ABSTRACT: Neuroblastoma (NB) is one of childhood's most common malignant tumours worldwide. Upon diagnosis, NB is categorized according to staging and risk, with treatment according to different risk categories. High-risk NB is treated with intensive chemotherapy, surgery, radiation therapy, bone marrow/hematopoietic stem cell transplantation, differentiation treatment of isotretinoin and antibody therapy that is usually administered with the cytokines GM-CSF and IL-2. To date, the genetic profile of NB is still being investigated. The most established gene associated with NB is the MYCN Proto-Oncogene, BHLH Transcription Factor (MYCN) amplification that contributes to the risk stratification of the disease. MYCN gene is an important foetal oncogene involved in cell proliferation for organ and tissue growth. Unfortunately, despite significant advances in the treatment of NB in recent decades, the prognosis for high-risk patients remains unfavourable since the overall 5-year survival rate, according to statistical data, does not exceed 40%. The use of cell technologies in paediatric oncology and haematology occupies a significant place and continues to improve. Since one of the leading causes of tumour development is an imbalance between cell death and cell survival, this paper aims to discuss treatment strategies to eliminate tumour cells using cell death pathways, including inducing apoptosis, necroptosis, autophagy, bioenergetics pathways, and immunotherapy. In conclusion, there is a need for a well-studied genetic profile of NB, which will allow the identification of new biomarkers, thereby contributing to the development of new therapeutic strategies. At the point of this review, immunotherapy seems to be the most promising treatment for high-risk NB as it has been highly effective in other kinds of cancer.

Keywords: High-risk neuroblastoma, treatment strategies, apoptosis, autophagy, immunotherapy

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Localization in the adrenal glands. At the time of diagnosis, about 40% of patients have the last stages of the disease, characterized by a disseminated tumour which occurs by lymphogenous and haematogenous metastasis to distant lymph nodes, skeletal bones, liver and skin (Gleissman, 2013).

The clinical course of the disease and prognosis varies widely from benign forms that spontaneously regressed or aggressive tumours characterized by rapid loco-regional spread, early appearance of distant metastases, poor response to ongoing intensive treatment and high relapse rates (Shimada & Ikegaki, 2019). The heterogeneity of clinical forms of NB is mostly due to the unpredictable biological behaviour of tumour cells, which includes a change in the number of copies of individual genes or chromosomal regions, mutations in oncogenes and tumour suppressor genes. A microarray study has demonstrated six-NB associated genes, which were MYCN, anaplastic lymphoma kinase (ALK), survivin, also called baculoviral inhibitor of apoptosis repeat-containing (BIRC5), cyclin D1 (CCND1), neurotrophic receptor tyrosine kinase 1 (NTRK1), and paired-like homeobox 2b (PHOX2B) that significantly discriminate into four molecular subgrouping of NB (Abel et al., 2011). Modern schemes of stratification of patients with NB into risk groups are based on a combination of clinical and molecular biological data. Age over 18 months, the last stages of the disease, and amplification of the MYCN gene are the main criteria for the distribution of patients into high-risk groups.

Factors of unfavourable prognosis also include segmental chromosomal aberrations such as deletions of the subtelomeric region of chromosome 1, the long arm of chromosome 11, and enlargement of the long arm of chromosome 17 (Tolbert & Matthay, 2018). However, the amplification of the MYCN gene, which is a cellular proto-oncogene of the transcription factors family, acquired the most significant importance in establishing a prognosis, choosing the optimal treatment strategy, and resistance to chemotherapy. Proteins in the MYC family are believed to play a key role in regulating the cell cycle and cell proliferation, as well as a regulatory role in stem-cell differentiation and cell-death modulation. MYCN amplification occurs in 20-30% of all NB patients and is one of the main markers of aggression and poor prognosis of the disease (Johnsen et al., 2018). Thus, high-risk NB remains a problematic link in childhood oncology, characterised by a low survival rate with long-term event-free survival (EFS) that does not exceed 34-38%, a high chance of relapses and a poorly studied biological, genetic and clinical basis (Valter et al., 2018). The treatment for NB includes five to six cycles of induction chemotherapy and surgery, followed by high-dose consolidation therapy and autologous hematopoietic stem cell rescue, including radiation, and post-consolidation treatment, which are part of today’s high-risk traditional therapies (Smith & Foster, 2018). Figure 1 shows the treatment for high-risk NB recommended by the Children’s Oncology Group (COG) (Smith & Foster, 2018).

Typically, during induction, patients receive 5-8 courses of intensive chemotherapy, including platinum, alkylating and topoisomerase drugs. Patients are given stem-cell harvest as part of the induction phase, anticipating autologous stem cell transplantation (ASCT). Another crucial part of high-risk NB treatments is surgery, generally performed at or near the completion of induction chemotherapy. Surgery is intended to minimise tumour size and surgical complications. According to the COG’s research, there was no marked improvement in survival for patients with complete resection compared to patients without complete resection in stage 4 tumours, as the five-year survival rate was 30% compared to 25%, respectively (Kreissman et al., 2013).

Following induction, the consolidation phase is then used to remove any residual tumour tissue. The latest clinical trial has demonstrated that high-dose chemotherapy followed by tandem ASCT has significantly improved the EFS to 61.6% compared to 48.4% in single transplant patients with high-risk NB (Park et al., 2019). A total of 176 patients were randomized to receive tandem transplants with thiotepa/cyclophosphamide followed by dose-reduced carboplatin/etoposide/melphalan, and another cohort of 179 patients received a single transplant with carboplatin/etoposide/melphalan. However, the authors cautioned that due to the low randomization rate, the result might not be representative of all high-risk NB cases.

The post-consolidation or maintenance phase was formulated to overcome the disease that remains despite intensive induction and consolidation therapies. The standard of maintenance protocol includes anti-disialoganglioside (anti-GD2) monoclonal antibody (mAb) immunotherapy together with immunostimulatory agents [interleukin-2 (IL2) and granulocyte-macrophage colony-stimulating factor (GM-CSF)] and the differentiating agent isotretinoin, which is used to normalize cells' redox and regeneration processes.
**Figure 1**: Treatment regimen for high-risk neuroblastoma patients recommended by the Children’s Oncology Group (COG) (created with BioRender.com).
Yu et al. (2010) first demonstrated that the EFS in high-risk NB patients receiving the combined immunotherapy (ch14.18) + GM-CSF+IL2 was higher than in isotretinoin-only groups, 66% versus 46%, respectively. These results from Phase III trials are the basis for prescribing maintenance therapy accepted by COG (Yu et al., 2010). Subsequently, other studies also shown that the combination of anti-GD2 with GM-CSF and isotretinoin also showed an improved progression survival rate of 62% compared to 46% of the control group (only anti-GD2 immunotherapy) (Cheung et al., 2012). The follow-up study from Yu et al. showed that combined immunotherapy (ch14.18 + cytokine) significantly improved 5-years EFS to 56.6% from 46.1% based on isotretinoin treatment only (Yu et al., 2021).

Due to the poor prognosis and high tendency of recurrence, the treatment of high-risk NB includes all possible therapeutic and surgical options currently available. An ongoing understanding of the biology of NB will help to identify the factors that alter the outcomes of patients in high-risk groups.

2.0 TREATMENT STRATEGIES

A common feature of all tumours, including high-risk NB, is alterations in cell death mechanisms. With the help of these alterations, tumour cells acquire the ability to survive in adverse conditions, avoiding programmed cell death, and in some cases acquiring resistance to chemotherapeutic agents. Previously, programmed cell death was mainly associated with apoptosis, the most well-studied and the main target of anticancer therapy. However, other mechanisms of cell death have been investigated recently, such as autophagy, bioenergetics pathways, and necroptosis. These pathways may be based on different biological mechanisms of cell death induction and specific sequences leading to cell death, including apoptosis (Pentimalli et al., 2019). The discovery of multiple death pathways has opened opportunities to develop new treatment strategies for high-risk NB. The study of the regulation of cell death enables a better understanding of how dysregulation affects the staging of disease, uncovering new molecular and genetic biomarkers of the disease that could be used as targets for cell death initiation. On this basis, a wide range of treatment strategies has been established.

Further studies of the morphological and biochemical mechanisms of cell death will provide an opportunity to better understand the pathogenesis of many diseases, improve their differential diagnosis, and open up potential new avenues of therapy (Strasser & Vaux, 2020). There were numerous research papers on current treatment strategies used. However, only a few are focused on summarizing cell-death pathways-based approaches. This review paper will focus on the latest advances in cell-death pathways approaches in treating high-risk NB, highlighting those treatment strategies undergoing pre-clinical and clinical trials demonstrating promising results.

3.0 APOPTOSIS

One of the most studied forms of cell death, apoptosis, is an antagonistic process of forming malignant neoplasms. The suppression of the mechanisms of apoptosis leads to the inability to eliminate cells potentially dangerous to the body and determines the resistance of tumours to therapy, while the induction of apoptosis is responsible for both tumour regression and its complete removal during anticancer therapy (Elmore, 2007; Jan & Chaudhry, 2019). There are two mechanisms for developing the apoptosis program - intrinsic (mitochondrial) and extrinsic (associated with signalling from death receptors). Cysteine-dependent aspartate-specific proteases, better known as caspases, are important participants in the signalling pathways of apoptosis. There are initiating caspases (caspase-2, -8, -9, and -10) and executive (effector, executor) caspases-3, -6 and -7 (Jan & Chaudhry, 2019).

The Bcl-2 family of proteins, characterised by at least one unique Bcl-2 homology (BH) domain, play an essential part in mitochondrial apoptosis control. This family is classified into two groups: anti-apoptotic proteins such as Bcl-2, Bcl-XL, Bcl-W, Bcl-B, A1 and Mcl-1, and pro-apoptotic proteins such as Bax, Bak, Bim, p53 upregulated modulator of apoptosis (PUMA), Noxa and others. Except for the Bax, Bim, Bak, and Bad proteins, which belong to the BH3 family, the pro-apoptotic group is normally found in the cytosol. Anti-apoptotic proteins Bcl-2 and Bcl-XL bind to these proteins on the mitochondrial membrane, effectively sequestering pro-apoptotic proteins from activating Bax/Bak. This leads to mitochondrial outer membrane permeabilization, the release of cytochrome c and induction of apoptosis. The BH3-only protein family regulates apoptosis by displacing anti-apoptotic proteins from the complex and replacing them with pro-apoptotic ones, allowing the latter to cause apoptosis. The ratio of pro- and anti-apoptotic Bcl-2-like proteins present during the development of such complexes defines whether apoptosis is implemented or inhibited in cells. Thus, the equilibrium of pro- and anti-apoptotic
proteins on the outer mitochondrial membrane is essential for the activation of apoptosis, and the imbalance of these proteins toward an increase in anti-apoptotic proteins is the source of the mitochondrial apoptosis pathway repression (Cory & Adams, 2002; Hartman & Czyz, 2020). Mutations passed on to the cell progeny prevent normal cells from undergoing apoptosis and are insufficient to induce full malignancy, and it does not cause neoplasia. It would require further mutations, including aberrant cell proliferation, enhanced survival and migration (Strasser & Vaux, 2020). Various studies have demonstrated NB-associated pathways that prevent apoptosis and enhance neoplasia, including overexpression of apoptosis inhibitors, such as Bcl-2 (Castle et al., 1993; Dole et al., 1994; Reed et al., 1991) and p53 inhibitor mouse double minute 2 homolog (MDM2) (Corvi et al., 1995; Rayburn et al., 2005) as well as epigenetic silencing of caspase-8 (Lázcoz et al., 2006; Teitz et al., 2000).

3.1 Bcl-2 mediated pathway

Tumour cells use anti-apoptotic proteins from the Bcl-2 family as a mechanism of cell death resistance, playing an essential part in the onset of oncological diseases, including NB and developing resistance to therapeutic stimuli by malignant cells (Hoehner et al., 1995; Lamers et al., 2012). The discovery of highly selective inhibitors of some anti-apoptotic family members, namely BH3 mimetics that targets Bcl-2, Bcl-xL and Mcl-1, has made a breakthrough due to a thorough analysis of the interactions between Bcl-2 proteins that underpin the modulation of apoptosis initiation in NB showed in Figure 2. One of the first BH3 mimetics was ABT-737, which is capable of causing apoptosis by releasing Bax or Bak from the complex with anti-apoptotic proteins in various NB cell lines (Goldsmith et al., 2010). A subsequent study demonstrated that ABT-737 co-administered with a retinoid derivative, fenretinide, synergistically induced caspase-dependent apoptosis in NB cell lines and xenograft models (Fang et al., 2011). Another study showed that ABT-737, together with the chemotherapy agent, melphalan (in vitro) or cyclophosphamide (in vivo), primed Bcl-2-dependent high-risk NB cell lines and xenograft model to Bim-activated apoptosis (Goldsmith et al., 2012). ABT-263, the orally available form of ABT-737, has been tested in a panel of NB cell lines, induced apoptosis in cells that overexpressed Bcl-2, with a xenograft model confirming this observation. The study also demonstrated that combination treatment with doxorubicin/vincristine/etoposide leads to greater synergistic effects (Lamers et al., 2012). The oral ABT-199 (venetoclax) has shown high efficiency in the induction of apoptosis together with Mcl-1 inhibition in Bcl-2-dependent NB cell lines (Bate-Eya et al., 2016); while Tanos et al. (2016) also demonstrated that ABT-199 + cyclophosphamide combination treatment induced apoptosis and tumour regression in Bcl-2 dependent NB cell lines and xenografts (Tanos et al., 2016). Detailed profiling analysis of ABT-263 and ABT-199 in a panel of NB cell lines showed that these drugs induced apoptosis according to the MYCN amplification status of the cell lines. Co-treatment of ABT-199 with aurora kinase A inhibitor MLN8237 further improved the anti-cancer effect, with increased apoptosis rate in in vitro study and tumour shrinkage in a xenograft model (Ham et al., 2016). The pre-clinical research also showed that γ-tocotrienol (γT3), a vitamin E analogue acts as BH3 mimetics to induce caspase-dependent apoptosis in SH-SY5Y cells (Tan et al., 2016).

One major limitation of these NB-associated pre-clinical studies with ABT-737, ABT-263 and ABT-199 is that cells quickly become resistant due to constitutive up-regulation of Mcl-1 to compensate for the loss of Bcl-2/Bax equilibrium. Another approach is to use a Bcl-2/Mcl-1 inhibitor, such as Tivantinib, which demonstrated single-agent cytotoxicity with high selectivity to MYCN amplification NB cell lines and xenograft models (Klenke et al., 2019). The latest study also shows that BH3 mimetics that target Bcl-2 (ABT-199), Bcl-xL (A1331852) and Mcl-1 (S63845) successfully induced BIM-dependent apoptosis in various NB cell lines but do not associate with MYCN status (Bierbrauer et al., 2020). Another study also reported that co-treatment of A1331852 and S63845 act synergistically to induce BIM-dependent apoptosis compared to single drug treatment highlighting that co-inhibition of Bcl-xL and Mcl-1 is the key to successful BH3 mimetics strategy (Kehr et al., 2020). In all of these BH3 mimetics studies, the authors highlight the need for molecular profiling of the NB tumours to ensure a successful treatment outcome, as the molecular heterogeneity nature of the tumour in patients can affect treatment outcomes. Other key findings from all studies demonstrated that Bcl-2 expression could be used as a predictive marker for ABT-199 sensitivity in NB cells. Although the findings are still preliminary in pre-clinical studies, they hold great potential to be translated into clinical studies. ABT-199 has been approved for chronic lymphocytic leukaemia (CLL) (Roberts et al., 2016). ABT-199 is currently on the RG7388/ Idasanutlin clinical Phase I/II trial as one of the experimental arms for NB, leukaemia and solid tumours (NCT04029688) and another clinical
Figure 2: Schematic representation of therapeutic targets to induce apoptotic cell death in NB, focusing on the (A) extrinsic pathway, (B) intrinsic pathway and (C) bioenergetics pathway. Proteins mediating similar functions are indicated by shading, a box with sharp edges indicating the pharmaceutical agent discussed in the text. Adapted from “Extrinsic and Intrinsic Apoptosis”, by BioRender.com (2022) (https://app.biorender.com/biorender-templates).
trial for relapsed or refractory NB and lymphoid malignancies (NCT03236857). Meanwhile clinical Phase I/II trial for ABT-263 for relapsed or refractory lymphoid malignancies (NCT00788684) and another Phase I/II trial for relapsed or refractory CLL (NCT00481091) is still ongoing (Montero & Haq, 2022). To date, no clinical trial of these BH3 mimetics targets neuroblastoma except for ABT-199.

3.2 p53/MDM2 mediated pathway
The p53 gene is the largest pro-apoptotic protein control unit of Bcl-2, and the key DNA damage sensor is located on the short arm of chromosome 17. The p53 protein is in a latent state. It is activated in response to DNA damage, exposure to hypoxia, activation of oncogenes, or exposure to other cytotoxic agents. Activation of p53 enhances Bax expression, shifting the Bcl-2/Bax ratio and eventually leading to apoptosis. Being the guardian of the genome, p53 mutations are rare in NB, with reported mutations of less than 2% (Vogan et al., 1993; Wolter et al., 2010), and various studies reported functional p53 signalling mechanism in NB (Chen et al., 2007; Tweddle et al., 2003). This implies the involvement of other mechanisms that suppress its activity. Accumulation of cytosolic p53 was noted in NB cell lines, which may be associated with its sequestration in the cytoplasm, leading to inactivation of p53 and the inability of cells to undergo G1 arrest following DNA damage (Moll et al., 1996). In NB, the mouse double minute 2 (MDM2) was first discovered as a p53 negative regulator that binds to the protein’s N-terminal region of p53, inhibiting its transcriptional activity while promoting its export from the nucleus to the cytoplasm. Under the unstressed condition, MDM2 primary function is to polyubiquitinate the p53 protein at many C-terminal lysines for subsequent degradation by proteosome (Zaika et al., 1999). Mutations of the MDM2 gene, for example, a single nucleotide substitution (SNP309) at the polymorphic region near the promoter – lead to greater gene activity. Consequently, it leads to a decreased level of p53, which is characteristic of NB and correlates with the aggressive course of the disease (Parodi et al., 2010).

Research by Slack et al. noted the correlation of MYCN amplification status with MDM2 expression since MDM2 is an MYCN transcriptional target (Slack et al., 2005). At the same time, MDM2 can also increase the translation of MYCN by binding to its mRNA. Among other things, MDM2 is associated with chemoresistance, and stabilization of the vascular endothelial growth factor (VEGF) was noted by increasing its translation by the protein, contributing to better adaptation of NB cells (Zhou et al., 2011). Another factor influencing reduced apoptosis is the inhibition of the p73 gene by MDM2, which is responsible for inducing apoptosis (Wu & Leng, 2015). Thus, MDM2 is an excellent potential target in treating high-risk NB, and its oncogenic roles are presented in Figure 2. The inhibition of MDM2 to increase the activity of the tumour suppressor p53 is a justified and promising therapeutic strategy. Nutlin-3, a p53/MDM2 antagonist, is highly efficacious in managing NB with the wild-type p53 phenotype. In a functional analysis with a panel of NB cell lines, results showed that nutlin-3 significantly induced apoptosis by disrupting p53 and MDM2 interactions in cell lines with wild-type p53, revealing upstream effector p14ARF to be a potentiating factor in the responses of NB cells to nutlin-3 (Van Maerken et al., 2011). Nutlin-3 activated the p53 pathway in mice with NB xenografts, encouraging apoptosis, increasing susceptibility to other chemotherapy medications, and reducing metastases and tumour development (Van Maerken et al., 2009). In addition, another study indicated a disruption in the interaction of MDM2 with p73, increasing the pro-apoptotic gene activity in NB cell lines (Lau et al., 2008). Pre-clinical evidence demonstrated that RG7388 / Idasanutlin (second generation Nutlin-3) elicit potent p53-dependent apoptosis in NB cell lines and xenograft regardless of p53 mutation status in NB cells, as a single agent (Lakoma et al., 2015) and in combination treatment with various chemotherapy drugs (Chen et al., 2015). However, RG7388 is currently at the recruiting stage of phase I/II of clinical trials with an improved efficacy and safety profile in treating p53 wild-type NB, leukaemias and solid tumours (NCT04029688). The study aims to recruit 220 participants. Interestingly, one of the experimental arms of the trial includes co-administration of RG7388 with ABT-199 (Zafar et al., 2021). This is based on a pre-clinical study that showed RG7388 re-sensitised NB cells previously resistant to ABT-199 (Vernooij et al., 2021).

3.3 Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated pathway
Targeting the intrinsic pathway of apoptosis is one of the most widely used approaches in treating high-risk NB. However, the extrinsic pathway also appears to be a potential target. The development of apoptosis along the extrinsic pathway begins with death receptors [tumour necrosis factor receptor (TNFR1), Fas receptor, death receptor 3 (DR3), DR6, TRAIL receptor (TRAILR-1) and TRAILR-2], whose function is to recognize the extracellular death ligand and activate the effector mechanisms of apoptosis in the cell. As the death receptor binds to the ligand, it becomes trimerized, and
the adapter molecule Fas-associated protein with death domain (FADD) is recruited, resulting in the development of a death-inducing signalling complex (DISC) (Sayers, 2011). Caspase-8 is activated due to the formation of DISC, leading to apoptosis in two ways. First, caspase-8 activates pro-caspase-3 in a sufficient amount, which in turn is capable of autocatalysis and activation of other effector caspses. The second way is that caspase-8 cleaves the Bid family protein (as a workaround required when its level is low in DISC) and activates the mitochondrial apoptosis pathway afterwards. A fragment of the Bid protein – tBid is capable of causing oligomerization and the incorporation of another pro-apoptotic protein Bak and/or Bak, into the outer mitochondrial membrane, forming pores through which proteins enter the cytoplasm from the intermembrane domain of mitochondria (Tummers & Green, 2017). Thus, the Bid protein is a link between the two apoptosis pathways (shown in Figure 2).

Studies have shown that in NB cells, the TRAIL receptor DR5 was upregulated by etoposide treatment (Kim et al., 2012; Tong et al., 2011) and doxorubicin (Tong et al., 2011), which sensitized the cells for TRAIL-mediated apoptosis. In addition, loss or epigenetic silencing of caspase-8 expression is significantly associated with MYCN-amplified and high-risk NB (Muhlethaler-Mottet et al., 2006; Teitz et al., 2001) and causes resistance for cells to undergo apoptosis (Teitz et al., 2000). TRAIL-based therapy is an attractive approach to treat cancer as the pathway specifically targets malignant cells and not normal cells (Raff & El-Deiry, 2018). The therapeutic strategy is either to target the TRAIL receptors or to target the caspase-8 expression in order to sensitize NB cells to TRAIL-induced apoptosis. One approach is to use HGS-ETR2, a monoclonal antibody targeting the TRAIL receptor, which was on phase I clinical trial for paediatric solid tumours, including NB (NCT00428272) that was terminated in 2019. At the point of this review, second-generation TRAIL receptor agonists such as single chain (sc) TRAIL fusion proteins ABBV-621 had just completed clinical trials in adult patients with previously-treated malignancies (NCT03082209) and had yet to start the trial in paediatric malignancy including NB. The clinical trial of ABBV-621 in adult solid tumours showed evidence of anti-tumour effect with manageable adverse reactions as monotherapy (LoRusso et al., 2022). Various studies demonstrate that hypermethylation of the regulatory regions of the caspase-8 gene leads to its inactivation in NB tumours (Grau et al., 2011; Kamimatsuse et al., 2009; Lázcoz et al., 2006). There are three strategies to increase caspase-8 expression and sensitise NB cells to TRAIL-mediated apoptosis: 1) IFNγ treatment that acts on the interferon-sensitive respond element within the caspase-8 promoter, thus restoring the expression of caspase-8 and therefore sensitise TRAIL resistant NB cells to apoptosis (Kim et al., 2004; Tong et al., 2009; Tong et al., 2011; Yang et al., 2003); 2) using methylation inhibitors to restore caspase-8 expression. For example, 5-Aza-2'-deoxycytidine (decitabine) was successfully shown to restore caspase-8 expression and sensitivity to TRAIL-induced apoptosis in NB cell lines (Egbert et al., 2001; Fulda & Debatin, 2006; Geiger et al., 2012). At the moment, IFN-γ has passed clinical trials and is available for use in the treatment of chronic granulomatous disease as well as malignant osteoporosis (Miller et al., 2009), however IFN-γ, in combination with HGS-ETR2, a monoclonal antibody did not pass the Phase I clinical trial for paediatric solid tumours including NB (NCT00428272). Clinical trials on low-dose decitabine with doxorubicin/cyclophosphamide on refractory NB patients under the COG has completed since 2013 (NCT00075634). The study reported that doses of decitabine capable of producing clinically relevant biologic effects were not well tolerated with this combination amongst patients (George et al., 2010). Subsequently, another clinical trial on decitabine with cancer antigen vaccine showed a promising result of eliciting T cells responses in most NB and sarcoma patients, and the treatment regimen is well tolerated (NCT01241162) (Krishandas et al., 2015). The third strategy to improve caspase-8 expression in NB cells is to use FLIP inhibitors. As various proteins modulate FLIP, there are various FLIP inhibitors at the transcriptional and post-transcriptional levels (Safa & Pollok, 2011). These are FLICE-inhibitory proteins, capable of inhibiting the activation of caspase-8 and-10 by competing for the connection with FADD. One example is depsipeptide, a histone deacetylase. As seen in multiple cancer cell lines, depsipeptide inhibits FLIP expression at the transcriptional and translational levels (Safa & Pollok, 2011). In NB cells and xenograft models, depsipeptide demonstrated encouraging anti-tumour effects such as inhibition of cell proliferation and tumour growth, increased apoptosis, decreased N-MYC protein expression and VEGF levels (Panicker et al., 2010). Depsipeptide has completed phase I clinical trials (NCT00053963), reporting no objective tumour response was found in paediatric solid tumours, although the drug is well tolerated (Fouladi et al., 2006). Another endogenous inhibitor of caspase is the inhibitors of apoptosis proteins (IAP) family proteins, which inhibit the activity of caspase-3 and -9 (Deveraux & Reed, 1999). Targeting IAP family proteins is a
potential target in the treatment of high-risk NB. Survivin (BIRC5, a member of the IAP family) is an oncogene which is overexpressed in NB and correlates with poor outcomes and is characteristic of high-risk groups (Islam et al., 2000; Wang & Zheng, 2004). Survivin binds to the spindle microtubules and blocks apoptosis through inhibition of caspase-3 and -9, as well as cell death in mitosis caused by spindle division failure. At the moment, there are many approaches targeting survivin. The low molecular weight antagonist teramprocol, or EM-1421, in phase I clinical trials in adult solid tumours (NCT00664586) leads to inhibition of survivin expression (Smolewski, 2008). Promising results have shown that survivin-based vaccines induce apoptosis in NB cell lines and animals using a DNA vaccine called pUS-high (Fest et al., 2009). Another survivin long peptide vaccine known as SVN53-67/M57-KLH (SurVaxM) is currently undergoing phase I clinical trials in neuroendocrine tumours (NCT03879694), with previous success in Phase I clinical trial outcome showing a favourable anti-tumour effect on gliomas (NCT01250470) (Fenstermaker et al., 2016).

Targeting induction of apoptosis is one of the therapeutic approaches in the treatment of NB to reduce tumour size and reduce the proliferation of malignant cells. To date, therapeutics that target the Bcl-2 and the p53/MDM2 mediated apoptosis- BH3 mimetics (ABT-199 and ABT-263), MDM2 antagonist (RG7388) and survivin inhibitors (EM-1421 and SurVaxM) holds great promise as potential therapeutics for the treatment of high-risk NB, with ongoing clinical trials. The other therapeutics targeting the TRAIL-mediated apoptosis pathway still have a long way to prove clinical efficacy in NB patients, although numerous pre-clinical studies show promising results.

### 3.4 Targeting bioenergetics pathways to induce apoptosis

Altered energy metabolism in tumour cells, known as the Warburg effect, is also demonstrated in many tumour types, including NB, which showed high glucose uptake under positron emission tomography (PET) examination (Freebody et al., 2014). As tumour cells favour glycolysis over oxygen, there is an increased uptake of glucose, excessive lactate production and a low rate of oxygen, which is also demonstrated in NB tumours (Levy et al., 2012). Strong evidence demonstrated that the Warburg effect of NB cells is not caused by lower mitochondrial mass. Mitotracker Green analysis showed a similar amount of mitochondria in NB compared to normal neuronal cells (Hoegger et al., 2008), while mitochondrial citrate synthase activities and porin expression is similar in human NB samples compared to normal kidney tissue (Feichtinger et al., 2010). Although there are no apparent changes to mitochondria mass and no pathological mutations detected in the succinate dehydrogenase (SDH) subunit genes, there are significant decreases in SDH activities and other oxidative phosphorylation (OXPHOS) complexes compared to normal kidney tissue. The mtDNA copy number was also significantly reduced in NB tumours (Feichtinger et al., 2010). Deletion of chromosomes 1 and 11 in primary NB tumours showed the loss of heterozygocity (LOH) of SDHB and SDHD genes, forming the important SDH enzyme in mitochondrial energy metabolism. The LOH of these genes in chromosome 1 correlates with MYCN amplification, while the LOH in chromosome 11 correlates with high-risk NB (Feichtinger et al., 2010; Guo et al., 1999; Martinsson et al., 1997). Low oxygen availability stimulates the stabilization of hypoxia inducible factor 1 (HIF-1α) for short-term exposure and HIF-2α for prolonged exposure to hypoxia. These two proteins are responsible for adapting cells to hypoxic conditions in NB tumours. In particular, HIF-1α regulates angiogenesis, energy metabolism, and apoptosis. HIF-1α is triggered at physiologically critical sites of oxygen pathway regulation, providing rapid and adequate responses to hypoxic stress (Edsjö et al., 2007). Increased HIF-1α expression is associated with aggressive types of NB and a poor prognosis since it enhances glycolysis and encourages improved adaptation. As in the case of high-risk NB, overexpression of HIF-1α significantly associates with MYCN overexpression (Dungwa et al., 2012), while HIF-2α is inversely correlated with MYCN expression, associated with poorer prognosis and advanced clinical stage (Zhang et al., 2014). Although VEGF is a downstream effector of HIF-1α and HIF-2α, conflicting studies regarding the association of VEGF levels with HIF-1α / HIF-2α, with VEGF is neither associated with the genotype, phenotype and staging of NB (Pålman & Mohlin, 2018). HIF-2α also targets transient receptor potential cation channel, subfamily M, member 2 (TRPM2), which modulates mitochondrial bioenergetics, cell viability and mitochondrial reactive oxygen species (ROS) level in NB cells. Depletion of TRPM2 using the CRISPR/Cas9 technique demonstrated inhibition of tumour growth, chemosensitivity and decreased level of HIF-1α / HIF-2α and other OXPHOS downstream proteins (Bao et al., 2016; Chen et al., 2014).

Thus, suppression of HIF-1α or HIF-2α is a promising strategy as its importance in the development of NB has been confirmed in many studies. One of the HIF-1α /
HIF-2α inhibitors is topotecan which inhibits HIF-1α/ HIF-2α and in turn, suppresses VEGF production and angiogenic activity. A completed clinical trial with topotecan as monotherapy demonstrated sufficient anti-tumour effect with NB tumours with manageable toxicity and suggested combining it with other anti-cancer drugs for future trials (Längler et al., 2002). A clinical trial of topotecan in combination with cyclophosphamide has been moderately response rate of 32% vs 19% for cyclophosphamide alone, therefore is cooperated into COG induction chemotherapy regimen for newly diagnosed NB since 2010 (London et al., 2010). A subsequent clinical trial of this regimen confirmed the efficacy of the topotecan + cyclophosphamide treatment and was recommended for the phase III trial (NCT00070200) (Park et al., 2011). There were clinical trials for other drug add-ins for the topotecan or topotecan + cyclophosphamide treatment in NB, but the trial outcome was not as encouraging. For example, tumour response to temozolomide + topotecan combination is not significantly different from temozolomide alone (NCT00918320) (Di Giannatale et al., 2014). Acriflavine, another HIF-1α inhibitor, has been shown to inhibit tumour growth and induce apoptotic death in glioma cell lines (Mangraviti et al., 2017), however pre-clinical data on the use of acriflavine to treat NB is lacking.

As the energy metabolism of cells depends on the integrity of the mitochondrial, any disruptor to the mitochondrial stability or inhibition of the mitochondrial metabolism will lead to apoptosis. Pre-clinical studies have also shown that vitamin E analogues α-tocopheryl succinate disrupt mitochondrial stability and induced apoptosis in NB cell lines (Swettenham et al., 2005). Other mitochondrial inhibitors to consider for the treatment of NB includes metaiodobenzylguanidine (MIBG), metformin and phenformin, which have demonstrated tumour suppressive effect of NB cells through destabilizing MYC/MYCN in in vitro studies (Wang et al., 2014). Among these mitochondrial inhibitors, MIBG is on a clinical trial as part of the regimen for refractory NB. MIBG is a noradrenaline analogue, and NB cells are known to uptake noradrenaline. MIBG labelled with $^{131}$I is commonly used for tumour-specific radioligand for NB PET imaging as it has better image quality (Qiu et al., 2021). Meanwhile, $^{131}$I-MIBG is being explored as a therapeutic adjuvant for chemotherapy and ASCT (NCT00253435). However, the trial outcome showed a poor response rate for refractory and relapse NB, while a higher response rate was noted in patients with poor response to conventional induction therapy, suggesting $^{131}$I-MIBG may have a better response rate at an early stage during induction for high-risk NB rather than at the later stage of salvage therapy (Yanik et al., 2015). A subsequent clinical trial of $^{131}$I-MIBG as induction therapy has demonstrated that early $^{131}$I-MIBG induction with topotecan has a significant and lasting effect on the overall response rate (57%) (Kraal et al., 2015).

Given the substantial rise in glycolysis in the bioenergetics of most tumours, inhibiting glycolytic pathways is a promising field of anticancer therapy. 2-Deoxyglucose (2-DG) is a glucose analogue that reaches the cell via hexose transporters (Pelicano et al., 2006). The inhibition of glycolysis occurs due to the accumulation of a non-metabolizable analogue. Using this approach is effective in several NB cell lines, especially in combination with other cytotoxic drugs (Chuang et al., 2013). In addition to the energy of the cell, 2-DG disrupts the N-glycosylation of proteins, which serves as a trigger for the initiation of the unfolded proteins reaction. If the cell cannot cope with the disturbances, this is followed by the induction of endoplasmic reticulum (ER) stress. Treatment of cells with 2-DG caused ER stress, leading to the initiation of both autophagy and apoptosis, which was observed in NB cells (Maximchik et al., 2018). In adult solid tumours, 2-DG + docetaxel treatment (NCT00096707) has completed phase I clinical trial, showing partial response in tumour reduction (Raez et al., 2013). Glycolysis inhibitor 3-Bromopyruvate (3-BP) is a synthetic analogue of pyruvate that attaches covalently to hexokinase 2 (HK2), contributes to the suppression of enzyme activity and thus the suppression of glycolysis in NB cells that has high glucose transporter (GLUT1) expression (Matsushita et al., 2012).

Further pre-clinical studies also showed that 3-BP enhance apoptotic cell death in doxorubicin-resistant NB cell lines (Bean et al., 2014), and combination treatment of 3-BP + rapamycin synergistically induced apoptosis, cell cycle arrest and inhibition of cytoprotective autophagy (which will be discussed in Section 5.0) in NB cells (Gan et al., 2020). The second generation of 3-BP, known as $^{177}$Lu-3-BP-227 in Phase I clinical trial (NCT03525392) for adult solid tumour expressing neurotransin receptor type 1 (NTR1) and showed an anti-tumour effect in metastatic pancreatic adenocarcinoma (Baum et al., 2018). PKD (pyruvate dehydrogenase kinase) inhibitors such as dichloroacetate (DCA), in addition to HK inhibitors, suppressing PKD lead to stimulation of mitochondrial activity, thereby restoring the phenotype characteristic
of normal cells. Stimulation of mitochondria and oxidative phosphorylation when cells are treated with DCA increases ROS production. This, in turn, can lead to mitochondrial damage and stimulation of apoptosis and mitophagy in SH-SYSY (Pajuelo-Reguera et al., 2015). Based on Niewisch et al., DCA successfully reduced the proliferation rate in selective NB cell lines (LS) over other NB cell lines (SK-N-SH and Kelly) (Niewisch et al., 2012). Completed phase I clinical trials of DCA in adult solid tumours did not show tumour reduction, most probably due to the short exposure of DCA being insufficient to induce response (NCT00566410) (Chu et al., 2015).

In addition to glucose, a wide range of tumour cells, including NB, depends on glutamine, which is used as both a substrate for energy and a regulator of the redox status of the cell. Glutamine is a carbon and nitrogen-rich amino acid needed for healthy and tumour cells to sustain biosynthesis, nutrition, and cellular homeostasis. Moreover, de novo glutamine metabolism provides alternative pathways to keep cancer cells alive, leading to chemotherapy and radiotherapy resistance in various tumours, including NB. Although glutamine deprivation can reduce cell proliferation and viability, combined therapy of irradiation and glutamine deprivation leads to radioresistance in MYCN-amplified NB cell lines (Le Grand et al., 2020). Glutamine supports cell survival, providing most of the important biosynthetic reactions in the cell and playing a vital role as the redox processes regulator in the Krebs cycle. The substrates of the Krebs cycle not only determine the adequate energy supply to the cell but can also serve as regulators of the cell death process. For some tumours, glutamine deprivation leads to cell death. Glutamine transporters are proteins of the SLC1, SLC6, and SLC38 families. Amongst these, great attention is focused on SLC1A5 (ASCT2), the target of MYC oncogene, that can increase the supply of glutamine to NB cells by promoting the expression of SLC5A1 and SLC7A1 (also called CAT1 glutamine transporters) (Wise et al., 2008). Furthermore, MYCN activates the expression of glutaminase-1 (GLS1) in NB cells, which is an enzyme of the first glutaminolysis reaction. Other studies also found that suppression of GLS1 expression inhibits NB cell proliferation in vitro and suppresses tumour growth in vivo (Xiao et al., 2015). In MYCN amplified NB cells, the MYCN expression is required to drive glutaminolysis, leading to a higher ROS burden in these cells (Wang et al., 2018).

Studies on the use of several allosteric glutaminase inhibitors have yielded encouraging results in other tumour models, but few studies focused on NB. Wang et al. (2018) discovered that dimethyl fumarate (DFM), a drug that treats multiple sclerosis, showed significant inhibition of cell proliferation in NB cell lines and suppression of tumour growth in xenograft models. DFM induced augmentation of ROS levels intracellularly and suppressed MYCN expression, which leads to decreased proliferation (Wang et al., 2018). Another pre-clinical study focused on an orphan drug candidate, 6-diazo-5-oxo-L-norleucine (DON), which strongly inhibits cell proliferation in various NB cell lines through glutaminase inhibition. DON suppressed tumour growth in xenograft models and initiated cell death in MYCN-amplified NB cells. Co-treatment with Bcl-2 inhibitor ABT-263 leads to additive and synergistic effects in mediating apoptosis across all the NB cell panels (Olsen et al., 2015). Targeting the downstream effector of the glutamine metabolism, the monocarboxylate transporter 1 (MCT1), which is also a transcriptional target of MYCN, is a feasible therapeutic approach for NB. A recent study showed that MCT1 inhibitor SR13800 elevates intracellular lactate levels and decreases glutathione levels while inhibiting cell growth. However, the inhibition of SR13800 can be circumvented by increased expression of MCT4, a homologue of MCT1 and is a known resistance factor to MCT1 inhibition. Co-treatment of SR13800 with lactate dehydrogenase A inhibitor FX11 can synergistically inhibit cell viability in NB cells (Khan et al., 2020). At the point of this review, the glutaminase inhibitor that has completed a clinical trial for adult solid tumours include CB-839, a Bis-2- (5-phenylacetamido-1,3,4-thiadiazol-2-yl) ethyl sulphide (BPTES) derivative (NCT02071862) with results yet to be published.

Taken together, targeting energy metabolism to sensitive tumours to cytotoxic treatments seems like a plausible approach to treating high-risk NB, as shown in Figure 2, especially these drug candidates - topotecan and MIBG. There is a need for a clinical trial on other drug candidates to be expanded to paediatric solid tumours, including NB, as most clinical trials focused on adult solid tumours.

4.0 NECROPTOSIS

On the other spectrum of cell death, necroptosis is a controlled cell death type with both characteristics of apoptosis and necrosis. Necroptosis is regulated necrosis mediated by death receptors, and it was first described by Degterev et al. (2005). Necroptosis is activated by cellular stress without caspase activity, which delayed the ischemic brain injury in the mouse in vivo model, and they demonstrated necrostatin-1 as a necroptosis inhibitor (Degterev et al., 2005). It was
found that necrostatins targets receptor-interacting protein kinase 1 (RIPK1), which was later found to be one of the key proteins in the initiation of necroptosis. Necroptosis is a caspase-independent mediated cell-death mechanism carried out by RIPK1, RIPK3, and mixed domain-like kinase domain (MLKL). It exhibits morphological signs similar to necrosis but at the same time has a clear regulation system, as in the case of apoptosis. Necroptosis can be classified into three types: 1) extrinsic necroptosis that is stimulated by tumour necrosis factor (TNFα); 2) intrinsic necroptosis stimulated by ROS and 3) ischemic mediated neuronal necroptosis. However, not all cells can undergo necroptosis. There are two criteria for necroptosis initiation: 1) cells must express RIPK3; 2) there is inhibition of caspase-8 activity (Dhuriya & Sharma, 2018). When activation of caspase-8 is inhibited, RIPK 3 will be recruited and phosphorylated by RIPK1 to form RIPK1/RIPK3 complex called ripoptosome (Cho et al., 2009; Li et al., 2012), which then recruits and phosphorylates MLKL to form necrosome (Murphy et al., 2013). Caspase 8 is an important switch between the two pathways because it binds to RIPK1 and blocks the necroptosis pathway. Thus, if the mechanisms of apoptosis are suppressed in proliferating cells, for example, caspases are inactivated, then the death of proliferating cells is carried out by the mechanism of necroptosis. Caspase-8 inhibits necroptosis mediated by RIPK1, RIPK3 and MLKL (Fritsch et al., 2019). Studies demonstrated that treatment of 24(S)-hydroxycholesterol (24S-OHC), a type of cholesterol metabolite in the brain, induced necroptosis-like cell death in NB cell line (SH-SY5Y), but the addition of necrostatin-1 attenuated cell death. Interestingly, the study found that caspase-8 was not expressed in cortical neurons and NB cells (Yamanaka et al., 2011).

Moreover, 24S-HC treatment in SH-SY5Y cells is dependent on phosphorylation of RIPK1 but independent of RIPK3 and MLKL to induce necroptosis-like cell death (Vo et al., 2015). In vitro studies demonstrate low expression of genes associated with necroptosis (caspase-8, RIPK1, RIPK3 and MLKL) in NB cell lines compared to other tumour cell lines used as control, as well as the resistance of certain NB cells to necroptosis. In this regard, it was suggested that this resistance is associated with hypermethylation in the regions where necroptosis genes are regulated. This problem can be solved using demethylating drugs/histone deacetylase (Nicolai et al., 2015). In addition, the effectiveness of increasing the level of calcium in the cytoplasm was established using a non-replicating virus (HVJ-E) as a carrier, which leads to the activation of calcium-calmodulin kinase (CaMK) II, which in turn phosphorylates RIPK1 and subsequently activating necroptosis in NB cell lines (Nomura et al., 2014). Another in vitro study demonstrated that D-galactose, a type of natural hexose transported in the cell via glucose transporter Glu3 in the brain, could induce necroptosis in NB cells compared to other tumour cell lines (Li et al., 2011). Figure 3 showed the involvement of the potential necroptotic activators that can be potentially therapeutic. Since necroptosis is a relatively new characterized programmed cell death, data on the effectiveness of necroptosis in the NB are insufficiently studied. Most of the potential anticancer therapeutics targeting activation of necroptosis is relatively new and mostly at pre-clinical studies, however using necroptosis as therapeutic target for high risk NB remains promising.

5.0 AUTOPHAGY

The third form of programmed cell-death is currently classified as autophagy. Autophagy is the process of degrading organelles and cytoplasmic content with the aid of lysosomes. Autophagosomes (two-membrane formations) are formed during de novo autophagy, and the cellular content (organelle or part of the cytosol) is inserted within and destroyed. When autophagosomes merge with lysosomes, autolysosomes are formed, where the cell components to be destroyed are cleaved. After autophagic destruction, intracellular material is removed from the lysosomal complex and recirculated in the cytoplasm (Glick et al., 2010). In the context of cancer progression and cancer therapy, autophagy can play the dual role of cytotoxic and cytoprotective in response to treatment. Within the cancer cell, there seems to be an autophagic switch that can shift from cytotoxic to cytoprotective autophagy. However, the molecular mechanism of this process is not well elucidated in NB compared to other cancer models (Frentzel et al., 2017). The phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian Target of Rapamycin (mTOR) kinase, which regulates translation, metabolism, and transcription in response to a lack of enhancement of nutrients and growth factors, is one of the primary regulators of autophagy in cells and is a key pro-survival pathway activated in NB. Therefore there is a possibility of crosstalk between apoptosis and autophagy in NB as the PI3K/AKT/mTOR pathway can activate both types of cell death, depending on the context of the treatment and cell type. In NB, the PI3K/AKT/mTOR pathway is closely associated with the ALK receptor, another NB-associated gene co-amplified with the MYCN gene due to close chromosomal
Figure 3: Schematic representation of the current knowledge of necroptosis in neuroblastoma cells and the necroptotic activators studied. Current studies revealed the use of HVJ-E to activate CaMKII to increase phosphorylation of RIP1, D-galactose and 24S-OHC acts on RIP1 and induced necroptosis in NB cells. Adapted from “Apoptosis Extrinsic and Intrinsic Pathways”, by BioRender.com (2022) (https://app.biorender.com/biorender-templates).
ALK mutations were also strongly associated with NB pathogenesis. Therefore PI3K/AKT/mTOR inhibitors and ALK inhibitors were considered potential therapeutic approaches for NB (De Brouwer et al., 2010; Frentzel et al., 2017; Zafar et al., 2021). However, studies revealed that these inhibitors do not sufficiently block NB growth due to the emergence of resistance; one of the factors is cytoprotective autophagy. Aveic et al. demonstrated that autophagy activation due to inhibition of ALK (entrectinib) in ALK-mutated SH-SYSY cells leads to poor efficiency of the drug (Aveic et al., 2016). The clinical trial outcome of ALK inhibitor crizotinib showed a poor response rate in refractory/relapsed NB patients (NCT00939770) (Foster et al., 2021). However, the newer ALK inhibitor, lorlatinib, has demonstrated better tumour inhibition in ALK-driven refractory/relapsed NB patients with manageable toxicity (NCT03107988) (Goldsmith et al., 2020); meanwhile, a clinical trial for entrectinib is ongoing (NCT02650401). Another study showed that third-generation ALK inhibitor ponatinib (PON) induced cytoprotective autophagy in human NB cell lines [SK-N-BE(2), SH-SYSY, and IMR-32] and co-treatment of autophagic inhibitor chloroquine (CQ) improves the anti-tumour effect of PON (Corallo et al., 2020). The clinical study of the combination treatment of ALK inhibitor MK2206 and autophagic inhibitor hydroxychloroquine (HCQ) in treating adult solid tumours is still ongoing (NCT01480154). Other studies demonstrated concurrent induction of apoptosis and autophagy through the PI3K/AKT/mTOR pathway, for example, mTOR inhibitor, AZD8055, in a panel of NB cell lines and xenograft model (Xu et al., 2018); VEGF inhibitor, apanitin, in SH-SYSY and BE(2)-M17 cells (Yu et al., 2020); a natural antioxidant oxyresveratrol in SH-SYSY and B103 cells (Rahman et al., 2017). Figure 4 shows an overview of the current understanding of the autophagic process in NB and the potential therapeutic target.

Another key regulator in the autophagy / apoptosis crosstalk is the interaction of Beclin-1 and Bcl-2, known as the “Beclin-1/Bcl-2 rherostat”. The pro-survival Bcl-2 and Bcl-XL inhibit autophagy by binding to Beclin-1. Therefore disruption of these interactions will release from sequestration and activates the autophagic process (Maiuri et al., 2007; Pattingre et al., 2005). In NB, several studies have demonstrated that Beclin-1 is a key modulator of cytoprotective autophagy that leads to resistance to treatment. Belounis et al. reported that in NB tissue, Beclin-1 is highly expressed in patients with poor prognosis, and there was a significant negatively correlated of LC3II expression, an autophagy marker with mTOR and pAKT expression in the tissue, suggesting that autophagy is activated through AKT pathway inhibition (Belounis et al., 2016). Xu et al. reported that under serum deprivation, cytoprotective autophagy was induced in NB cell lines with upregulated Beclin-1 and Bcl-2 expression, with autophagic cell death induced when Bcl-2 expression was inhibited by siRNA (Xu et al., 2013). Besides CQ and HCQ, there are other autophagic inhibitors such as 3-MA (3-methyladenine) and BafA (bafilomycin A1). 3-MA, including LY29402/wormannin/spautin-1, are inhibitors of Vps34 kinase (PI3K class III) and block autophagy at the earliest stages (Wu et al., 2013). These autophagic inhibitors are usually used in combination with other inhibitors to enhance apoptotic cell death, leading to tumour reduction. For example, CQ and spautin-1 are used together with tyrosine kinase inhibitors (RTKi) to improve the killing efficacy of NB cells (Aveic et al., 2016). At the point of this review, there are no studies reported on the use of wormannin, LY29402 and BafA in NB models. Current clinical trials on the single treatment of autophagic inhibitors have yet to demonstrate positive outcomes, such as HCQ in melanoma patients has been completed, but no result being released (NCT00962845); CQ in breast cancer patients showed no impact on cell proliferation and significant toxicity (Arnaout et al., 2019). There is an ongoing trial of combination MEK inhibitor trametinib and HCQ in neuroblastoma RAS melanoma patients (NCT03979651).

6.0 IMMUNOTHERAPY

The era of immunotherapy for NB started with the demonstration of the efficacy of monoclonal antibodies against GD2, which was approved by the Food and Drug Administration (FDA) in 2015. It has been shown that GD2 can have an immunosuppressive effect, affecting the number and function of tumour-infiltrating myeloid suppressor cells which are immature immune cells capable of suppressing the immune response, including those against tumours (Wondimu et al., 2014). GD2 is considered an element of mammalian cells’ immunological identity and is not immunogenic. Thus, the humoral immune response in producing antibodies to GD2 does not develop in vivo. Early studies 30 years ago have already demonstrated the overexpression of GD2 on NB tumour tissue regardless of the stage, including patients with the latest stages. Wu et al. revealed the expression of GD2 on all 36 tissue samples of NB, with GD2 accounting for 38% (Wu et al., 1986). Expression of GD2 is also specific to NB tumours and not found in normal Schwannian stromal cells, thus...
Figure 4: Schematic representation of the autophagy process in NB. Autophagy consists of several stages, each regulated by specific proteins and protein complexes. Only ALK inhibitors that successfully demonstrated anti-tumour effects in NB are shown here. CQ, HCQ and Spautin-1 inhibit the fusion of autophagosome and lysosome. Adapted from “Autophagy in Cancer Pathways” and “Autophagy Process”, by BioRender.com (2022) (https://app.biorender.com/biorender-templates).
making it easier to differentiate NB tumours from benign ganglioneuroma and intermediate-grade ganglioneuroblastoma tumours that either do not express GD2 or express it at low levels (Terzic et al., 2018). These features have led GD2 to be listed as the top tumour antigen under the National Cancer Institute programme and are one of the excellent diagnostic markers for NB (Cheever et al., 2009).

Currently, both murine anti-GD2 monoclonal antibodies, in particular m3F8 and 14.G2a, and chimeric mouse/human antibodies, in particular ch14.18 (also known as dinutuximab), have been developed. In this review, we focused more on the ch14.18 mAb and ch14.18 produced from CHO cells (ch14.18/CHO). The effectiveness of the clinical application of the latter has been demonstrated in the framework of clinical trials, including randomized ones. As mentioned in the earlier section, the regimen of ch14.18 mAb with an alternating cycle of GM-CSF and IL-2 followed by isotretinoin has changed the course of high-risk NB treatment and have vastly improved patients’ outcome (Yu et al., 2021). In addition, this study and a similar European randomized trial showed IL-2 cycle has no added benefit to the EFS and demonstrated significant toxicity. Therefore COG has dropped IL-2 from the anti-GD2 post-consolidation protocol but retained GM-CSF to augment the anti-GD2 activity (Ladenstein et al., 2018; Yu et al., 2021). Preclinical studies have demonstrated the anti-tumour activity of ch14.18 antibodies on NB cell lines, which served as the basis for clinical studies. Cell death mechanisms upon exposure to anti-GD2 monoclonal antibodies include antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and direct cytotoxicity, which triggers apoptosis in tumour cells. The main driver of the anti-tumour effect of ch14.18 mAb is likely to be ADCC mediated through natural killer (NK) cells and CDC (Mueller et al., 1990; Perez Horta et al., 2016; Zeng et al., 2005). However, CDC has been associated with adverse reactions such as IL-2-associated neuropathic pain; therefore, clinical trials on ch14.18 focus on reducing adverse reactions by improving the current regimen. This includes an investigation of a 10-day constant infusion of ch14.18 mAb instead of four daily infusions of the antibody with and without IL-2 subcutaneous regimen (NCT01701479) based on a previous report of lesser neuropathic pain with a 10-day infusion (Lode et al., 2015). Another approach to improve the efficacy of humanized ch14.18 mAb is to include K322A point mutation, known as 14.18K322A, to increase ADCC by lowering fucosylation and removing CMC, which significantly reduced adverse effects of pain in the pre-clinical study (Sorkin et al., 2010) and clinical study (Navid et al., 2014). A recent trial evaluating the potential use of ch14.18 mAb during chemotherapy instead of post-chemotherapy reported a favourable overall response rate of 53.9 %, exceeding the 40% set by the research group, and this rate of response is compared to the commonly used treatment of chemotherapy and 131-MIBG (NCT01767194) (Mody et al., 2020).

Strategies to further improve the efficacy of anti-GD2 immunotherapy with adoptive cell therapy that has reached clinical trials are the incorporation of NK cells with anti-GD2 immunotherapy and anti-GD2 chimeric antigen receptor T-cells (CAR T-cells). There were seven clinical trials on NK cells with either m3F8 (NCT00877110), hu3F8 (NCT02650648), hu14.18K322A (NCT01576692, NCT02130869 and NCT01857934) and ch14.18/CHO (NCT03242603 and NCT02258815) with various combination of treatment at various stage of completion. The current publications from these trials indicating the NK cells infusion as consolidation therapy exhibit increased NK cells cytotoxicity and anti-tumour effect, indicating such cell therapy is feasible and well tolerated in NB patients (Federico et al., 2017; Furman et al., 2021; Modak et al., 2018; Nguyen et al., 2020; Seitz et al., 2021). The other adoptive cell therapy approach is to produce GD2-specific CAR T-cells by genetically transducing CAR into autologous T-cells irrespective of HLA expression. There are six surface targets on neuroblastoma GD2, L1-CAM, GPC2, B7H3, and ALK, and NCAM have been developed for CAR T cell therapy. Out of these, GD2 and L1-CAM are in clinical trials. The first generation of anti-GD2 CAR T cells trial (containing only the CD3ζ endomodulin but no costimulatory domain) showed promising anti-tumour activity with no neuropathic pain and long term T cells persistence (NCT00085930) (Louis et al., 2011; Pule et al., 2008). Subsequent trial on a third-generation GD2 CAR in which the specific single-chain variable fragment (scFv) was derived from the murine 14.G2a mAb coupled with the ζ-chain endomodulin and two costimulatory endomodulins in tandem (CD28 and OX40) with lymphodepletion demonstrate some measurable response (NCT01822652) (Hecezy et al., 2017). A change in the costimulatory domain to 4-1BB instead of OX40 improved the anti-tumour efficacy, T cells activation and long-term persistence (Quintarelli et al., 2018). The latest clinical trial with incorporated IL-15 and inducible caspase-9 within the GD2 CAR construct demonstrated that transduced T cells promote significant anti-tumour activity, T cells expansion and survival (NCT03721068) (Chen et al., 2019). Several
ongoing clinical trials related to CAR T cells were excellently reviewed by Morandi et al. (2021) and not listed in the summary table (Table 1). Figure 5 shows the summary of immune-centric therapy in NB.

7.0 CONCLUSION
The biological heterogeneity of paediatric neuroblastic tumours, especially high-risk NB has contributed to a complicated treatment strategy according to risk stratification. High-risk NB is characterized by suppressed apoptosis, hyperactivation of proliferation signalling, altered cellular energetics, immune surveillance avoidance, genome instability and tumour-associated inflammation, which leads to increased proliferation and metastasis. Working within this complex disease framework, developing effective therapy while considering the increased resistance of tumour cells to therapeutic agents is a great challenge in NB research.

Ongoing research is revealing more and more molecular signatures and dysfunctional pathways, many of which are already being used as targets in NB treatment. Targeting induction of diverse cell death pathways shows promising results as a potential new treatment for high-risk NB. Although many of the approaches are still very much in pre-clinical studies, some of the potential therapeutics has gone through an early clinical trial, with some drugs such as topotecan and anti-GD2 immunotherapy showing significantly improved survival rate in clinical trials. The inclusion of anti-GD2 immunotherapy and topotecan in high-risk NB treatment has vastly improved the survival rate compared to a decade ago.

The current clinical trials in NB also look into combining different therapeutic targets instead of monotherapy, such as the combination of BH3 mimetics ABT-199 with MDM2 antagonist RG7388 and decitabine with cancer antigen vaccine to see if there is improved efficacy with such combinations. The clear trends in NB therapeutic approach include optimising treatment efficacy, minimising adverse reaction and improving pre-treatment tumour profiling. From this review, it is clear that genetic profiling of NB tumours is essential for a successful outcome of targeted therapy such as BH3 mimetics, MDM2 antagonists and ALK inhibitors. Table 1 summarizes all the previously discussed cell death-based treatment approaches used to treat high-risk NB and solid tumours.

However, despite comprehensive multimodal therapy, more than half of children with advanced NB have relapses. Therefore, it is imperative to bring more potential therapeutics that target different cell death pathways to clinical trials to expand the repertoire of treatment options for high-risk NB patients. It is also important to have a better studied genetic profile of the disease to identify new markers, thereby contributing to developing new strategies. Today, the methods of immunotherapy are the central object of study, since targeting the immune system to eliminate tumour cells is very promising, not only in the treatment of high-risk NB but also in other types of cancers.

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Figure 5: Immunotherapy approach to treat high-risk NB, including anti-GD2 monoclonal antibody, IL-2 infusion to activate NK cells, and GM-CSF treatment to activate granulocytes and macrophages (created with BioRender.com).
Table 1. Previously discussed cell-death based treatment strategies for neuroblastoma (the order of treatment strategies is according to their order in the text). The year of clinical trials is according to the last modifications made in the findings.

<table>
<thead>
<tr>
<th>Treatment strategy</th>
<th>Drug</th>
<th>Target</th>
<th>Development status</th>
<th>Disease</th>
<th>Notes</th>
<th>Clinical trial identifier/ Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH3 mimetics: Bcl-2 mediated apoptosis</td>
<td>ABT-737</td>
<td>Bcl-2, Bcl-W, Bcl-xL</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Clinical utility hampered by lack of oral bioavailability</td>
<td>(Fang et al., 2011; Goldsmith et al., 2012; Goldsmith et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>ABT-263</td>
<td>Bcl-2, Bcl-W, Bcl-xL</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>The orally available analogue of ABT-737, also known as navitoclax</td>
<td>(Ham et al., 2016; Lamers et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>ABT-199</td>
<td>Bcl-2</td>
<td>Preclinical</td>
<td>NB</td>
<td>Also known as venetoclax</td>
<td>(Bate-Eya et al., 2016; Ham et al., 2016; Tanos et al., 2016)</td>
</tr>
<tr>
<td></td>
<td>TW-37</td>
<td>Bcl-2, Mcl-1</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>A second-generation of benzenesulfonyl derivative of gossypol.</td>
<td>(Klenke et al., 2019)</td>
</tr>
<tr>
<td></td>
<td>A1331852</td>
<td>Bcl-xL</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Re-engineering of previously BCL-XL inhibitor A-1155463</td>
<td>(Bierbrauer et al., 2020; Kehr et al., 2020; Tanos et al., 2016)</td>
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<tr>
<td></td>
<td>S63845</td>
<td>Mcl-1</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>First-generation Mcl-1 inhibitor</td>
<td>(Bierbrauer et al., 2020; Kehr et al., 2020; Tanos et al., 2016)</td>
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<tr>
<td></td>
<td>γT3</td>
<td>Bcl-2</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Vitamin E analogue</td>
<td>(Tan et al., 2016)</td>
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<td>p53/MDM2-mediated apoptosis</td>
<td>Nutlin-3</td>
<td>MDM2 antagonist</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Also known as Idasanutlin, a second-generation nutlins.</td>
<td>(Lau et al., 2008; Van Maerken et al., 2009; Van Maerken et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>RG7388</td>
<td>MDM2 antagonist</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Also known as leptomiumab. The trial was terminated in 2019.</td>
<td>(Chen et al., 2015; Lakoma et al., 2015; Vernooij et al., 2021)</td>
</tr>
<tr>
<td>TRAIL-mediated apoptosis</td>
<td>HGS-ETR2</td>
<td>Anti-TRAIL receptor mAb</td>
<td>Phase I</td>
<td>Paediatric solid tumours</td>
<td>Also known as lexatumumab. The trial was terminated in 2019.</td>
<td>NCT00428272 (Merchant et al., 2012)</td>
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<tr>
<td></td>
<td>ABBV-621</td>
<td>sc TRAIL fusion protein</td>
<td>Phase I</td>
<td>Adult solid tumours</td>
<td>Also known as eftozanermin alfa. Second-generation TRAIL receptor agonist</td>
<td>NCT03082209 (LoRusso et al., 2022)</td>
</tr>
<tr>
<td>Drug Name</td>
<td>Mechanism</td>
<td>Phase</td>
<td>Tumor Type</td>
<td>Other Notes</td>
<td></td>
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<tr>
<td>IFN-γ caspase 8 inducer</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Paediatric solid tumours</td>
<td>Co-treatment with HGS-ETR2 in a clinical trial (NCT00428272)</td>
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<tr>
<td>5-Aza-2’-deoxycytidine DNA methyltransferase inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Also known as decitabine. (NCT00075634, NCT01241162)</td>
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<tr>
<td>Dapsipeptide Histone deacetylase/FLIP inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Also known as romidepsin. (NCT00053963)</td>
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<tr>
<td>EM-1421 Survivin inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Also known as terameprocol (NCT00664586)</td>
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<tr>
<td>pUS-high Survivin minigene DNA vaccine</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>The trial was terminated due to funding constraints (NCT01250470)</td>
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<tr>
<td>SVN53-67/M57-KLH peptide vaccine</td>
<td>Phase I</td>
<td>Glioma</td>
<td>Also known as SurVaxM (NCT03879694)</td>
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<tr>
<td>Bioenergetic pathways-mediated apoptosis Topotecan HIF-1α inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Also known as Hycamtin and Potactasol. It is known as a topoisomerase inhibitor, used as a chemotherapy drug. (NCT00070200)</td>
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<tr>
<td>α-tocopheryl succinate Disrupt mitochondrial stability</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>A vitamin E analogue (NCT00918320)</td>
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<tr>
<td>MIBG Mitochondrial inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Also known as Iobenguane, it is an aralkylguanidine analogue of noradrenaline, typically used as a radiotracer in PET scans. (NCT00253435)</td>
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<tr>
<td>Compound</td>
<td>Type</td>
<td>Phase</td>
<td>Tumour Type</td>
<td>Reference</td>
<td></td>
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</tr>
<tr>
<td>2-DG Glycolysis inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Solid tumours</td>
<td>NCT0096707 (Raez et al., 2013)</td>
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<tr>
<td>3-BP Glycolysis inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Adult solid tumours</td>
<td>NCT03525392 (Baum et al., 2018)</td>
<td></td>
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<tr>
<td>DCA PKD inhibitors</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Adult solid tumours</td>
<td>NCT00566410 (Chu et al., 2015)</td>
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<tr>
<td>DFM Glutaminase inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Also used for the treatment of multiple sclerosis and psoriasis</td>
<td>(Wang et al., 2018)</td>
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<tr>
<td>DON Glutaminase inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Originally isolated from Streptomyces in a sample of Peruvian soil</td>
<td>(Olsen et al., 2015)</td>
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<tr>
<td>SR13800 MCL1 inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Co-treatment of SR13800 with lactate dehydrogenase A inhibitor FX11</td>
<td>(Khan et al., 2020)</td>
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<tr>
<td>CB-839 Glutaminase inhibitor</td>
<td>Phase I</td>
<td>Adult solid tumours</td>
<td>Also known as Telaglenastat</td>
<td>NCT02071862</td>
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<tr>
<td>24S-OHC RIPK1 inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>24S-OHC is an endogenous oxysterol to maintain the brain’s cholesterol homeostasis</td>
<td>(Olsen et al., 2015; Vo et al., 2015)</td>
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<tr>
<td>HVJ-E CaMK II activator</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>HVJ-E is a UV-treated, nonreplicating Sendai virus</td>
<td>(Nomura et al., 2014)</td>
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<tr>
<td>D-galactose Disrupt sugar metabolism</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>D-galactose is a form of sugar that will lead to galactitol formation, which is harmful to cells.</td>
<td>(Li et al., 2011)</td>
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<tr>
<td>Lorlatinib ALK inhibitor</td>
<td>Phase I</td>
<td>NB</td>
<td>A second-generation ALK inhibitor</td>
<td>NCT03107988 (Goldsmith et al., 2020)</td>
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<tr>
<td>Entrectinib ALK inhibitor</td>
<td>Phase I</td>
<td>NB</td>
<td>Also known as Rozlytrek.</td>
<td>NCT02650401</td>
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<tr>
<td>CQ/HCQ</td>
<td>Authophagic inhibitor</td>
<td>Phase</td>
<td>Tumour Type</td>
<td>Treatment</td>
<td>Reference(s)</td>
<td></td>
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<tr>
<td></td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Co-treatment of CQ + ponatinib (ALK inhibitor)</td>
<td>(Corallo et al., 2020)</td>
<td></td>
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<tr>
<td></td>
<td>Phase I</td>
<td>Adult solid tumours</td>
<td>Co-treatment of HCQ + MK2206 (ALK inhibitor)</td>
<td>NCT01480154</td>
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<tr>
<td></td>
<td></td>
<td>NB RAS melanoma</td>
<td>Co-treatment of HCQ with trametinib (MEK inhibitor)</td>
<td>NCT003979651</td>
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<table>
<thead>
<tr>
<th>Spautin-1</th>
<th>Authophagic inhibitor</th>
<th>Pre-clinical</th>
<th>NB</th>
<th>Co-treatment with CQ</th>
<th>(Aveic et al., 2016)</th>
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</thead>
<tbody>
<tr>
<td>Immunotherapy</td>
<td>Anti-GD2 mAb</td>
<td>GD2 inhibitor</td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Ch14.18 mAb</td>
</tr>
<tr>
<td></td>
<td>Phase I</td>
<td>NB</td>
<td>Ch14.18K322A mAb</td>
<td>(Sorkin et al., 2010)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phase II/III</td>
<td>NB</td>
<td>Ch14.18 mAb</td>
<td>(Gilman et al., 2009)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Ch14.18K322A mAb</td>
<td>(Navid et al., 2014)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phase II/III</td>
<td>NB</td>
<td>Ch14.18 mAb was approved by FDA in 2015.</td>
<td>(Yu et al., 2010; Yu et al., 2021)</td>
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<td></td>
<td></td>
<td></td>
<td>Test if 10 days continuous IL-2 infusion will improve the efficacy of ch14.18 mAb treatment.</td>
<td>NCT01701479</td>
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<td></td>
<td></td>
<td></td>
<td>Ch14.18 mAb as an adjuvant with chemotherapy.</td>
<td>NCT01767194 (Mody et al., 2020)</td>
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<tr>
<td></td>
<td>Pre-clinical</td>
<td>NB</td>
<td>Proof of concept for GD2-specific CAR T cells</td>
<td>(Pule et al., 2008)</td>
<td></td>
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<tr>
<td></td>
<td>Phase I</td>
<td>NB</td>
<td>m3F8 mAb CAR T cells</td>
<td>NCT02650648, NCT00877110 (Modak et al., 2018)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>hu14.18K322A CAR T cells</td>
<td>NCT01576692 (Federico et al., 2017) NCT01857934</td>
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<td></td>
<td>Phase II</td>
<td>NB</td>
<td>hu14.18K322A CAR T cells</td>
<td>(Furman et al., 2021; Nguyen et al., 2020) NCT02130869</td>
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<td></td>
<td></td>
<td></td>
<td>ch14.18/CHO CAR T cells</td>
<td>NCT02258815 (Seitz et al., 2021) NCT03242603</td>
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</tr>
</tbody>
</table>

Abbreviation: CAR = Chimeric antigen receptor; CLL = chronic lymphocytic leukaemia; mAb = monoclonal antibody; NB = neuroblastoma; TRAIL = TNF-related apoptosis inducing ligand.
References


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suitable drug for neuroblastoma therapy? Cellular Physiology and Biochemistry, 29(3-4), 373-380. https://doi.org/10.1159/000338492


Valter, K., Zhivotovsky, B., & Gogovadze, V. (2018). Cell death-based treatment of neuroblastoma. *Cell Death & Disease*, 9(2), 113. [https://doi.org/10.1038/s41419-017-0060-1](https://doi.org/10.1038/s41419-017-0060-1)


