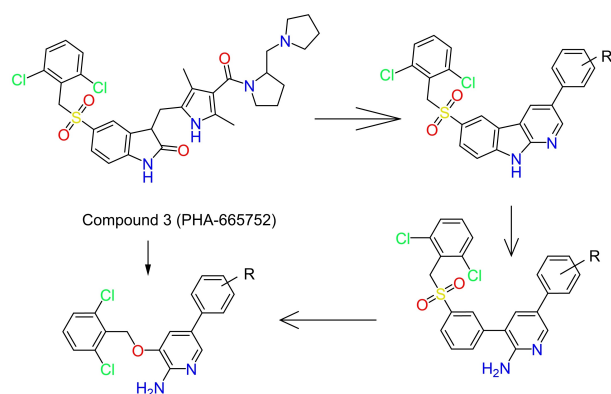
Figure 7. Design of 5-aryl-3-benzyloxy-2-aminopyridine scaffold from compound 3 for c-MET inhibition.[30]
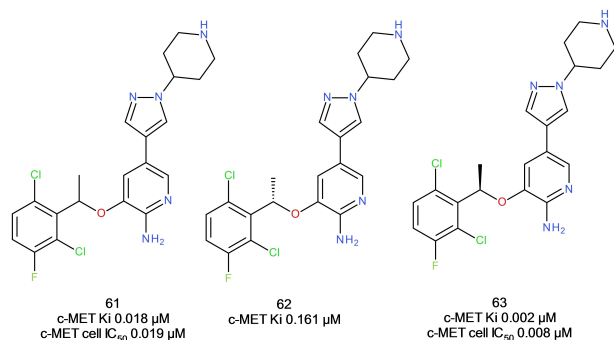

Figure 8. Structure and activity of 61, 62, and 63 (Crizotinib).

Kinase Selectivity Profile of Crizotinib

Crizotinib was assessed against a panel of more than 120 human kinases, the results of which are summarised in Table 6 and the synthesis is in Figure 9.

Four clinical trials on Crizotinib have been identified:
– NCT03107988 phase 1 started July 2009, still active in October 2023.
– NCT01121588 phase 1 started May 2010, terminated November 2023.
– NCT01606878 phase 1 started May 2012, completed January 2024.
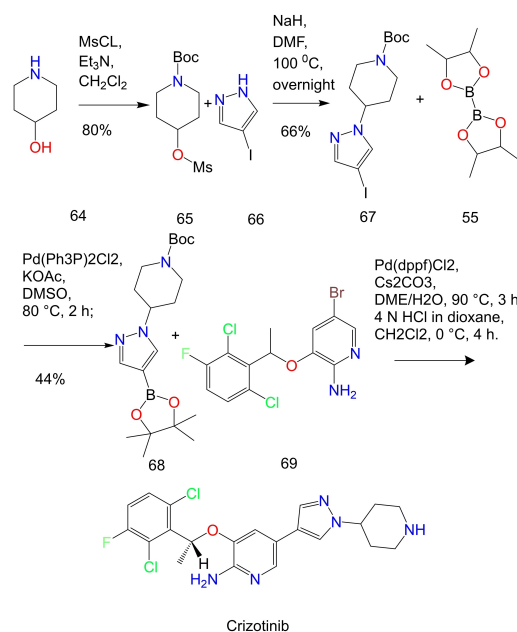– NCT00939770 phase 2 started July 2009, completed June 2020.

Figure 9. Synthesis of Crizotinib.[30]

4.3. Roscovitine/Seliciclib (CYC202)

The discovery of cyclins, proteins that regulate the CDK function has been attributed to the work of Timothy Hunt. Hunt was employed by the Imperial Research Fund in London and discovered cyclin-dependent kinases (CDKs) in 1982.[36] These are protein kinases characterised by needing a separate subunit - a cyclin - that provides domains essential for enzymatic activity. CDKs play an important role in the control of cell division and modulate transcription in response to several extra and intracellular cues. The drug Roscovitine was develop in response to the discovery of CDKs as an anti-cancer drug because of its role in cell division. According to Lee et al. in 2015[37] regulation of the cell cycle (Figure 10) involves cyclin-dependant kinase (CDK), which are activated by cyclins and inhabited by CDK inhibitors (DKIs) that control levels of phosphorylated retinoblastoma suppressor gene protein (RBI), transcriptional factors, and the activity of tumour suppressor gene TP53 in some cases.[37] Goga et al. in 2007 also showed that CDK1 inhibition can be a potential therapy for tumours over-expressing MYC.[38] A detailed account is provided by Zhelev et al. on the role of CDKs, particularly, CDK1, through to CDK9 in cell cycle control and cell growth.[37]

The discovery of Roscovitine was a collaborative approach between Pierre Guerrier at the *Roscoff Biological Station* in Lille
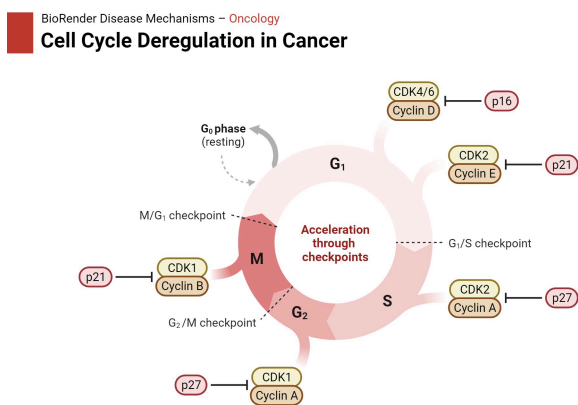
Table 6. Kinase selectivity of Crizotinib.

Kinase										
parameter	C–MET	ALK	RON	AXL	TIE2	TRKA	TRKB	ABL	IR	LCK
% inhibition (1 μM)[a]	97.0	99.0	97.0	93.0	97.0	99.3	99.7	91.5	67.7	96.5
Enzyme IC$_{50}$ (nM)[a]	<1.0	<1.0	NA	<1.0	5.0	<1.0	2.0	24	102	<1.0
Cell IC$_{50}$(nM)[b]	8.0	20	80	294	448	580	399	1159	2887	2741

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Figure 10. Cell Cycle Regulation in Cancer.[39] With permission from BioRender.com.

and David Epel at *Stanford University*.[40] Taking into consideration previous work on the role of CDK1 and its requirement for cell division, the collaborators began with *in vitro* studies, and developed the radioactive kinase assay to identify kinase inhibitors.[41] Using Staurosporine as the control compound (Figure 11A), a non-specific kinase inhibitor that was available at the time, all initial *in vitro* tests with current chemotherapeutic agents were negative. It was only 6-dimethylaminopurine (DMAP) that was found to be a kinase inhibitor (Figure 11B), a purine base that was discovered and structurally identified as a Puromycin analogue, a potent inhibitor of mitosis in the sea urchin embryo, but it did not inhibit protein synthesis.[42] Further work confirmed that DMAP has an inhibitory potency on the M-phase. *In vitro* kinase assays on the maturation factor-CDK1/Cyclin B[43] proved the inhibitory effect of DMAP but were neither selective nor potent (55 μM and $IC_{50} = 120$ μM).[40]

Further work at the *Institute of Experimental Botany* in Olomouc in the Czech Republic in collaboration with the *Biological Station*, Roscoff in France, produced more than 30 kinase inhibitors for MAPK and CKD. After testing the compounds, Olomoucine[44] (Figure 11C) was found to have a higher potency than isopentenyladenine (Figure 11D). Additional col-
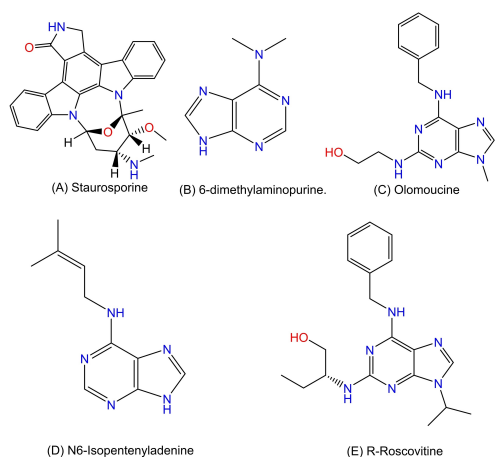
laboration between Michel Legraverend at the *Institute of Marie Curie* in Orsay (Institut Curie - Centre de recherche d'Orsay, France) and researchers at *MiroslavStrnad* in Olomouc (Czech Republic) on Olomoucine attempted to improve selectivity, and the compound Roscovitine was selected ($IC_{50} = 0.45$ μM) (Figure 11E). The synthesis of Roscovitine is described in Figure 12.

The studies showed that the (R)-enantiomer is about twice as potent as the (S)-enantiomer in inhibiting cdc2/Cyclin B (Table 7).

Using the compound Roscovitine, inhibition studies on cell growth were done *in vitro* and *in vivo*. For *in vitro* studies, the average IC_{50} was 16 μM (from 100 different cancer cell lines such as lung, breast, NB etc). When using the cell line L1210 it was found that the compound Roscovitine arrested the cells in the G2/M phases. *In vivo* studies of Roscovitine[45] in combination with ionising radiation, Erlotinib, and PIK-90[45] using single cancerous tissues from the colon, uterus, breast, sarcoma, lung, nasopharyngeal, and brain, showed anti-tumour activity.[45]

One clinical trial on Roscovitine was identified:
– NCT00999401 phase 1, started October 2009, completed December 2021. This was a study of oral Roscovitine and oral Sapacitabinen in patients with advanced solid tumours, including patients with NBS4.

4.4. Alisertib (MLN8237)

Aurora Kinases A or B are enzymes that are essential for normal cell division. The over-expression of Aurora Kinases A or B found in solid and haematological malignancies, leading to cell arrest and apoptosis, inspired scientists to search for inhibitors for Aurora Kinases A and B as an anti-cancer drug.[46] Work by Wang et al. in 1984[47] reported that Benzodiazepines (BZD) that bind at peripheral sites inhibit cell proliferation. The antiproliferative potencies of a series of 15 compounds were subsequently
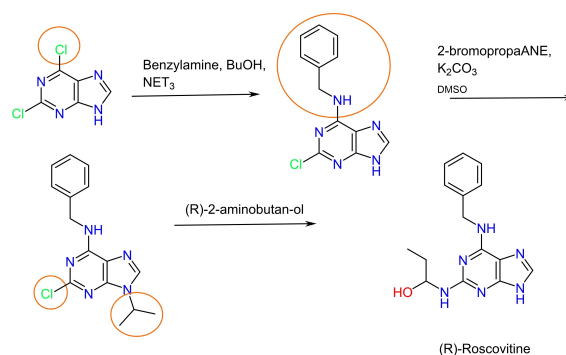

Figure 12. Synthesis of both (R)-Roscovitine and its (S)-enantiomer.[40]

Figure 11. The development of R-Roscovitine/Seliciclib.

Table 7. The IC_{50} for the Roscovitine *in vitro* assays with a 100 mM ATP.

CDK1/CyclinB	$IC50 = 2.69$ μM
CDK2/CyclinA	$IC50 = 0.71$ μM
CDK2/CyclinE	$IC50 = 0.10$ μM
CDK7/CyclinH	$IC50 = 0.49$ μM

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tested for the peripheral-type BZD and their binding constants, suggesting the possibility of involvement in the control of cell proliferation. Antiproliferative potencies have been observed in thymoma cells lines, as well as Swiss 3T3 cells, B103 and B104 NB cells, and Friend erythroleukemia cells.[47]

The discovery of Benzazepine BBL22 (Figure 13A) was reported by Xia et al.[48] and was found to induce arrest in the G2/M phase in cancer cells. Further research revealed that BBL22 inhibited Aurora Kinase A at micromolar concentration in enzymatic assay[48–49] with a log P of 4.1 and Aurora Kinase A ligand efficiency (LE) of 0.34. Sells et al., in 2015 using a high throughput screening for BBL22 with some modification of the aromatic ring, and substitution on the pyrimidine amine, produced a generation of compounds (Table 8), including Alisertib (compound 10). From these compounds, compound 7 showed better selectivity for Aurora A over Aurora B (33 nM). This was done by comparing phosphorylation of direct substrates, Aurora A autophosphorylation (pT288) and Aurora B phosphorylation of histone H3 (pHisH3) on Ser-10, in HCT116 cells. The compound's effect on cellular proliferation was measured by Bromodeoxyuridine (BrdU) incorporation assay.[49] Compound 7 was found to be stable in human S9 fraction, cell-permeable, and soluble as the sodium salt.[30]
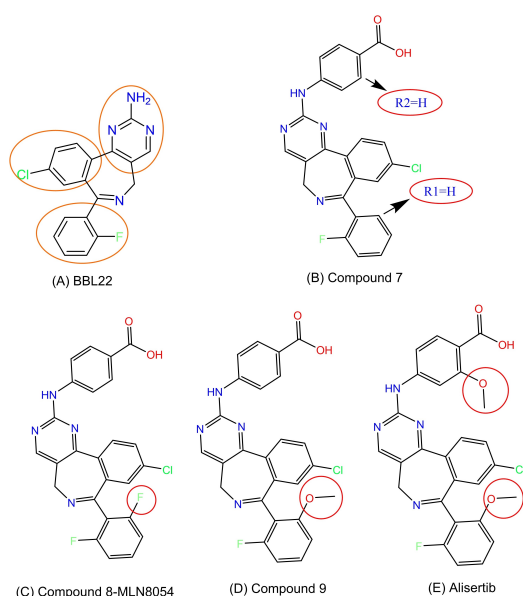
Optimisation of compound 7 using structured-activity relationship (SAR) lead to the development of other compounds, compound 8 (MLN8054) and compound 9 (Figure 14). Compound 8 was developed by adding fluorine at the R1 position with a 150-fold potency for Aurora A over Aurora B in HCT116 cells. Further screening on MLN8054 revealed a potential off-target binding activity and is the only one found with GABA$_A$ α-1 BZB (IC$_{50}$ = 330 nM). Compound 9 was produced by replacing the fluorine on the 7-phenyl ring with methoxy group and showed a three-fold brain area under the curve (AUC) reduction (Table 8). Further research lead to the development of compound 10 (Figure 14), Alisertib (MLN8237) that demonstrated favourable tumour growth inhibition in multiple human tumour xenograft models.[50] Maintaining valuable pharmacokinetic (PK) and physiochemical properties, Alisertib is also highly protein-bound (97%).[51] Furthermore, pharmacodynamic effects in tumour biopsies and skin, reflecting Aurora A inhibition, were observed at doses below the maximum tolerated dose.[46]

Tree clinical trials on Alisertib have been identified:
- NCT02444884 phase 1, started May 2015, completed February 2016.
- NCT01154816 phase 2 started 2010, completed May 2020.
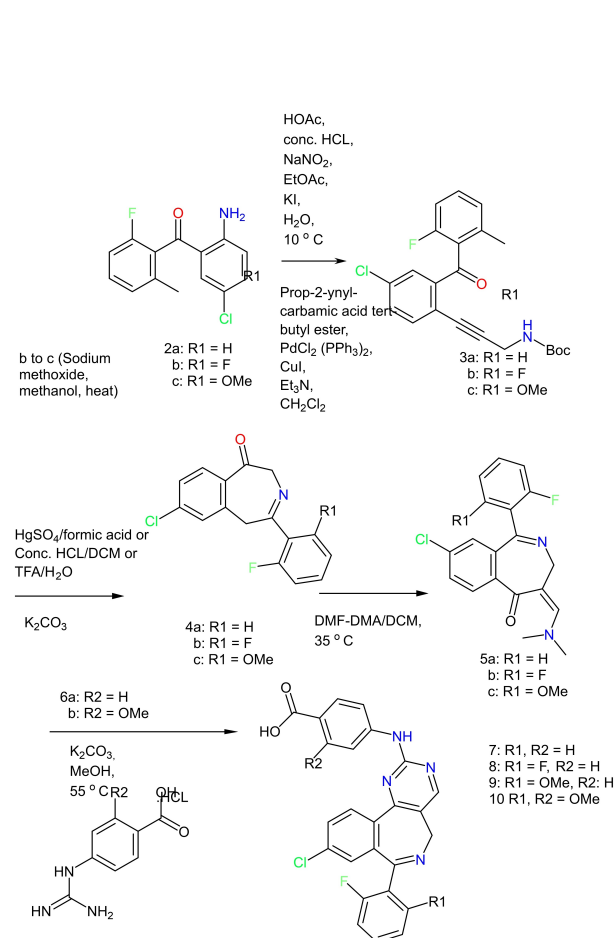- NCT01601535 phase 2 started May 2012, completed July 2019.

Figure 13. The development of Alisertib.

Table 8. Enzyme and cellular activity ([a]IC$_{50}$ in HCT116 cells. [b]GI$_{50}$ values).

Compound	AurA (nM)	pT288 (nM)[a]	pHisH3 (µM)[a]	HCT116 BrdU (µM)[b]
1	1700	6000	>10	11
7	33	170	>10	0.95
MLN8054 (8)	31	34	5.2	0.22
9	10	18	2.5	0.13
Alisertib (10)	1	7	1.5	0.03

Figure 14. Synthesis of compound 7 to compound 10.[49]

4.5. Sorafenib (BAY 43–9006)

The discovery of Sorafenib (ISIS 5132) was initiated by Kasid et al.[52] a compound found to inhibit the growth of human ovarian, breast, and lung xenograft in athymic mice.[52] ISIS 5132 was able to interrupt the Raf1 gene, offering a new target for cancer therapy. Further collaboration on ISIS 5132 between two pharmaceutical companies, Onyx and Bayer, confirmed the finding.[53] In 1994, Bayer and Onyx collaborated to discover novel Raf/MEK/ERK inhibitors and in 1995, produced 200,000 compounds using high-throughput screening (HTS) for Raf kinase inhibitory activity. These compounds were also tested as a targeted Ras–Raf–MEK–ERK pathway therapy. As a result, a lead compound 1 was found (Figure 15A) with a Raf1 IC_{50} of 17 μM.[54] This was followed by structured-activity relationship (SAR) studies that led to the development of Sorafenib.[55] Figure 15 summarises the chemical optimisation of Sorafenib.

Sorafenib was a potent inhibitor of Raf1 kinase in *in vitro* studies with $IC_{50} = 6$ nM. More X-ray crystallographic studies on Sorafenib showed the formation of a complex between *b-raf* V600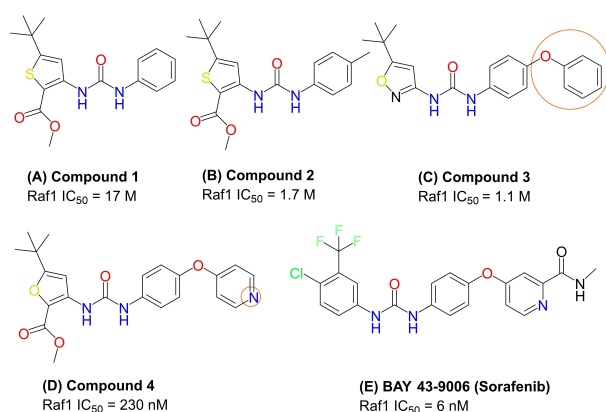E, wild-type B-Raf and Raf1[56] and its inhibitory ac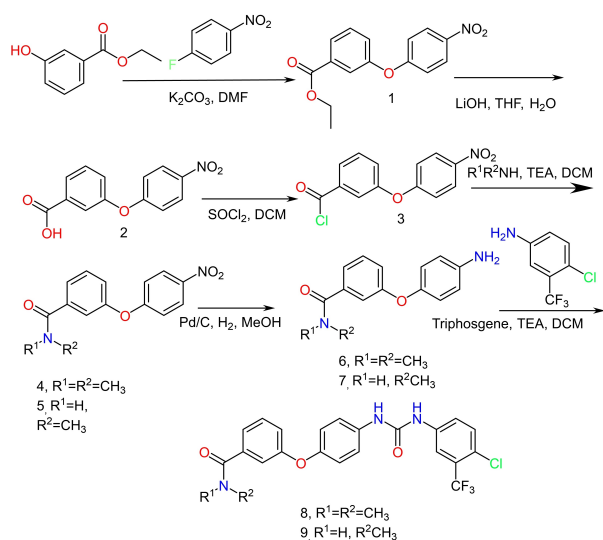tion on several receptor tyrosine kinases.[55] The development of Sorafenib took nearly 11 years, and was approved in 2005 by the Federal Drug Agency (FDA) for the treatment of advanced renal cell carcinoma (RCC).[55] The synthesis of Sorafenib is described in Figure 16.

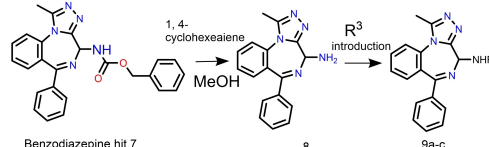There have been three clinical trials of Sorafenib:
- NCT01518413 phase 1 started January 2012, completed February 2015.
- NCT02298348 phase 1 started November 2014 and is currently active, but not recruiting in January 2023.
- NCT02559778 phase 2 started September 2014 and was still recruiting in September 2023.
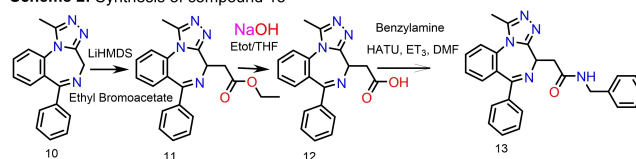
4.6. GSK525762/Molibresib (I-BET762)

In 2001 researchers managed to generate a stable human HepG2 hepatocyte cell-line containing a ApoA1 luciferase reporter.[57] This ApoA1 Luciferase reporter was then used to screen compounds capable of upregulating reporter gene activity.[57] Researchers using diversity and targeted screening approaches, discovered the lead compound benzodiazepine (BZD) hit 7 (Figure 17, scheme 1) with Apo A-1 Luc $EC_{170} = 0.22$ μM, Brd2/3/4 pIC_{50} 5.9/6.2/6.3.[58] From this, a medicinal chemistry program was developed to optimise the potency against ApoA1 upregulating assay. Schemes 1 to 7 of this program (Figures 17, 18, 19, and 20) are summarised below.[58]

Having synthesised the compounds through the seven schemes, researchers started to perform SAR studies. The first SAR was around the BZD ring RHS substituents (Figure 21,

(A) Compound 1
Raf1 IC_{50} = 17 M

(B) Compound 2
Raf1 IC_{50} = 1.7 M

(C) Compound 3
Raf1 IC_{50} = 1.1 M

(D) Compound 4
Raf1 IC_{50} = 230 nM

(E) BAY 43-9006 (Sorafenib)
Raf1 IC_{50} = 6 nM

Figure 15. The development of Sorafenib.

Figure 16. Synthesis of Sorafenib.

Scheme 1: Synthesis of compounds 7, 8, and 9a-c.

Scheme 2: Synthesis of compound 13

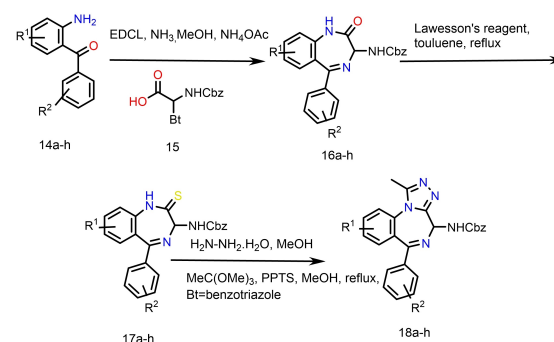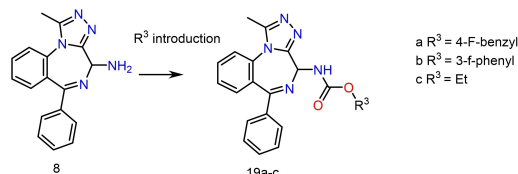**Scheme 3**: Synthesis of 14a-h, 16a-h, 17a-h, and 18a-h.

Figure 17. Schemes 1 to 3.

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Scheme 4. Synthesis of compound 19a-c.

Figure 18. Schemes 4 and 5.
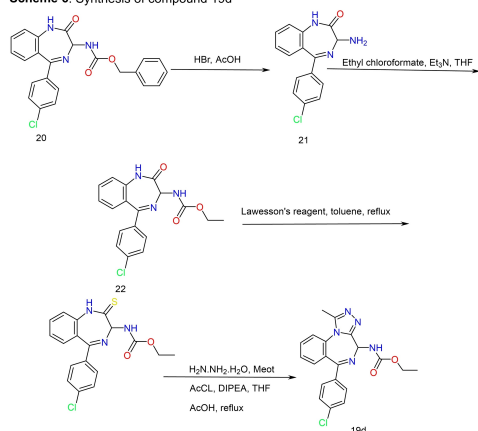
Figure 19. Scheme 6.

Figure 20. Scheme 7.

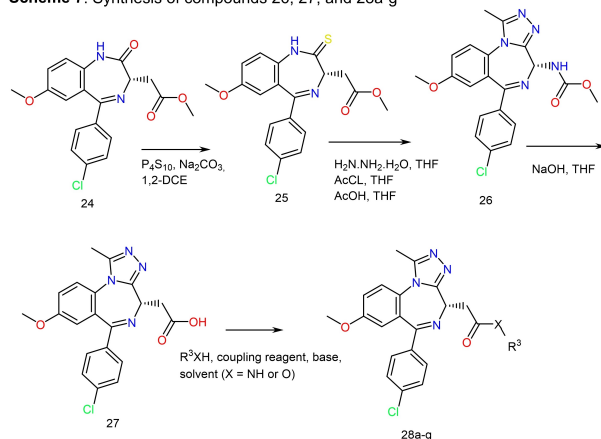compounds 7, 8, 9a, 9b, 9c, and 13). They also looked at the anti-inflammatory properties of the compounds by looking at the inhibition of IL-6 production after a Lipopolysaccharide (LPS) challenge in peripheral blood mononuclear cells (PBMC). It was found that compound 9b is much weaker than compound
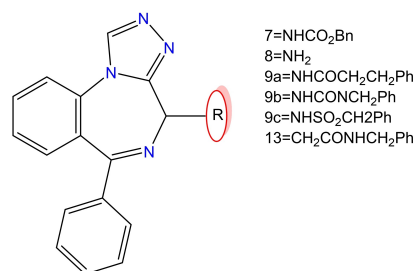

Figure 21. Compounds 7, 8, 9a, 9b, 9c, and 13.

7, which was put down to the higher solubility of the carbamate compared to urea.

Substituting the fused phenyl ring on the benzodiazepine, 8-ethoxy compound 18d showed the best potency (Figure 22).

Working on the *ortho*-chloro substituent and the methoxy substituent in the cell assay, it was found that 18f and 18 g (Figure 22) had the same potency and that the *para* position of the pendant phenyl ring tolerates most substitutions. This result showed that the carbamate functioned best with the linker for the benzodiazepine ring on position 3. The next step was to work on the physicochemical properties (Figure 23).

Reducing the lipophilicity (clogP) or the molecular weight (MM) led to a drop in potency. The Bromodomain (BRD) affinity study showed that (−)-19c had no significant activity, and (+)-19c was active. Also, testing compound 19d in the central Gamma-aminobutyric acid (GABA) receptor binding assay was found to be inactive, which meant that the pendant ring plays an essential part in the selectivity of the GABA receptor. This led to further SAR studies on the amide linker in compound 13 (Figure 24).

Compound 1 (R1 = 8-OMe, R2 = 4Cl, X = NH, and R3 = Ethyl), BET762; BRD4 $pIC_{50} = 6.2$, PBMC (IL-6) $pIC_{50} = 6.5$, and clogP = 2.4, was selected for its good properties.

Compound	R1	R2
7	H	H
18a	N	H
18b	8-Cl	H
18c	9-Me	H
18d	8-OMe	H
18e	H	2′-OMe
18f	H	3′-OMe
18g	H	4′-OMe
18h	H	4′-Me
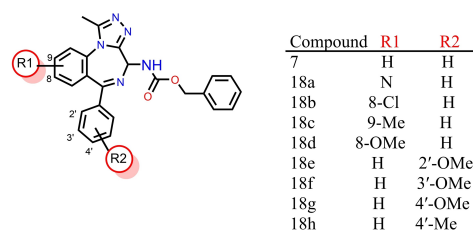
Figure 22. Substitution of R1 and R2.

Compound	R2	R3
7	H	Bn
19a	H	4F-Bn
19b	H	3F-Ph
19c	H	Et
(−)-19c	H	Et
(+)-19c	H	Et
19d	4′Cl	Et
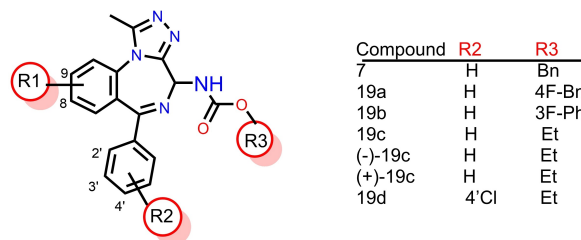
Figure 23. Substitution of R2 and R3 for optimisation.

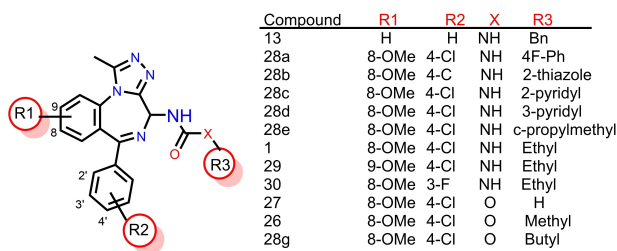| Compound	R1	R2	X	R3
13	H	H	NH	Bn
28a	8-OMe	4-Cl	NH	4F-Ph
28b	8-OMe	4-C	NH	2-thiazole
28c	8-OMe	4-Cl	NH	2-pyridyl
28d	8-OMe	4-Cl	NH	3-pyridyl
28e	8-OMe	4-Cl	NH	c-propylmethyl
1	8-OMe	4-Cl	NH	Ethyl
29	9-OMe	4-Cl	NH	Ethyl
30	8-OMe	3-F	NH	Ethyl
27	8-OMe	4-Cl	O	H
26	8-OMe	4-Cl	O	Methyl
28g	8-OMe	4-Cl	O	Butyl

Figure 24. SAR around the BZD Acetamide Template.

As a consequence of these results, testing began on primates, including dogs, rats, and mice. The data on blood clearance, blood distribution volume, $t_{1/2}$, oral bioavailability, solubility, and permeability and in vivo studies demonstrated that BET762 can be used as an anti-cancer drug.[57]

Two clinical trials on GSK525762 were identified:
– NCT01587703 phase 1 started April 2012, completed March 2020.
– NCT03925428 phase 1 started April 2019, withdrawn September 2020.

4.7. Trametinib (GSK1120212)[50]

The work by Abe et al, 2011 led to the discovery of Trametinib.[59] The team started with compound 2 (Figure 25B), a compound that expressed the cyclin-independent kinase (CDK) 4/6 inhibitor p15^{INK4b} during a high-throughput screening programme.[59] Compound 2 was also illustrated to have antiproliferative activity against HT-29 (colorectal adenocarcinoma) and human cancer cell lines ACHN (renal adenocarcinoma) with IC$_{50}$ values of 990 and 4800 nM, respectively.[59] Optimisation of compound 2 to improve its antiproliferative activity led to synthesising the orally bioavailable GSK1120212 (JTP-74057DMSO solvate) compound 1 (Figure 25A).[59] In a diverse
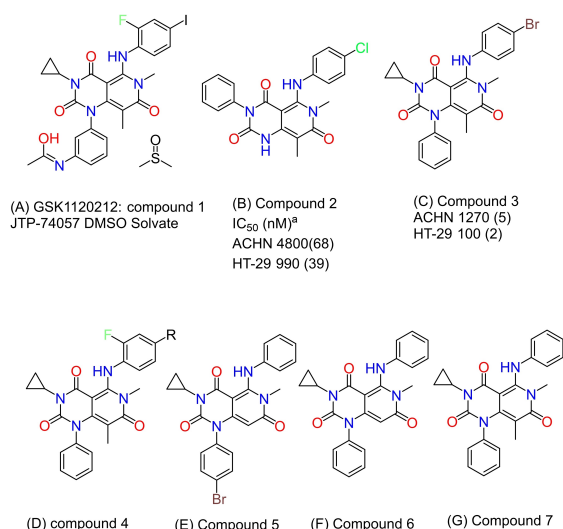
BRAF of mutant cancer cell lines, the compound exhibited selective inhibition of proliferation. In addition, molecular targeting revealed that the compound is selective and a highly potent inhibitor of MEK1/2.[59]

Compound 2 was not the best option for optimisation as it has high hydrophobicity (ClogP 6.3) due to its three aromatic rings. Optimisation started by placing an alkyl group instead of each aromatic group, to reduce the hydrophobicity. By inserting at the 3-position of the pyridopyrimidine core, a benzene ring with a cyclopropyl ring, and a chlorine atom on the aniline ring with bromine, showed an improved potency (4-fold) with a reduction in the hydrophobicity (ClogP 5.0). In the in vivo model, compound 3 (Figure 25C) showed some anti-cancer effects in the nude mouse HT-29 xenograft model.[59] However, improved potency (4-fold) was observed when placing a fluorine atom at the 2-position of the aniline ring with a slight increase in hydrophobicity (ClogP 5.2) (Table 9).

Further optimization resulted in a potency improvement of 500 folds, but the compounds in Table 9 were unstable under weak basic conditions.[59] Additional optimization led to the production of compounds 5, 6, and 7 (Table 10).

Figure 25. The development of Trametinib.[59]

(A) GSK1120212: compound 1 JTP-74057 DMSO Solvate

(B) Compound 2 IC$_{50}$ (nM)^a ACHN 4800(68) HT-29 990 (39)

(C) Compound 3 ACHN 1270 (5) HT-29 100 (2)

(D) compound 4

(E) Compound 5

(F) Compound 6

(G) Compound 7

Table 9. Substitution at the 4-Position of Aniline SAR.[59]

	R	IC$_{50}$ (nM) ACHN	IC$_{50}$ (nM) HT-29
4a	H	10000 (1)	1300 (1)
4b	Me	1300 (2)	110 (2)
4c	Et	185 (3)	27 (3)
4d	Pr	2100 (2)	230 (2)
4e	cPr	290 (2)	39 (2)
4f	Vinyl	104 (3)	15 (3)
4g	C≡CH (ethynyl)	42 (2)	5.9 (2)
4h	C≡C–Me	>3000 (1)	>1000 (1)
4i	CN	>10000 (1)	1600 (1)
4j	F	>3000 (1)	>1000 (1)
4k	Cl	>4600 (2)	102 (2)
4l	Br	325 (4)	15 (4)
4m	I	13 (2)	1.5 (2)
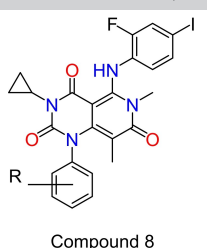
Table 10. IC$_{50}$ Results from compounds 3, 5, 6 and 7.

Compound	ACHN IC$_{50}$ (nM)	HT-29 IC$_{50}$ (nM)
3	1270 (2)	100 (2)
5	>3000 (1)	>1000 (1)
6	>3000 (1)	>1000 (1)
7	830 (2)	135 (2)

Compound 5 (inactive) was produced after treating compound 3 with potassium carbonate in methanol/chloroform. The highest potency was achieved (compound 8) by adding 2-F,4-I aniline moiety in the same substitution fashion as 4 m (Table 11).

When comparing the compounds in Table 11, compound 8a showed a low IC_{50} value and a low solubility with a ClogP of 6.0. Further optimisation to improve potency focused on replacing the phenyl ring at the 1-position with methanesulfonamide, and acetamide analogues showed that the best candidate for further studies was compound 8b (JTP-74057). Abe et al., 2011 found compound 8b to have poor oral exposure, a weak base and was unsuitable for stable salt formation.[59] However, two solvates, DMSO and acetic acid, were found, and showed similar oral bioavailability to compound 8b. Due to the better solid-state properties of DMSO solvate 1, it was selected for further
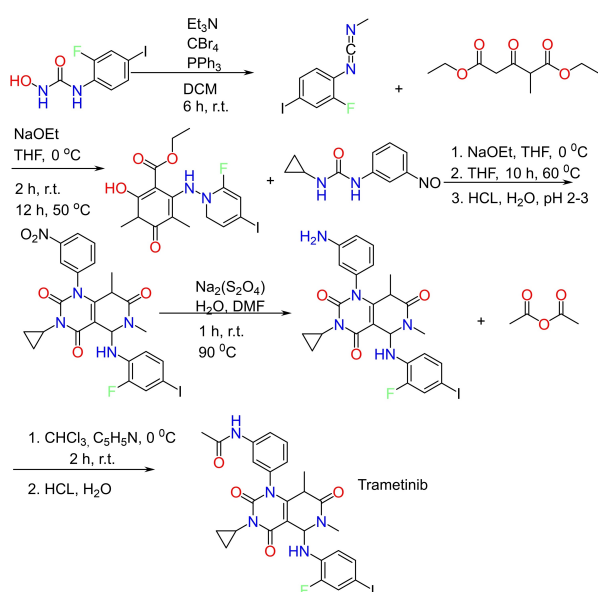
development. Further testing of GSK1120212 (JTP-74057 DMSO Solvate) on KRAS-mutant HCT116 and BRAF-mutant SKJ-ML-28 cells, caused dose-dependent inhibition of ERK1/2 phosphorylation and dose-dependent inhibition of growth.[59] The synthesis of Trametinib is described in Figure 26.[60]

One clinical trial of Trametinib was identified:
– NCT03434262 phase 1 started February 2018, completed November 2023.

To conclude this section Figure 27 represents a summary of the seven drug's interactions with their receptor protein. Understanding the properties of each binding may help identify possible multi-target drugs.

Since 2003 a total of 19 drugs aimed at treating NBS4 have made it to clinical trial. This review selected seven of the 19 drugs (Table 5), none of which progressed beyond phase two clinical trial for NBS4. The seven drugs represent three common subgroups used for investigating NBS4 treatments along with a summary of the medicinal chemistry approach to drug discovery in each of the three groups. The three common subgroups include A-MYC driven (GSK525762, Roscovitine, Alisertib); B-Telomere aberrations (Sorafenib) and C-ALK (Crizotinib, Gefitinib, Trametinib). With none of the seven drugs progressing beyond phase two, it is clear that more research is needed.

4.8. Immunotherapy as Therapeutic Treatment for NBS4

With the lack of success using therapeutic agents, immunotherapy, which has been successful in the treatment of several cancers, is currently used in the treatment of NBS4. Using monoclonal antibodies this treatment has led to an improved 5-year survival rate of NBS4 from below 40%[62] up to 50%.[20,25] Monoclonal antibodies are not drug-based treatments but are part of cancer immunotherapy, and Dinutuximab (UNITUXIN), is an example of this approach. Dinutuximab was approved by
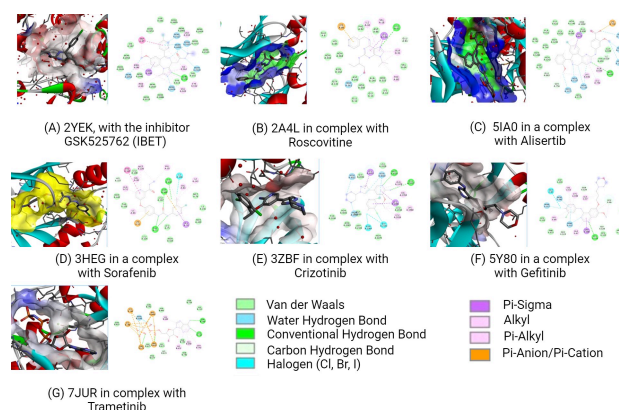
Table 11. Introduction of 2-F,4-I aniline moiety.

Compound 8

Compound	R	ACHN IC_{50} (nM)	HT-29 IC_{50} (nM)
8a	H	25 (2)	1.7 (2)
8b	3-NHCOMe	9.8 (5)	0.57 (10)
8c	4-NHCOMe	195 (2)	14 (2)
8d	3,5-diNHCOMe	1000 (1)	>100 (1)
8e	3-NHSO2Me	6.4 (5)	0.52 (5)
8f	4-NHSO2Me	22 (5)	1.4 (5)

Figure 26. Synthesis of Trametinib[60]

(A) 2YEK, with the inhibitor GSK525762 (IBET)
(B) 2A4L in complex with Roscovitine
(C) 5IA0 in a complex with Alisertib
(D) 3HEG in a complex with Sorafenib
(E) 3ZBF in a complex with Crizotinib
(F) 5Y80 in a complex with Gefitinib
(G) 7JUR in complex with Trametinib

Van der Waals · Water Hydrogen Bond · Conventional Hydrogen Bond · Carbon Hydrogen Bond · Halogen (Cl, Br, I) · Pi-Sigma · Alkyl · Pi-Alkyl · Pi-Anion/Pi-Cation

Figure 27. A summary of the seven selected drugs' interactions with their receptors. Where (A) 2YEK is the crystal structure of the first bromodomain of human Brd2 (2YEK) with the inhibitor GSK525762 (IBET). (B) 2 A4 L Human cyclin-dependent kinase 2 in complex with Roscovitine. (C) 5IA0-Crystal Structure of Ephrin A2 (EphA2) Receptor Protein Kinase with alisertib (MLN8237). (D) 3HEG- P38 in complex with Sorafenib. (E) 3ZBF Complex with Crizotinib. (F) 5Y80- Complex structure of cyclin G-associated kinase with Gefitinib, and (G) 7JUR in complex with Trametinib. Created using Biovia Discovery Studio[61] and produced by BioRender.com.

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the FDA for patients with NBS4 in 2015 in the US and is used in combination with other therapeutic agents to improve survival rates for this group.[25]

There are two main strategies of cancer immunotherapy, active and passive.[63] Active cancer immunotherapy aims to harness the host's immune system to attack cancer cells, whereas passive immunotherapy delivers tumour antigen-specific monoclonal antibodies to kill cancer cells directly or through complement dependent cell-mediated cytotoxicity (ADCC). Immunotherapy for paediatric malignancies has mostly focused on targeting nonmutated antigens with a differential expression on malignant versus normal cells.[64] Cell surface antigens, the most commonly targeted molecules in NB immunotherapy, are not major histocompatibility complex (MHC)-restricted and are optimal for targeting by monoclonal antibodies (mAbs) or engineered immune effector cells. Intracellular tumour antigens usually require processing by antigen-processing cells for presentation to cytotoxic lymphocytes and are MHC-restricted. mAbs can target intracellular antigens if they internalize after binding. Tumour antigens targeted for immunotherapy in patients with NB thus far are chiefly expressed on the cell surface, the gangliosides GD2 being the most common with GD2 mAbs Dinutuximab being an example of this.[65]

Gangliosides are glycosylated lipid molecules containing sialic acid residues in their carbohydrate structure.[66] In normal
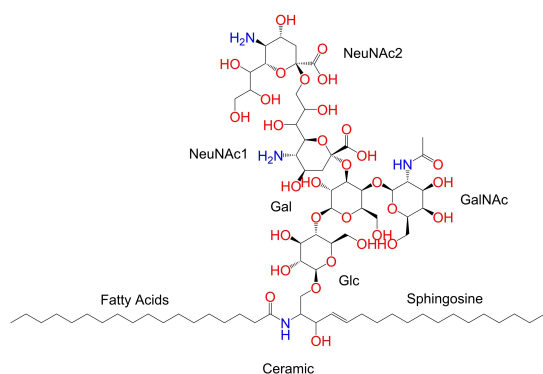

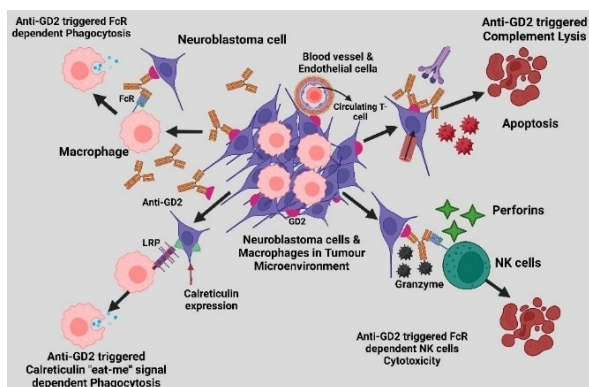**Figure 28.** Structure of GD2 Ganglioside.[67]

Figure 29. Neuroblastoma tumour microenvironment and cytotoxic action induced by anti-GD2.[71] Reproduced from [73] and recreated with BioRender.com. https://www.nature.com/articles/nrm2510

cells, GD2 (Figure 28) is not expressed or only to a low extent, and is largely limited to neuron, skin melanocytes, and peripheral pain fibers. In contrast, it is highly expressed in tumour cells,[67] and therefore, it has become an attractive target for cancer immunotherapy and NBS4 in particular.[68] A feature seen in NBS4 is the GD2 expression in neural progenitor cells[69] (Figure 29) and various types of stem cells.[70]

Since 2018 in the UK, the drug regulator NICE has approved the use of Dinutuximab, a chimeric monoclonal antibody produced in the Chinese hamster ovary cell line that targets GD2, a glycolipid overexpressed in certain tumours such as NB.[19] It is administered intravenously and has been studied in clinical trials as a single agent, as well as in combination with isotretinoin with or without aldesleukin (also known as interleukin 2) in people between the ages of 1 month and 21 years with high-risk NB who received myeloablative therapy and autologous stem cell transplant.[19]

One clinical trial on Dinutuximab was identified:

– NCT05421897 phase 4 started October 2022, with estimated completion 2024.

Despite combining monoclonal antibodies with therapeutic agents, the survival rate for NBS4 remains at 40 to 50%[20] indicating other molecular targets or target therapies need to be considered.[62] A further difficulty with cancer immunotherapy is cost. In the UK, which operates a universal tax-based health care system, the drug regulator NICE, approved use of Dinutuximab in August 2018 with the following caveat: "Dinutuximab beta does not meet NICE's criteria for a life-extending treatment at the end of life. Also, the range of cost-effectiveness estimates presented is higher than what NICE usually considers a cost-effective use of NHS resources. But taking into account the uncaptured health-related benefits, the rarity and severity of the disease, and the potential lifetime benefit for children with neuroblastoma, Dinutuximab beta can be recommended for high-risk neuroblastoma."[19]

In the next section, we review recent work on other possible targets including histone deacetylase inhibitors (HDACIs), and hedgehog inhibitors (HHIs). The section will conclude with a review of retinoic acid derivatives (RADs) which are currently in use for the treatment of NBS4.

5. Recent Research on NBS4

5.1. Histone Deacetylases and Histone Deacetylase Inhibitors (HDACIs) in NBS4[72]

What are histone deacetylases (HDACs)? According to Bolden et al. in 2006 the HDAC family comprises 18 enzymes divided into four classes according to their enzymatic activities, subcellular localisation, and homology to yeast HDCA.[73] Class I HDACs are found in the nucleus and are involved in the transcriptional repression of several genes that contain a deacetylase domain and share homology with the yeast protein RPD3. Class II shows homology to yeast Hda1 and is divided into class IIa and IIb which are located in the nucleus/cytoplasm. Class III, known as sirtuins (SIRT 1–7), are based on NAD$^+$

cofactors and are homologues of the yeast protein Sir2. Finally, class IV is the HDAC family's smallest isoform and shares class I and class II features.[73–74]

Why HDACs? Historically, the search started in 2009, when HDAC 8 were found to be downregulated in NBS4.[75] The development of two compounds by Heimburg et al. in 2017 showed that the two compounds, 8b and 20a (Figure 30), are potent and selective inhibitors of HDAC 8 with $IC_{50} = 69$ nM and $IC_{50} = 27$ nM, respectively.[76]

In another study by Thole et al. in 2017 it was found that NBS4 cells depend on HDCA 11 for mitotic cell cycle progression and survival of MYCN-amplified NB cells, suggesting that HDCA 11 could be a valuable drug target.[77] In Germany, Körholz et al. in 2021,[78] showed that the broad-spectrum HDACs, Panobinostat and Vorinostat (Figure 31) induce autophagy in NB cells at the transcriptional level, and they were able to sensitise NB to treatment with HDACs, in vitro and in vivo.[78] Deacetylation of HDAC 8 along with its inhibition is shown in Figure 32

Four clinical trials of HDACs and NBS4 have been identified:
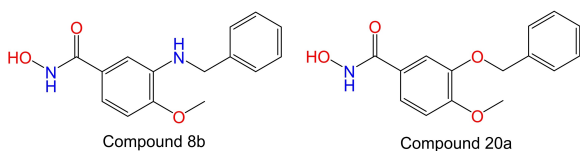– NCT03332667 phase 1 began September 2018, still active;
– NCT01208454 phase 1 began 2010, completed 2014. Vorinostat (HDAC) in combination 13-Cis-retinoic acid (RA);
– NCT04308330 phase 1 began 2017, still recruiting.
– NCT02035137 phase 2 began 2014, completed 2021.

5.2. Inhibitors of the Hedgehog (HH) Signalling Pathway

According to Peukert et al. Hedgehog Inhibitors (HHIs) have become a promising new target for cancer therapy.[79] The signalling pathway was discovered in 1980[80] and was an essential regulator of growth, patterning formation and cell migration during embryonic development.[81] The components of the HH signalling pathway are involved in signalling to the Gli transcription factors. Models of HH pathway activation in cancer are demonstrated in Figure 33.[82] Another study by Goodrich et al. in 1998 found that deregulation of the HH signalling was observed with Gorlin syndrome and cancers.[83] In 2018, Skoda et al. reported that HH signalling contributes to one-third of all malignant tumours.[82]

Three types of mechanisms of HH signalling activation in cancer were proposed by Rubin et al. in 2006:[84] Type I is an autonomous and ligand-independent type of HH signalling. This type is caused either by the inactivating mutations in *Ptch1* or *Sufu* or by the activation mutations in SMO, which results in the activation of HH signalling in the absence of ligand.[85] Type II is a ligand-dependent oncogenic HH signalling in an autocrine/juxtacrine manner that has been observed in a number of studies.[86][87][88] Type IIIa/b is a ligand-dependent HH signalling in a paracrine or reverse paracrine manner. It was found that HH ligands secreted by tumour cells were associated with various types of cancer.[89][90][91] Recent studies show that apart from mutations, the HH signalling pathway can also be disrupted by epigenetic changes.[92] More than 50 inhibitors of the HH signalling pathway have been identified.[82]

Three clinical trials of HH signalling and NBS4 have been identified:
– NCT03434262 phase 1 began 2018, still active;
– NCT01125800 phase 2 began 2011, completed 2014;
– NCT05199584 phase 2 began 2022, still active.
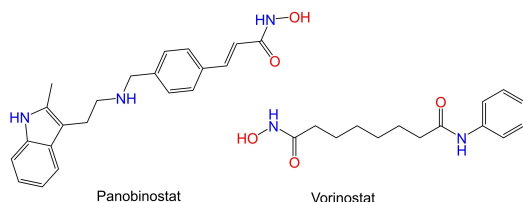

Figure 30. compounds 8b and 20a.[76]

Figure 31. Structure of Panobinostat and Vorinostat.[78]

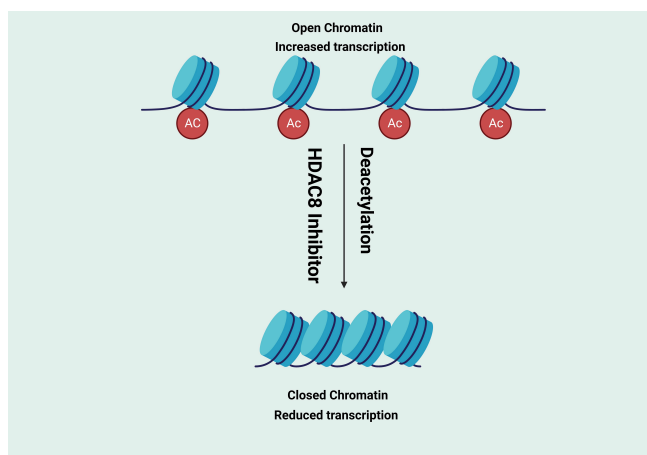**Figure 32.** Deacetylation by histone deacetylase 8 (HDAC8) along with inhibition of HDAC8 by HDAC8 inhibitor. Created with BioRender.com.
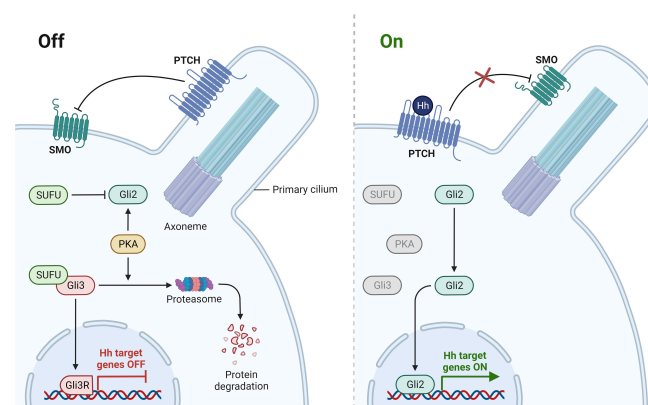

Figure 33. Models of HH pathway activation in cancer. Created with BioRender.com.

5.3. Retinoic Acid Derivatives (RADs)

Retinoic acid derivatives (RADs) act as ligands for RA nuclear receptors (NRs) and transfer them from transcriptional repressors to activators.[93] RAs are involved in maintaining the differentiation state of neural stem cells and adult neurons.[94] In the case of NB, research has demonstrated that the clonal human NB cell line, SH-SYFY, differentiates and expresses neural markers upon exposure to RA.[95] RA exists in various isomeric forms: 13-cis-retinoic acid (13cisRA), all-trans retinoic acid (ATRA), and 9-cis-retinoic acid (9cisRA).[96] RA functions through its two receptors, Class 1: Retinoic Acid Receptors (RAR) and Class 2: Retinoic X Receptors (RXR), each class containing three different subtypes-α, β, and γ.[97] Interestingly, the RARβ expression gene is lost in various tumours during early development. Several studies have demonstrated its unique physiological role as a regulator of retinoid-induced inhibition of cell proliferation and a tumour suppressor protein in multiple cancers.[98][99][100][101]

According to Bayeva et al. the use of 13-cis RA, also known as isotretinoin, or retinoic acid (RA) isoform, is used in NB therapy; however its efficacy is restricted to maintenance therapy of minimally residual disease.[101] Studies have looked into RA derivatives that may have an enhanced anti-NB effect or have investigated possible synergies between RA and other drug classes like immune modulators, cellular process mediators, and epigenetic modifiers.[101] In the study by Bayeva et al, in 2021, they attempted to systematically review retinoic acids, their derivatives and synergistic interactions in NB, collecting data from 1980 to 2020. The study concluded that more studies need to be conducted *in vivo* to test the signalling cascade effector drugs in combination with other anti-NB drugs in animal models and 2D cell cultures.[102] Some examples of RA metabolites tested in NB cell lines are Fenretide, 9-cisUAB30, 6-Methyl-UAB30, LG153, LG69, CD437, Ro 13–6307, RA-Triazolyl derivatives, Geranylgeranoic acid, All-trans-retinoyl β-glucuronide, TTNB, N-(4-hydroxyphenyl) amido, and 4-hydroxybenzyl.[101]

One clinical trial of RA used in first line treatment (that is, disease that is not refractory/recurrent) of NBS4 has been identified:

– NCT01208454 phase 1 began 2010, completed 2014. Vorinostat (HDAC) in combination 13-Cis-retinoic acid (RA).

6. Future Work on NBS4: A Multi-Targeted Approach

Developing a multi-functional drug was discussed by Morphy et al. in 2004[103] and is described as taking "a primary target that is well validated for a given disease and adding secondary activities, to enhance efficacy and reduce side effects." To design a drug of that nature, according to Li et al. in 2021, involves combining two or more distinct inhibitory activities of two or more drugs, into a single molecule.[104] The hope is that such a drug will have better therapeutic effect, less chance of drug-drug interaction, more predictable pharmacokinetics (PK), pharmacodynamics (PD), and could be a novel approach to lowering the prevalence of drug resistance.[104]

Ramsay et al. in 2018 suggest that the tradition of designing drugs with the aim of targeting a biological entity, usually a protein, "with high selectivity to avoid any unwanted effects arising from mis-targeting other biological targets" have been demonstrated as inadequate to achieving a therapeutic effect, particularly in complex diseases.[105] Since 2017 drug discovery has increasingly focused on the concept of multi-target drugs to improve specificity of the drug on the target to achieve better outcomes (the term multi-target is now more commonly used, replacing the term multi-functional). A range of multi-target drugs are already available on the market including drugs for the nervous system as well as multi-target drugs for treating schizophrenia and major depressive disorders.[105]

In the case of cancer, where several aberrant proteins and pathways concur to initiate tumour growth and also facilitate progression, the FDA in the US have approved multi-target drugs including Lenvatinib, a reversible muti-tyrosine kinase receptor inhibitor approved for the treatment of radioiodine-refractory thyroid cancers. The drug Lenvatinib targets growth factor receptors (GFR) and has five targets: 1) it modulates the activities of vascular endothelial growth factor receptors (VEGFR); 2) 1–3, fibroblast growth factor receptors (FGFR) 1–3; 3) RET; 4) mast/stem cell growth factor receptor kit (SCFR), and 5) platelet-derived growth factor receptor (PDGFR) beta, all implicated in pathogenic angiogenesis, tumour growth, and cancer progression.[105] Other examples include Midostaurin, which is used to treat adult patients with newly diagnosed acute myeloid leukaemia who have a particular variant of the FLT3 gene and Palbociclib, Abemaciclib, and Ribociclib as breast cancer therapy.[105] Designing multi-target drugs is not without difficulties and Ramsay et al. have outlined some of these including the need for: 1) a strong understanding of adverse event profiling, 2) target-disease associations, and, 3) pathway-target-drug-disease relationships. The potential for synergistic or additive effects from varying the selected targets should guide the decision.[105]

According to Savelieff et al. in 2019.[106] the design of multi-target molecules[96] fits into two categories that are connected: *library screening* and *rational design*. Library screening is a collection of stored chemicals usually used in high-throughput screening (HTS) or industrial manufacture, for identifying novel drugs (hits). These HTS hits are then combined by rational design to produce multi-target molecules. Rational design is based on combining two or more compounds with a known target and properties, into one single molecule and can be achieved in three different ways: by linking, fusing, or merging the compounds. However, the disadvantages of rational design are the large molecular size, the loss of some pharmacological properties (bioavailability and blood-brain barrier permeability), and the reduction in biological activity and specificity of the original scaffolds.

In the case of NBS4, a multi-target drug approach, similar to the one suggested by Savelieff et al.[106] offers a possible way forward in the search for an effective treatment. Having

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investigated a range of drug targets for NBS4 from a medicinal chemistry perspective, this review has demonstrated a good understanding of the targets and binding modes already operationalised within the field. The section below proposes how this could be achieved using some of the programs freely available to academics. The following method is suggested:

Utilising OpenEye Scientific programs that come with different applications and can be used at various stages to achieve multi-target compound/s, it is suggested the following steps be taken:

- Having identified the receptor's binding mode, use the SiteHopper application[107] to compare the binding mode between proteins. This would help in selecting the correct receptor for the docking studies.
- MakeReceptor application to prepare the receptor for docking.
- BROOD application[108] to help search for analogous molecules to help find a lead molecule by changing a part or parts of the molecules, with the new compounds having some desired properties slightly different from those of the lead compound. In BROOD, the user can combine two or more compounds into a single molecule. This can be done through linkage, fusion, and incorporation. Taking the new compound/s and running each separately in BROOD, the application creates bioisosteric compounds with similar shapes and electrostatics but potentially unique connectivity and chemistry. Fragments and scaffold couplings are derived within the framework of a known chemical space, either user-specific or generally related to medicinal chemistry. The druglike preset filter sets the property filters to the following ranges: Synthetic Accessibility $= \geqslant 0.1$, Heavy Atom Count $< = 30$, Molecular Weight $< = 500$, Rotor Count $< = 10$, LogP $< = 5.0$, Polar Surface Area $< = 150$, Lipinski Donor Count $< = 5$, Lipinski Acceptor Count $< = 10$, Lipinski Failures $< = 1$, ABS $> = 0.25$, Fraction sp3 Carbons $> = 0.3$, Aromatic Freres Jacques $< = 4$, Egan egg is not required, and Veber bioavailability is not required. The programme will produce a hitlist that can be viewed;
- Having identified a hitlist, new compounds, an OEDocking suite of programs (FRED,[109] HYPRID,[110] and POSIT[111]) and tools that dock small molecules into a protein receptor, the user will be able to select the most promising ones;
- Another available tool is the AFITT[112] application, a crystallographic tool for accurately placing small molecules in the density of real space. The AFITT GUI also has an interface to external refinement programs and refinement validation tools, including real-space correlation coefficient calculation (RSCC) and interactive Ramachandran plots.
- Selected compounds can be tested *in vitro* and *in vivo* on a single molecule basis.[113]

7. Summary and Outlook

This review has highlighted the need to find a treatment for high-risk NB that increases survival rates beyond 40 to 50%. In the UK approximately 100 children are diagnosed with NB each year and it is the most common cancer in the first year of life, accounting for approximately one fifth, to one quarter of all cancers in this age group. NB is often present at birth with the natural history of NB ranging from highly malignant tumours to biologically benign variants that regress without active treatment, the prevalence of the latter being inversely related to age.[18] According to Chamberlain[18] in 1994 "biological markers of the disease, including ploidy, chromosome 1p deletion, and NMYC amplification, performed within the same patient at different times, indicate that malignant potential does not progress over time." The distribution of these markers in cases detected by initial screening in those under one year, shows that they are characteristically tumours with a good prognosis, unlike interval cases that have not been detected. Because initial screening differentially picks up tumours that are least likely to progress, and at the same time, fails to detect tumours that lead to poor outcomes, the UK National Screening Committee in 2015 has not recommended universal screening for NB. The screening of unborn children has also been discussed in this review. In light of the genetic abnormalities associated with NB, including gains of whole chromosomes and large-scale chromosomal imbalances, the similarities between DS and NB, in this respect, have been noted. With chromosome 17q gain found to be the parameter for poor outcome according to Inomistova et al. this finding has significant implications for obstetric ultrasound screening and the identification of those with high-risk disease.

For all of these reasons, there is an urgent need to find a more successful treatment for high-risk NB and improve survival rates. While improved outcomes associated with Dinutuximab (mAb), are encouraging, advances in medicinal chemistry and the focus on developing multi-target drugs with multiple efficacy producing actions, should be considered as the way forward in the treatment of NBS4. This review has considered a number of biological entities that have been used for targeted cancer treatment and assessed in clinical trials. Since 2003, a total of 19 drugs aimed at treating NBS4 have made it to clinical trial. This review selected seven of the 19 drugs (Table 5) according to the three most common subgroups used for investigating NBS4 treatments, along with a summary of the medicinal chemistry approach to drug discovery in each group. With none of the 19 drugs progressing beyond phase two clinical trial, it is clear that further work is urgently needed.

The review also pinpointed targets that are increasingly the focus of NBS4 research including: inhibitors of the Hedgehog (HHIs) signalling pathway, histone deacetylases and inhibitors of histone deacetylases (HDAIs) and retinoic acid derivatives (RADs). To enhance efficacy in drug treatment, multi-target drugs are increasingly the focus of research.[103] Despite an ongoing trend identified by Morphy et al. in 2004, of "the deliberate and rational design of ligands that act on multiple targets [and] by the substantial increase over the past few years in the number of publications describing such approaches" it is only since 2017 that multi-target drug development has accelerated. However, the authors of this review found no evidence of a similar approach being adopted for NBS4. Having reviewed the process used by Savelieff et al. in their search for a

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therapeutic drug for neurodegenerative diseases, this review advocates a similar approach be adopted for NBS4. On examining B-Telomere maintenance/elongation aberrations, one of the examples reviewed, Sorafenib, showed some promising results and is a strong contender to be the lead compound in the pursuit of a multi-target drug to be used in the treatment of NBS4. C-ALK Mutation/Amplification also showed promising results in the form of Crizotinib and could also be used as a lead compound. Using these examples and others, the outcomes to be pursed would include: an improved therapeutic effect resulting in improved survival rates, less chance of drug-drug interaction, and a lowered prevalence of drug resistance.

Abbreviations

EGFR   Epidermal growth factor receptor
GPCR   G-protein Coupled Receptor
HDACI   Histone deacetylase Inhibitor
HHI   Hedgehog Inhibitor
HTS   High Throughput Screening
ICR   Ion Channel Receptor
mAb   Monoclonal antibody
NB   Neuroblastoma
NBS4   Neuroblastoma Stage 4
NR   Nuclear Receptor
PKA   Protein Kinase A
PM   Plasma Membrane
RAD   Retinoic Acid Derivative
TK   Tyrosine Kinase
TKR   Tyrosine Kinase Receptor

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Conflict of Interests

The authors, Amgad Gerges and Una Canning are husband and wife. Their daughter, Isabella, was diagnosed with NBS4 in January 2003. Isabella relapsed in March 2005 and died in July 2005 a week after her seventh birthday.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Keywords: Neuroblastoma · Receptors · Stage · Target

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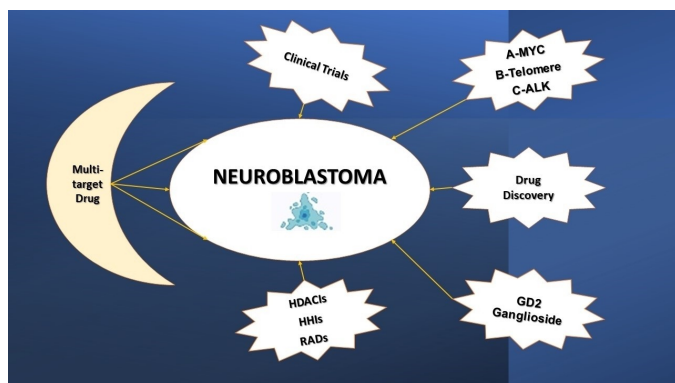
REVIEW

A. Gerges*, Dr. U. Canning

1 – 23

Neuroblastoma and its Target Therapies: A Medicinal Chemistry Review

Searching for a cure for neuroblastoma: This review highlights the urgent need to find a cure for high-risk neuroblastoma. It provides an overview of the current treatment available and the drug discovery approach of seven drugs that reached clinical trial. Since 2017 there has been an acceleration in multi-target drug development, and the review concludes by arguing for a similar approach to be adopted in finding a cure for NB.