



Nanomedicines and cell-based therapies for embryonal tumors of the nervous system

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ABSTRACT

Embryonal tumors of the nervous system are neoplasms predominantly affecting the pediatric population. Among the most common and aggressive ones are neuroblastoma (NB) and medulloblastoma (MB). NB is a sympathetic nervous system tumor, which is the most frequent extracranial solid pediatric cancer, usually detected in children under two. MB originates in the cerebellum and is one of the most lethal brain tumors in early childhood. Their tumorigenesis presents some similarities and both tumors often have treatment resistances and poor prognosis. High-risk (HR) patients require high dose chemotherapy cocktails associated with acute and long-term toxicities. Nanomedicine and cell therapy arise as potential solutions to improve the prognosis and quality of life of children suffering from these tumors. Indeed, nanomedicines have been demonstrated to efficiently reduce drug toxicity and improve drug efficacy. Moreover, these systems have been extensively studied in cancer research over the last few decades and an increasing number of anticancer nanocarriers for adult cancer treatment has reached the clinic. Among cell-based strategies, the clinically most advanced approach is chimeric-antigen receptor (CAR) T therapy for both pathologies, which is currently under investigation in phase I/II clinical trials. However, pediatric drug research is especially hampered due not only to ethical issues but also to the lack of efficient pre-clinical models and the inadequate design of clinical trials. This review provides an update on progress in the treatment of the main embryonal tumors of the nervous system using nanotechnology and cell-based therapies and discusses key issues behind the gap between preclinical studies and clinical trials in this specific area. Some directions to improve their translation into clinical practice and foster their development are also provided.

1. Introduction

Cancer is the second leading cause of death after domestic accidents in children and adolescents in high-income countries [1]. Contrary to adult cancers, which are mostly related to environmental factors, pediatric tumors are mainly caused by genetic and epigenetic factors [2].

Among them, embryonal neural tumors (ENTs) are especially difficult to treat and consequently very lethal. They arise from embryonal tissues of the nervous system through aberrations in the very early stages of development [3]. ENTs include neuroblastoma neuroblastoma (NB), retinoblastoma, atypical teratoid rhabdoid tumor, embryonal tumors with multilayered rosettes, ependymoblastoma, medulloblastoma (MB)

Abbreviations: BBB, blood-brain barrier; DOX, doxorubicin; ENT, embryonal neural tumor; EPR, enhanced permeation retention; HR, high-risk; hNET, human norepinephrine transporters; INRG, international Neuroblastoma Risk Group; MB, medulloblastoma; MIBG, meta-iodobenzylguanidine; NB, neuroblastoma; NCAM, neural cell adhesion molecule; NP, nanoparticle; NSCs, neural stem cells; SHH, sonic hedgehog; SMO, smoothened; STING, stimulator of interferon genes; PTT, photothermal therapy; PTX, paclitaxel; WNT, *Wingless-int1*.

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and primitive neuroectodermal tumors, among others. We focus on MB and NB to illustrate ENTs of the central and peripheral nervous system respectively, as they are the most common ENTs and often present unfavorable prognosis. A major problem faced by ENT patients is treatment resistance and the complex tumor biology encountered [4,5]. The etiology of ENTs is associated with apoptotic machinery failures and genetic aberrations, which leads to increased treatment inefficacy in comparison to other tumors. MB and NB present similarities but also face different challenges. As NB affects mostly neonates and infants, therapeutic protocols are especially difficult to establish [6]. Dose estimation in infants represents a major problem due to the rapid changes in their development and the aggressive nature of the disease. Moreover, for MB, overcoming the blood-brain barrier (BBB) is one of the main difficulties in drug delivery [7]. The BBB protects the central nervous system (CNS) from substances circulating in the blood that may be neurotoxic, but, at the same time, prevents entry of most drugs into the brain. As a result, very few therapeutic molecules reach the brain effectively.

Intensive chemotherapy is often the only tool left for patients presenting the worst prognosis. However, high dose chemotherapy causes severe side effects and may have long-term consequences that can endanger patients' survival in adulthood [8,9]. Studies on the long-term consequences of pediatric cancer treatments emphasize this problem, showing higher mortality and comorbidities in these populations [10–12]. Although survival has certainly increased over the last few decades, there is an urgent need to ameliorate the overall health and quality of life of these pediatric patients throughout their lifetime. In this regard, nanomedicines and cell-based therapies could improve the treatment of ENTs by improving the specific accumulation of drugs in tumors and reducing the appearance of toxicities [13,14]. Nowadays, nanomedicine offers a wider horizon, with the possibility to modulate tumor microenvironment, improve anti-cancer drug efficacy and reduce toxicity. Similarly, cell-based therapies are changing the current landscape of cancer treatment with groundbreaking results [15,16]. The objective of this review is to present the progress made in nanotechnology and cell-based therapies for NB and MB over the last 5 years and highlight the challenges that need to be overcome in order to achieve further clinical translation. We chose to focus on nanomedicines and cell-based therapies to give a wide framework for the most promising novel treatments available for ENTs. First, nanomedicines could help to ameliorate the therapeutic response to conventional drugs but also to new drugs and fragile molecules. This is a major aspect to consider for pediatric population, which are especially fragile and prone to develop long-term toxicities associated with anticancer treatments in 70% of survivors. On the other hand, cell therapy technologies have the potential to offer targeted treatment with high degrees of personalization.

2. Current treatments for embryonal neural tumors

ENTs are disorders of the embryonal development of the nervous system that appear when a pluripotent stem cell proliferates in an uncontrolled manner and does not differentiate to correctly accomplish organogenesis [3]. The embryonal origin of the tissue will determine the type of tumor and the treatment provided. In the case of NB, tumor cells arise in the neural crest, while for MB they originate in the external granular layer. This section will summarize the background and main approaches that are currently employed for the treatment of ENTs.

2.1. Current treatments for neuroblastoma

NB is the most frequent extracranial malignancy affecting between 8 to 10 % of pediatric cancers [17]. This pathology is usually diagnosed in children under 2 years old and represents 15 % of cancer-related pediatric deaths. NB tumors arise from the adrenal gland and the paraspinal chains of the sympathetic nervous system. NB severity is classified by the International Neuroblastoma Risk Group (INRG) staging system, where some patients will spontaneously be cured with no or a very small

amount of treatment and other more severe cases will receive multimodal high-dose therapies. For instance, the survival rate of low-risk NB is above 90 % while it drops drastically to 40 % for high risk (HR)-NB patients [18]. Several genetic abnormalities have been described including the loss of heterozygosity in chromosome 1 [19]. Moreover, NB frequently concurs with abnormalities on chromosomes 11q and 14q [20,21]. Mutations in gene ALK are also very frequent in NB patients and are found in 12 % of familial NBs [22,23]. Finally, the gold standard in NB classification is the amplification of MYCN oncogene, which is strongly associated with HR-NBs and unfavorable prognosis [24].

Surgical resection is the treatment of choice in low-risk patients with localized disease. Patients with intermediate-risk NB may receive chemotherapy to shrink the tumor for surgical resection. Whenever there is a risk of spinal cord compression or respiratory compromise and surgical resection is not possible, chemotherapy will be administered. HR patients receive aggressive multimodal treatment including surgery, chemotherapy, radiation therapy, and consolidation with high-dose chemotherapy. In contrast, the current recommendation for congenital NB is conservative treatment with close follow-up [25]. HR-NB has benefited in the last decade from aggressive multimodal therapy including chemotherapy, primary tumor surgery, high-dose chemotherapy followed by autologous stem cell transplantation, radiotherapy and immunotherapy. Immunotherapy has emerged as a promising approach in the treatment of NB. So far, only two drugs are available for targeted immunotherapy in NB, and both are specific anti-GD2 monoclonal antibodies (dinutuximab and naxitamab), but their application still has many limitations. For instance, naxitamab, which was recently approved in 2020, is indicated only for patients with relapsed or refractory HR-NB in combination with GM-CSF [26]. The development of effective and safe anti-GD2 targeted immunotherapies and the analysis of other potential molecular targets for the treatment of NB is therefore crucial [27–29].

NB can show different levels of genomic instability and harbor a wide variety of numerical and structural genetic abnormalities that reflect the heterogeneous clinical and biological behavior of the disease. Homogeneous amplification of the MYCN oncogene is present in 25% of all NBs. MYCN analysis is routinely performed in clinical practice for treatment stratification and is considered a HR factor. Among patients with stage 4 NBs, it has been possible to identify a subgroup with a very poor prognosis which is characterized by the presence of two or more of the following alterations: deletion 1q, 17p, 19q, ATRX and / or alterations of telomerase reverse transcriptase (TERT) [30]. Activating point mutations (in 10% of cases), as well as amplification of the ALK gene in patients with NB, have also been described. This positions ALK as a promising target for NB treatment, especially considering the positive outcomes observed with ALK inhibitors in malignant lung tumors that are not small cell and that present ALK fusions [31]. HR-NB is the group with the highest mortality within NBs. For those patients who complete treatment, event-free survival at 3 years is currently 40-55% [32,33]. However, 60% of patients relapse and their survival falls below 10% [34,35]. The improvement in the prognosis of these patients is conditioned by a better understanding of the biological subgroups currently included in the so-called HR group and refinement of current treatments within international cooperative clinical trials for rare diseases [27,36].

2.2. Current treatments for medulloblastoma

MB develops in the cerebellum and represents more than 60 % of childhood intracranial embryonal tumors. It is usually diagnosed in children between 6 and 8 years old but can also occur in younger patients and more rarely in early adulthood [37]. The latest classification includes 4 subgroups of MB: sonic hedgehog (SHH), WNT(Wingless/int1), group 3 and group 4 [38]. In the case of WNT, the upstream signaling pathway is upregulated while SHH presents an aberrant activation of the SHH signaling cascade. The SHH group represents 30 % of all MB and has a variable prognosis [39]. Group 3 presents the worst

outcome and the amplification of MYCN is found in almost 20 % of cases. This also classifies the MYCN amplification as a characteristic oncogenic biomarker of MB. Group 4 does not present any specific well-established genetic aberrations, but abnormalities associated with chromosome 17 are found in some patients. MB represents almost 20 % of brain tumors in children and the survival rate is very variable depending on the tumor biology. Risk stratification is based on age, molecular group, presence of metastasis at diagnosis and the amount of tumor left after surgery [40].

There has been a consensus on MB treatment based on a triple approach: surgery, radiotherapy, and chemotherapy. Although Cushing had already pointed out that the survival increase for patients undergoing complete resection compared to those undergoing a biopsy, the importance of the degree of resection has been analyzed subsequently [41]. In general, all studies have shown a similar prognosis for patients without tumor remains after surgery and those undergoing gross total resection. The recommendation is to reach maximum tumor resection with minimal risk for the patient. Currently, although gross total resection is the surgical goal, similar results can be achieved with minimal tumor remnants. Therefore, neurosurgeons must weigh up the potential neurological morbidity of a broader resection against leaving tumor remains. In addition, surgery should also enable the obtention of sufficient tumor tissue for both histopathological and molecular diagnosis [42].

For decades, in children older than 3-5 years with standard risk MB and complete tumor resection, treatment has been completed with radiotherapy and chemotherapy to prevent recurrence [43–45]. Patients receive 23.4 Gy craniospinal radiotherapy with a 54 Gy boost on the tumor bed. In addition, in most current protocols the approach includes chemotherapy, initiating weekly vincristine during radiotherapy followed by several cycles of cisplatin, vincristine cyclophosphamide or lomustine [44–46]. For patients with metastatic disease, the recommended doses of radiotherapy are more variable, although the most widely recommended doses are 36 Gy craniospinal radiotherapy with a 54 Gy boost on the tumor bed and 50 Gy on metastatic nodules [43,47].

However, without molecular classification, chemotherapy for patients with metastatic disease is not yet well established. Therefore, the current objective is the stratification of patients both by histological subtype and molecular biological factors that can determine clinical behavior and help to optimize the treatment stratification [42]. The analysis of these variables and their clinical relevance can allow prognosis and risk estimation, and condition a wide range of disease stratification. About 30% of patients diagnosed with MB are considered HR-MB based on one of these risk factors: metastatic disease, anaplastic or large-cell histology, MYC amplification or significant post-surgery tumor remains.

There is an urgent need to improve survival and quality of life for patients with HR-MB. For this reason, it is essential to carry out biological analyses in tumor samples to identify which patients have HR disease but with a good prognosis and can be treated as standard risk MB, and to identify those patients who will not respond to conventional therapies, who might be candidates for phase I-II clinical trials. To date, the best approach to these new treatments includes high-dose chemotherapy pre (or post) craniospinal radiation therapy, hyperfractionated and accelerated radiation therapy (HART twice daily), and conventional craniospinal radiation therapy (once daily), most commonly before maintenance chemotherapy. The relative benefits of these therapeutic strategies have not been systematically evaluated and have not taken into account the biological heterogeneity of the disease. In addition, the toxicities and the derived side effects have not been assessed either. This makes clear the need for multicenter international trials to assess which of these strategies offers survival advantages.

The role of surgery for relapsed or recurrent MB has not yet been defined. Some studies point to the possible benefit of subsequent resections. In any case, given the divergent evolution and clonal selection derived from the treatment, surgical resection or a new biopsy may

allow a new molecular perspective to be developed that can guide treatment for relapse.

Many current clinical trials are incorporating new approaches derived from the molecular knowledge of MB. One of the challenges is treatment de-escalation in the WNT MB to reduce toxicities derived from the treatment and improve quality of life while maintaining survival rates. For example, NCT02066220, NCT01878617 and NCT02724579 trials evaluate the reduction of craniospinal radiation therapy and tumor bed boost with reduced chemotherapy. Another trial, NCT02212574, which assessed the combination of surgery and radiotherapy without radiotherapy, was discontinued [42]. Other current studies are evaluating the incorporation of targeted therapies for SHH MB. For years, SHH signal inhibition has been considered a potential therapeutic target for those MBs with alterations in the genes of the SHH pathway. Previous studies have shown improved survival in patients with SHH MB using vismodegib (GDC-0449) and sonidegib (LDE225), competitive antagonists of the smoothed (SMO) receptor, a protein of the SHH pathway [48–50]. Nonetheless, SMO receptor antagonists such as vismodegib can cause adverse reactions [51] and should be considered carefully before use in pediatric populations. As another example, the NCT01878617 trial provides a model for incorporating subgroup and risk stratification into molecular studies. The trial combines molecular subgroups and clinical and cytogenetic characteristics to stratify patients. Patients with low-risk WNT MB receive reduced-intensity therapy, patients with mature SHH tumors receive vismodegib (in addition to standard treatment, and patients with standard-risk, HR non-WNT/non-SHH tumors receive intensified treatment with pemetrexed and gemcitabine) [48].

That being said, childhood cancer survivors still face many challenges including fertility problems and physical dysfunctions but also severe psychological distress [52]. The health dysfunctions described among childhood cancer survivors are usually caused or associated with curative treatments, mostly chemotherapy and radiotherapy [53]. The St Jude Lifetime Cohort Study has demonstrated that almost all childhood cancer survivors develop a severe chronic health condition in adulthood [10]. The prognosis is significantly worse for patients presenting recurrence and who have undergone high-dose treatments [54], suggesting the need to develop novel treatments with an improved therapeutic index.

3. Nanomedicines and cell-based therapies for embryonal neural tumors

Nanomedicine is a medical field that employs nanometric systems for the treatment, diagnosis and prevention of diseases [55,56]. In past decades, nanomedicine has provided major contributions to oncology by improving the therapeutic index of anticancer drugs by enhancing safety [57,58]. The efficacy of nanomedicines for cancer research relies on three distinct principles: indirect targeting through the enhanced permeation retention (EPR) effect, direct targeting with specific ligands, and stimuli-responsive targeting. Although many studies suggest that the EPR effect is not the general rule for nanomedicine delivery, most commercialized nanomedicines for cancer treatment are based on this principle [59]. It should be noted that all the nanomedicine-based approaches mentioned in the following sections are in the preclinical stage, as none have reached the clinic for either NB or MB. On the other hand, cell-based therapies can be defined as the administration of living cells into a patient's organism to produce a biological effect. Both adult stem cells and chimeric-antigen receptor(CAR) T cells have emerged as promising approaches to tackle ENTs. Adult stem cells have intrinsic tumor-trophic properties and have been used to facilitate the tumor-targeted delivery of anticancer drugs. Additionally, CAR T are genetically engineered T cells that enable us to selectively attack cancer cells that express specific antigens. CAR T therapy has been preferentially investigated for blood malignancies and its efficacy in solid tumors has recently begun to be investigated. In this section, we summarize recent advances in nanomedicine and cell-based therapies for ENTs treatment

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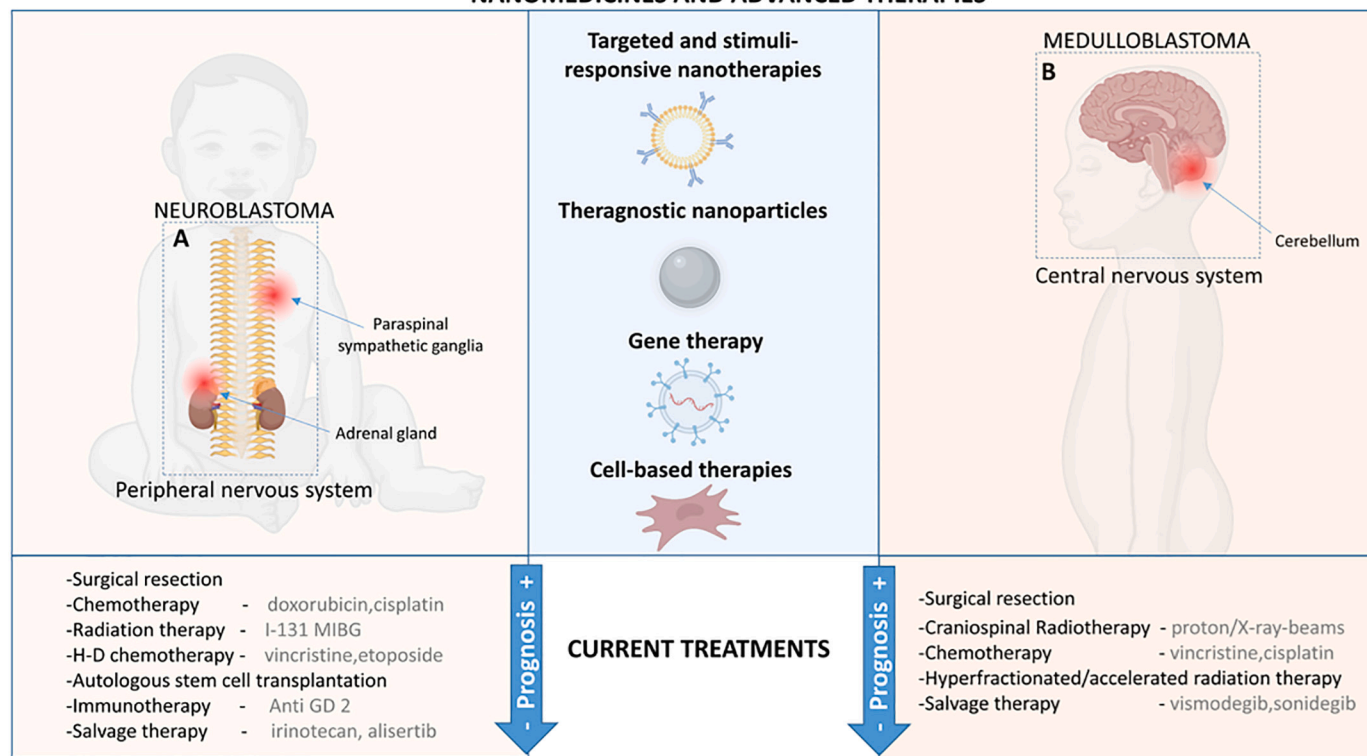


Fig. 1. Prognosis-driven current treatment for embryonal tumors of the peripheral (A) and central nervous system (B) combines the application of surgery, chemotherapy, radiation and immunotherapy. The most advanced therapies tested in NB and MB tumor models are mainly directed to the design of targeted/stimuli-responsive or theragnostic nanoparticles, gene and/or cell-based therapies.

(Fig. 1).

3.1. Nanomedicines and cell-based therapies for neuroblastoma treatment

Considerable advances in the development of nanomedicines and cell-based therapies for treating NB more effectively have been made in recent years. However, the need for valid preclinical NB models has not been met so far, and the translation to clinical practice is slow [60]. A general overview of different types of nanomedicines including non-targeted, targeted, stimuli-responsive, and theragnostic nanomedicines for NB treatment will be provided in this section. Then, the potential use of cell-based therapies will also be covered.

3.1.1. Non-targeted nanomedicines

There are numerous studies involving non-targeted cancer nanomedicines for NB, with polymeric nanoparticles (NPs) being investigated most (Table 1). Notably, nanomedicines can be employed to encapsulate many well-established anticancer drugs such as doxorubicin (DOX), 5-fluorouracil, temozolomide and etoposide [61–65], but also more innovative therapeutics such as the small molecule withalongolide, the bioactive compounds from *Posidosina oceanica* [66,67], or nucleic acids [68,69].

siRNA has taken on crucial importance in cancer therapy as it suppresses the carcinogenic expression of oncogenetic markers [70]. However, these negatively charged molecules are highly unstable under physiological conditions and are poorly taken up by cells. For this reason, many nanomedicines, in particular, cationic and especially ionizable lipid-based particles, have emerged to deliver nucleic acids [71]. In the area of NB, MXD3 was identified as a highly expressed oncogene in cell lines and primary tumors [68]. Therefore, MXD3 siRNA was encapsulated in superparamagnetic iron oxide NPs and tested pre-clinically (Table 1). MXD3 siRNA was electrostatically attached to the NP surface obtaining positively charged particles (42.2 mV) of around

56 nm with high loading efficiency. The lockdown of MXD3 gene was associated with an increase in annexin V and caspase activity, thus causing apoptosis in NB cell lines.

One of the potential applications of nanomedicines involves the co-loading of various drugs for synergistic combination therapy. For instance, the novel molecule cantharidin was conjugated with platinum derived (IV) (picoplatin (IV)) within liposomes to enhance efficacy in a Neuro-2a NB mouse model [72]. Platins are well known chemotherapeutic agents and among them, picoplatin can overcome chemotherapy resistance when compared to cisplatin or carboplatin [73]. Interestingly, picoplatin is only active in its (II) form but also very toxic. For this reason, platinum (IV) was encapsulated and meant to be reduced in cancer cells to its (II) form. Similarly, cantharidin is a molecule derived from the poison of blister beetles and is an inhibitor of phosphatases 1 and 2a, provoking cycle cell arrest and apoptosis [74]. Two hydrophobic cantharidin molecules were attached in an axial position to the platinum (IV) to improve the loading in the lipid bilayer of liposomes, reaching 85% of encapsulation efficiency. Tumor biodistribution was enhanced and consequently higher tumor reduction was observed, suggesting that these combinative nanomedicines are effective for NB therapy. Moreover, combining synergistically drug molecules can increase efficacy while reducing the dose of chemotherapy administered [75]. As both picoplatin and cantharidin are highly toxic, even at low concentrations, toxicity studies were performed in mice showing lower systemic toxicity for NP formulation. AST, ALT, BUN and CK levels were significantly reduced for NPs when compared to the equivalent free treatment of picoplatin and cantharidin at a 1:2 ratio.

Immunotherapy's response directly depends on tumor immunogenicity. MYCN amplified HR-NB concur with low tumor immunogenicity, making them much less sensitive to immunotherapy [76]. For this reason, the induction of immunogenic responses specifically against tumors sparing healthy tissues is an interesting solution to this problem. This idea is addressed in an insightful study involving CCL21 chemokine

Table 1
Non-targeted nanomedicines for neuroblastoma treatment under preclinical evaluation.

Therapeutic molecule	Type of NP	Main outcomes	Ref
Inorganic NPs			
	Graphene oxide nanoribbons	Graphene oxide nanoribbons increase ROS production and induce autophagy in vitro in NB only in the first 48h of exposure. NPs are preferentially internalized in NB cells (SK-N-BE(2) and SH-SY5Y)	[82]
	Graphene oxide AgNPs	In vitro efficacy on SH-SY5Y differentiation of pluripotent NB cells with the action of graphene oxide and silver.	[80]
	Zinc oxide	Zinc oxide NPs induce SH-SY5Y cell death by autophagy with a TRPC6 Ca ²⁺ influx.	[83]
	Zinc oxide	Zinc oxide NPs extracted from Chinese fruit <i>Clausena lansium</i> have a cytotoxic activity towards SH-SY5Y.	[84]
Curcumin	Cerium oxide	Curcumin ceria NPs coated with dextran proved to be effective against MYCN amplified cells. Ceria coated dextran nanocarriers enable a slow and controlled release of curcumin.	[85]
Retinoic acid	Ag-Bi ₂ Se ₃ with RNA three-way junction-RA on the surface	Combination of RNA and retinoic acid to boost cell differentiation in SH-SY5Y cells in vitro.	[81]
Polymeric NPs			
Anthocyanin	PAMAM and silica	Nanomedicines induce cytotoxicity in Neuro2a cells but not in Vero (not carcinogenic) cells.	[86]
CCL21	Alginate, CaCl ₂ , protamine sulfate and Pluronic F-127	CCL21 was successfully encapsulated in alginate nanomedicines and increases CCL21 immunotherapy and efficacy.	[77]
DOX	PLGA, PLGA-PEG or PLA	A pilot study where different polymers are compared to deliver DOX. PLGA presented better physicochemical properties and good in vitro efficacy. Transport-mediated drug efflux could not be avoided.	[62]
DOX	Cyclodextrin-fibrin gels	Cyclodextrin nanomedicines loaded in fibrin gels improve DOX efficacy and reduce toxicity in vivo locally in an SH-SY5Y model of NB.	[61]
5-fluorouracil	Poly(Am-coDADMAC) nanogels	Hydrogels in the nanosized range seem hemocompatible and enhance efficacy in vitro.	[63]
GAMP	Polymer-PEG	Activation of the STING pathway generates an immunogenic response and increased tumor reduction in vivo due to cGAMP-nanomedicines.	[78]
mi RNA	PAMAM dendrimers	The combination of miRNA nanomedicines with NK exosomes enhances in vivo antitumor efficacy.	[87]
MXD3 SiRNA	PEI with SPIONs	Identification of MXD3 as a therapeutic target in NB. Use of SPIONs-PEI nanomedicines to carry MXD3 SiRNA as a	[68]

Table 1 (continued)

Therapeutic molecule	Type of NP	Main outcomes	Ref
<i>Psidonia oceanica</i>	Chitosan or soluplus polymer	combination therapy with DOX, vincristine, cisplatin and maphosphamide.	[67]
Paclitaxel	PLGA	Soluplus nanomicelles maintain <i>Psidonia oceanica</i> efficacy and improve the inhibition of cell migration. Pilot study of PLGA-paclitaxel-NPs that maintained anticancer efficacy in vitro and provoked DNA damage in SH-SY5Y cells.	[88]
SN-38	PLA nanofiber	Nanofiber matrices are used as adjuvants in surgery to treat cancer cells locally at the tumor site.	[89]
SN-38-TOA	PLA-PEG	Conjugation of SN-38 with TOA increases efficacy (mitocan action of TOA) in xenograft NB model when compared to commercially available form irinotecan.	[75]
Temozolomide	Alginate-TiO ₂	Hybrid nanomedicines reduce oxidative and inflammatory markers in NB models while maintaining efficacy in vitro and reducing MAPK and NK- κ B expression.	[64]
Lipid NPs			
Cantharidin-platinum (IV) conjugate	Cholesterol, DSPC, DPPC, DSPE-MPEG _{2k}	The combination of two anticancer agents in liposomes enhanced tumor accumulation and growth inhibition.	[72]
Etoposide	Solid lipid (Precirol®)	Combination therapy with RGD peptide (cilengitide) that targets the tumor cell-ECM interaction.	[65]
siRNA-Dy677, DNA-Cy3	DOTMA and DOPE	Nanovesicles that showed good transfection capability and good siRNA delivery to tumor in a Neuro2a model.	[69]
Sphingadienes	DMPC	Sphingadiene nanomedicines inhibit tumor growth in NB preclinical models by suppressing the AKT pathway at a dose of 5 mg/kg BID.	[90]
TLR-9 agonist and PC7A	Bacterial membrane nanoparticle and surface decorated with maleimide	Radiation therapy is followed by intratumoral injection of nanomedicines that capture neoantigens boosting their internalization into dendritic cells and enhancing T cell activation.	[91]
Protein NPs			
5 fluorouracil	Lactoferrin-TC in a ZIF 8 framework	Lactoferrin-TC (a photosensitizer) and 5 fluorouracil carried in a ZIF-8 framework present anticancer activity in vitro and are safe in vivo in rats.	[92]
Withalongoide	sHDL	sHDL-withalongoide-NPs have antitumoral properties in vitro towards various NB cells and these NPs efficiently deliver withalongoide to NB tumors in vivo.	[66]

BID, twice a day; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine DOTMA, 1,2-di-O-octadecyl-3-trimethylammonium propane, DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DSPE-MPEG_{2k}, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Methoxyl(polyethylene glycol)-2000]; DOX, doxorubicin; ECM, extracellular matrix; GAMP, guanosine monophosphate-adenosine

monophosphate; $Gd(NO_3)_3$, gadolinium oxide; NB, neuroblastoma; NP, nanoparticle; PAMAM, polyamidoamine; PEG, polyethylene glycol; PEI, polyethylenimine; PLA, Poly(lactic acid); PLGA, poly(lactic-co-glycolic acid); Ref, reference; RGD, Arginylglycylaspartic acid; SPION, superparamagnetic iron oxide nanoparticles; TC, Titanocene dichloride; TOA, D- α -Tocopheryloxyacetic acid; ZIFs, zeolitic imidazolate frameworks.

loaded in alginate NPs [77]. CCL21 was efficiently loaded and presented a slow-release profile at high drug loading, good stability and increased antitumor efficacy *in vivo* when compared to controls and free CCL21, demonstrating the potential of nanotechnology for cancer immunotherapy. Nevertheless, these nanomedicines were injected intratumorally, limiting their applicability for disseminated and metastatic NBs. Nanomedicine can also help to stimulate tumor immunogenicity. The stimulator of the interferon genes (STING) pathway is expressed in most cancer cells and its activation enhances the capacity of the innate immune system to sense and target tumors. For example, by encapsulating a STING analogue called cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) in PEG-coated polymeric NPs (STING-NPs) promising results were found [78]. STING-NPs delivered cGAMP intracellularly and induced the activation of the STING signaling pathway, which was confirmed by a quantitative qRT-PCR of the transcript levels of downstream antitumor effectors in both MYCN amplified (9464D, LAN-1) and MYCN non-amplified cell lines (Neuro-2a, SK-N-SH). Moreover, STING-NPs increased caspase 3 levels in all cell lines, demonstrating their potential use as apoptosis inducers. *In vivo*, these NPs triggered immunogenic cell death through ATP release, HMGB-1 release, the surface expression of calreticulin, dendritic cell activation and phagocytosis.

Since cancer cells can lose their high replication capacity and pluripotency by differentiation, some therapeutic strategies for NB follow this approach. In the clinics, 13-cis retinoic acid is already used in HR-NB as differentiation therapy in the maintenance stage of the treatment process [79]. As an example, when SH-SY5Y cells were exposed to graphene-oxide silver NPs, a neurite outgrowth was observed with no

sign of apparent cytotoxicity [80]. In another study, bismuth selenide core-silver NPs were combined with a novel three-way junction RNA conjugated to retinoic acid [81]. The three-way junction RNA had three specific missions: to boost retinoic acid's efficacy, cell-penetration and inhibition of micro-RNA-17 that promotes cell differentiation and consequently enables retinoic acid release by strand displacement. After 6 to 9 days of exposition to NPs, SH-SY5Y cells reached and maintained maximum levels of neuronal differentiation markers (MAP2, nestin and Tuj 1), which suggests that NPs enhanced cell differentiation when compared to free retinoic acid, which poorly differentiated SH-SY5Y cells. As depicted in this section, nanotechnology could greatly contribute to treating NB more effectively than conventional therapeutic agents by using non-targeted NPs, which rely mostly on the EPR effect.

3.1.2. Targeted nanomedicines

NB cells can be actively targeted by linking specific surface receptors or ligands to NPs. For instance, GD 2 ganglioside, neural cell adhesion molecule (NCAM) and human norepinephrine transporters (hNET) are overexpressed in $\geq 90\%$ of NB cancer cells (Fig. 2) [93]. Nevertheless, we should always bear in mind that these molecules are also expressed at low levels in healthy tissues. SSR receptor is also overexpressed in a considerable number of NBs [94], although other receptors have also been described as shown in Table 2. For instance, nipecotic acid is commonly employed to inhibit GABA uptake [95] and can be used as a targeting moiety for NPs [96]. Bhunia *et al.* used a nipecotic acid-derived cationic amphiphile to surface decorate liposomes containing paclitaxel and CDC20siRNA [97]. CDC20siRNA is a key cell cycle molecule needed to complete mitosis which is overexpressed in numerous types of cancer cells. However, efficacy and targeting were only demonstrated in a preclinical model of NB that overexpresses GABA_A receptor (IMR-32), which is not representative of the heterogeneity of NB, and therefore the active targeting could be minimal in the clinic.

hNET is overexpressed in most NB tumor cells [98], hence it has been extensively exploited as a therapeutic target and diagnostic tool. Meta-

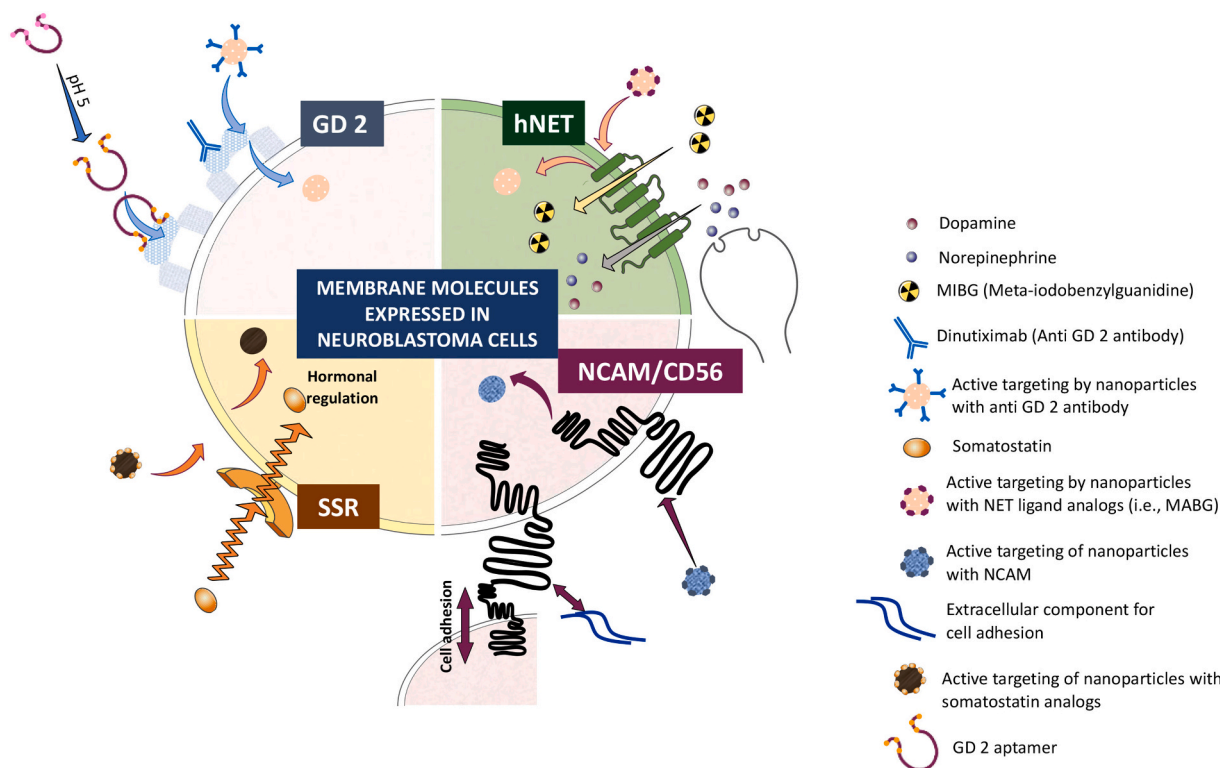


Fig. 2. Graphical representation of the main membrane molecules expressed in neuroblastoma cells. GD 2, hNET, SSR and NCAM/CD56 are represented together with the main physiological and therapeutic molecules or diagnosis tools used for NB management.

Table 2
Targeted nanomedicines for neuroblastoma treatment under preclinical evaluation.

Drug	Type of NP	Targeting agent	Main outcomes	Ref
Inorganic NPs				
	Mesoporous silica-PEG	MABG	MABG targeting enhanced NP uptake in NB xenograft mice model by Y-shaped structured scaffolds that have 2 MABG based binding points.	[100]
Camptothecin	SiO ₂ microtubes	anti-p75NTR antibody	Microtubes were modified by silanization and by the attachment of antibodies to the surface. The antibody specifically targeted p75NTR overexpressed in hNET receptor and increased specifically efficacy in NB cells.	[102]
DOX	SiO ₂ -LDH	Bevacizumab	Bevacizumab surface modification enhances tumor targeting by increasing delivery to tumors overexpressing VEGF in vitro and in vivo	[108]
Polymeric NPs				
DOX	PLG and calcium carbonate	RVG	Calcium carbonate in acidic conditions generated gas, enabling an accelerated release of DOX. RVG enabled to specifically target NB cells	[109]
DOX	PLGA	DAS	DAS is a peptide derived from RVG structure that enhances NB targeting and efficacy in vitro.	[110]
PTX	PGA	NTP	NTP-NPs enable to increase the dose of paclitaxel, augmenting tumor reduction without increasing toxicity	[106]
PTX	PG	NTP	NTP is attached to nanomedicines by a union with PEG improving tumor targeting in vitro but does not significantly improve efficacy in vivo.	[107]
siRNA cocktail (siMyc, siBcl-2, siVEGF)	PLGA	RVG	RVG was coupled to NPs by covalent conjugation using carbodiimide chemistry. RVG-PLGA increased cell uptake and	[111]

Table 2 (continued)

Drug	Type of NP	Targeting agent	Main outcomes	Ref
			tumor targeting. RVG-PLGA significantly increased efficacy in vivo in mice when compared to naked PLG.	
Lipid NPs				
CDC20siRNA + paclitaxel	Cholesterol, DCA and DSPE-(PEG) ₂₇ -Amine	NACA	NACA helped to specifically distribute NPs towards tumors sparing organs in biodistribution studies in mice. The combination of paclitaxel and CDC20siRNA enhances tumor growth inhibition in mice xenografted human NB.	[112]
DOX	Cholesterol And DSPE-(PEG) ₂₀₀₀	TP pep	TP-pep homing peptide increases drug delivery in NB tumors and increases tumor reduction in different types of NB mice models.	[113]
Protein NPs				
DOX	GD2-Aptamer	GD2	GD2 aptamer increases the tumor targeting efficiency when compared to conventional GD2 antibody	[105]
Ellipticine	Ferritin	GASNGINAGYL and SLWERLAYGY	Surface decoration by homing peptides increases efficacy in vitro by targeting hNET. GASNGINAGYL presented better results in terms of cell uptake.	[101]

DCA, Dicationic amphiphile((n-C₁₆H₃₃)₂N⁺(CH₃)CH₂CH₂N⁺(CH₃)₃·2Cl⁻); DOX, doxorubicin; DSPE-(PEG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)]; MABG, meta-aminobenzylguanidine; NACA, nipe-cotic acid-derived cationic amphiphile; NB, neuroblastoma; NP, nanoparticle; NTP, neural cell adhesion molecule targeting peptide; Ref, references; RVG, rabies virus glycoprotein; PTX, paclitaxel; PG, dendritic polyglycerol; PGA, poly (glycolic acid); PLGA, poly(lactic-co-glycolic acid); TP pep, tissue-penetrating peptide; VEGF, vascular endothelial growth factor.

iodobenzylguanidine (MIBG) is a radioactive analogue of norepinephrine that binds hNET and is approved as an imaging agent able to target NB tumors (Fig. 2). Nevertheless, MIBG has poor retention in NB cells and can be toxic to healthy tissues at high doses due to its radiotoxicity [99]. MIBG non-radioactive analogues can also be employed as targeting agents for nanomedicines, MABG, for example, contains an amino group instead of the conventional radioactive iodine [100]. Finally, hNET overexpression is a versatile feature that can be targeted with other molecules such as homing peptides [101] or antibodies [102].

GD 2 ganglioside is highly expressed in neuroectodermal tumors [103,104] but also in healthy tissues, which causes unavoidable side effects. This can be solved by slightly modifying the recognition domain of the anti GD2 antibody, for example by using aptamers [105]. pH-sensitive anti-GD2-aptamers carrying DOX were synthesized to specifically target NB cells overexpressing GD2 ganglioside and spare healthy

tissues which have normal/low expression of GD 2 ganglioside. The aptamer is pH sensitive which means that due to the low pH of the tumor microenvironment, the conformation of the binding site changes and can bind GD2 ganglioside only in this specific environment, sparing healthy tissues (Fig. 2). Interestingly, this treatment was also compared to dinutuximab in terms of efficacy and nerve damage. Notably, there was no difference between the two treatments in terms of efficacy in mice, but the nerve damage score was much higher in the dinutuximab group indicating that the aptamer formulation maintains efficacy and reduces nerve damage toxicity *in vivo*.

Efforts have also been devoted to the development of nanomedicines targeted to NCAM, which is often associated with metastatic and aggressive NBs with poor prognosis [106,107]. In one example, NCAM targeting peptide (NTP) was synthesized and bound to polymeric NPs. PTX was conjugated to poly(glycolic acid) (PGA) with a hydrolysable ester link, while NTP was bound to PGA with a non-cleavable amide bond. These nanomedicines maintained their cytotoxic activity and inhibited IMR-32 cell migration. Moreover, the maximum tolerated dose was augmented *in vivo* thanks to PGA-PTX-NTP from 15 mg/kg up to 30 mg/kg. No relevant side effects were reported for a dose of 30 mg/kg when administrating PGA-PTX-NTP in mice. The targeted nanomedicines were more effective in terms of anticancer activity *in vivo* when compared to non-targeted ones allowing an increase in the dose of chemotherapy administered while reducing the appearance of toxicities. Despite the great progress achieved with targeted nanomedicines, several challenges remain. For instance, the evaluation of targeted nanomedicines often involves only one or two cell lines in both *in vitro* and *in vivo* animal models of the disease. Thus, translation to clinical practice is very limited due to the high heterogeneity found in NB patients.

3.1.3. Stimuli-responsive nanomedicines

There has also been work towards the development of stimuli-responsive nanomedicines for NB. Stimuli-responsive nanomedicines are based on the principle that NPs' content is released in response to a specific stimulus or specific environmental conditions [114]. A stimulus can be originated inside the body (endogenous stimuli) but also from exogenous sources such as light, ultrasounds or temperature. Table 3 summarizes the stimuli-responsive nanomedicines studied for NB treatment in the last 5 years. Most of these studies involve exogenous sources such as light, temperature and magnetic fields. For endogenous stimuli, the principle relies on specific tumor features such as tumor microenvironment markers or low pH. For example, a pH-sensitive nanovesicle loaded with DOX and gefitinib and coated with an amphipathic polymer mPEG-P(Asp(DBA-co-MEA)-Phe) released both drugs inside tumor cells responding to increased levels of GSH and lysosomal pH (pH = 5) [115]. Interestingly, nanovesicle stability was partially attributed to the cross-linking of disulfide bonds in oxidizing conditions. At low pH levels, amino groups of P(Asp (DBA-co-MEA)-Phe) protonated causing hydrolyzation and disaggregation of the block. This is a pH-responsive feature that enables the release of both drugs intracellularly. *In vivo*, the nanovesicles accumulated in the tumor and significantly enhanced tumor reduction when compared to either free or single treatments. Another interesting pH-responsive nanomedicine was proposed by adding mineralized calcium carbonate within the core of the nanomedicine. Calcium carbonate ionizes at low pH, triggering the generation of carbon dioxide gas within the nanomedicine and therefore the release of the drug (DOX) [109]. To enhance tumor targeting, a peptide was attached to the NP surface. Results showed that efficacy was maintained *in vivo* while the toxicity (*i.e.*, body weight) was decreased with stimuli-responsive and targeted stimuli responsive nanomedicines when compared to free DOX.

Exogenous stimulation can lead to structural changes in the nanomedicine's conformation leading to a specific antitumor action. For instance, localized hyperthermia treatment can cause cell death in NB tumors thanks to magnetic nanomedicines through the application of an

Table 3

Stimuli-responsive nanomedicines for neuroblastoma treatment under preclinical evaluation.

Type of NP and drug content	Main outcomes	Ref
Photothermal		
PBNPs	Photothermal therapy using PBNPs in a Neuro-2a preclinical model of NB improves long term survival <i>in vivo</i> when administered in the optimal temperature range.	[118]
PBNPs	Intratumor administration of PBNPs and PTT followed by immunotherapy anti-CTLA-4 led to complete tumor remission <i>in vivo</i> and long-term survival in 55% of mice compared to 12% in CTLA-4 treated animals and 0% in PBNPs -PTT treated and untreated groups.	[122]
PBNPs coated with CpG oligodeoxynucleotide	Combination of Prussian blue and CpG with layer-by-layer coating technique increase tumor immunogenicity and enables complete tumor remission by an intratumoral administration.	[121]
UV radiation		
DOX loaded in ZIFs-TNT	TNTs have a photocatalytic activity ZIFs and release DOX <i>in vitro</i> when exposed to UV radiation.	[125]
Alternating magnetic field		
PEI-MNPs	Magnetic hyperthermia therapy <i>in vitro</i> enhances cytotoxicity when compared to exogenous hyperthermia therapy in NB.	[123]
PEI-TPP-IONPs	Magnetic nanocarriers loaded with miRNA-34a enhance miRNA expression while suppressing MYCN <i>in vitro</i> and triggering heat.	[117]
Iron oxide-FDG	Magnetic hyperthermia cytotoxicity <i>in vitro</i> in NB cells.	[116]
Fe ₃ O ₄ core-polymeric shell nanospheres, PVP-gold NPs and cisplatin polymeric NPs	Combination of three NPs to achieve photo-magnetic irradiation and sensitization to tumors combined with thermoresponsive cisplatin nanospheres.	[124]
Ultrasounds		
DOX-Liposomes	Sonoporation with microbubbles increases nanomedicines uptake and efficacy <i>in vivo</i> .	[126]
External beam radiation Fe ₃ O ₄ -TiO ₂ .DOPAC or DOPAC/MIBG	DOPAC helped to attach MIBG to nanoparticles. Nanodevices increase radiation toxicity after beam radiation, acting as radiosensitizers.	[119]
CeO ₂ monodisperse	pH <i>In vitro</i> characterization of ceria NPs boosted by Mn that induce preferential cytotoxicity in cancer NB cells.	[127]
DOX-GFP-PCL	Improved antitumor activity <i>in vitro</i> and enhanced drug release in acidic conditions (pH=5).	[128]
DOX- IONPS-Pluronic F127	Hybrid pH-sensitive nanomedicine delivers DOX at low pH (pH=5).	[129]
DOX and gefitinib- Nanovesicle with mPEG-P(Asp(DBA-co-MEA)-Phe)	pH-responsive NPs tested in an <i>n</i> -Neuro-2a preclinical model. Drug content is released by lysosomal escape and reduction in acidic extracellular media.	[115]
Withaferin A- Polymer [Poly(<i>N,N</i> -dimethylacrylamide)]	Withaferin induces ferroptosis and withaferin pH-sensitive NPs are a suitable carrier for this novel molecule.	[130]

DBA, dibutyl ethylenediamine; DOPAC; 3,4-Dihydroxyphenylacetic acid; DOX, doxorubicin; FDG, fluorodeoxy glucose;IONPs, iron oxide nanoparticles; MIBG, metaiodobenzylguanidine;MNPs, magnetic nanoparticles; NB, neuroblastoma; NP, nanoparticle; Prussian blue NPs PBNPs; PCL, polycaprolactone PEI, polyethylenimine; PTT, photothermal therapy; PVP, polyvinylpyrrolidone;Ref,

reference; TNTs, titanium dioxide nanotube; TPP, Sodium tripolyphosphate; ZIFs, zeolitic imidazolate frameworks.

alternating magnetic field [116,117]. This concept is also illustrated by photothermal therapy (PTT), where nanodevices are exposed to specific light and generate heat able to destroy cancer cells but also stimulate tumor immunogenicity by the release of tumor endogenous elements and antigens [118]. Phototherapy can be used in many settings, as for Fe₃O₄-TiO₂ nanocomposite surface coated with 3,4-dihydroxyphenylacetic acid and MIBG used as a radiosensitizer [119]. When exposed to photon energy these nanocomposites have catalytic properties able to produce ROS species and enhance cell death when exposed to radiation in mice. This type of photosensitizing treatment, administered before radiotherapy, could help to reduce radiation exposure which could entail long-term benefits for NB patients.

Prussian blue PTT is very versatile as it can be combined with magnetic targeting agents such as iron oxide in a theragnostic setting [120] or with conventional chemotherapy or immunotherapy [121]. For instance, one study involves the combination of PTT and immune therapy through Prussian blue NPs and anti-CTLA-4 checkpoint inhibition antibodies [122]. Prussian blue has already been approved by the FDA for oral delivery to treat radioactive poisoning, including in pediatric populations. In the first study, Prussian blue NPs were administered intratumorally in mice, reducing tumor growth and followed by an immunostimulatory reaction with anti-CTLA-4. PTT was unable to ameliorate long-term survival when administered as a single treatment. However, tumors were completely eradicated, and long-term survival was increased when PTT was combined with anti-CTLA-4 immunotherapy. It has also been stated that PTT can eliminate tumors in other types of cancers, but complete remission has not been described in NB bearing mice using this agent. This reinforces the need to use PTT in combination with other treatments such as radiotherapy and immunotherapy when treating NB. Remarkably, this nanomaterial is approved by the FDA for pediatric populations, and this is a major positive aspect that must be taken into consideration. Other safe materials have been suggested to induce PTT such as indocyanine green, which is also approved and used for biomedical purposes. Additionally, there are other types of thermal therapies under investigation, such as magnetic hyperthermia [123] or the combination photomagnetic therapy and thermoresponsive nanomedicines [124] (Table 3), although all of them are in the early stages of development.

3.1.4. Theragnostic nanomedicines

Theragnostics enable monitoring tumor progression while treating it, avoiding extra-invasive procedures [131]. The concept of theragnostics has gained some interest in NB nanomedicine. For instance, Gd(NO₃)₃ coated with rabies virus glycoprotein can target NB tumors and be used for MRI/fluorescence imaging to enable precise tumor removal by surgery [132]. Similarly, Fe₃O₄-Gd Prussian blue NPs are used as imaging agents and PTT [120]. On the other hand, gold NPs are considered versatile carriers that can be used in computerized tomography as contrast agents. For example, the Anti GD 2 coated gold nanomedicines were specifically bonded to NB1691 cell line at 4h and were accumulated in endosome-like vesicles at 12h sparing non-NB cells. Natural killer cells can recognize the Fc portion of Anti GD 2 antibodies, further releasing cytokines able to enhance cancer cell death [133].

The combination of ultrasound and microbubbles make it possible to permeate membranes and increases nanomedicine uptake while having an imaging action as contrasting agent. Microbubbles have been used for a long time in clinics to augment echogenicity and now have gained interest in nanomedicine, notably to permeate biological barriers. After exposure to ultrasound and microbubbles, there is a sonopermeation phenomenon that makes it possible to open tight junctions and increases membrane permeability, facilitating uptake [134]. For instance, by increasing tumor permeability with microbubbles, the uptake of DOX liposomes can be significantly enhanced in an NGP orthotopically mouse

model, and can be directly followed by imaging [126]. Briefly, a focused therapeutic ultrasound transducer was applied, while microbubbles and DOX liposomes were administered by a 3D printed rotating syringe platformed to assure a sustained infusion of an evenly dispersed mix. Here, imaging techniques were used to increase drug delivery. However, microbubbles are not always able to cross the leaky tumor vasculature due to their micrometric range, which can limit their use as theragnostic agents for NB. Ultrasound based theragnostics can also be achieved with calcium carbonate-based pH-sensitive nanomedicines [135]. The dual mechanism of action relies on the induction of cell death by the release of carbon dioxide in cancer cells in acidic conditions, while generating gas that enables tumor localization by ultrasound. Results showed that NPs accumulated in tumor sites, aggregated and led to the formation of microbubbles that were detectable by ultrasound in an *in vivo* tumor mice model of NB. In another study, SPION coated with Pluronic F127 loaded with DOX showed important release in acidic and neutral media but was demonstrated to be safe *in vitro* [136]. As depicted in this section, although MIBG and PET scanning are still the gold standard, other strategies employing nanomaterials are made possible by using computerized tomography, ultrasound or MRI/fluorescence. In that sense, nanotechnology is likely to play a major role in the development of new theragnostic techniques for NB treatment.

3.1.5. Cell-based therapies

Cell therapy, such as CAR T cell therapy, is a new domain increasingly explored for NB treatment. Remarkably, some CAR T therapies entered clinical trials and have proven to be safe in phase I/II for NB patients [137]. Most of the ongoing clinical trials on CAR T cells involve GD2 ganglioside (Table 4). At present, CAR-T manufacturing is a complex and expensive process, and challenges associated to scalability and manufacturing of CAR T under cGMP remains [138]. CAR T cells present limitations in the duration of the effect and can cause toxicities such as cytokine release- and macrophage activation- syndromes [139]. The limitation in the duration of the effect is attributed to the complex tumor microenvironment of some neoplasms such as NB, and more specifically to a phenomenon named T cell exhaustion caused by excessive and continuous stimulation. To overcome this issue, a gated and specific GD2-B7H3 CAR T cell therapy was tested preclinically [140]. This novel therapy has two targets, GD2 and B7H3, which is an immune checkpoint overexpressed in many pediatric solid tumors. This specific GD2-B7H3 CAR T was prepared using a novel synthetic Notch technology that enables CAR T cell activation only when recognizing the tumor-associated antigen in NB cells. Therefore, GD2-B7H3 CAR T cells avoid T cell exhaustion, which makes this treatment metabolically active for a longer period, resulting in prolonged anti-cancer activity *in vivo*.

Notably, cell therapy is increasingly being investigated in combination with nanomedicine to improve tumor targeting and decrease tumor growth. Additionally, nanotechnology can also be used to boost CAR T cells by making treatment less expensive and more effective facilitating the genetic modification of T cells with economical nanocarriers [141]. In the field of NB, one example is illustrated by NK cells that are highly effective against solid tumors and represent a new therapeutic opportunity for NB patients [142]. By incubating biohybrid NK cells extracted from umbilical cord blood mononuclear cells with streptavidin-coated iron oxide NPs, NK-iron oxide NPs can be generated [143]. NK-iron oxide NPs bioconjugation magnetically guided NK cells to the tumor site and enhanced cytotoxicity in 3D *in vitro* models of NB. In another study, the combination of radiation, nanomedicine and dendritic cell recognition has been explored to improve tumor recognition and growth inhibition *in vivo* by using patient's own neoantigen [91]. In this case, bacterial membrane nanomedicines are meant to be injected intratumorally, adsorb neoantigens released by tumor cells after radiation and enter dendritic cells by endocytosis to finally activate them. Interestingly, the membrane was extracted from a non-pathogenic but very immunogenic bacteria and was added to the nanomedicine by extrusion. The treatment increased tumor growth inhibition in a syngeneic model

Table 4
CAR T therapies in clinical trials for neuroblastoma and medulloblastoma

CAR T cell	Type of study	Status	Condition	First posted date	Identifier
NEUROBLASTOMA					
GD 2 CAR T cells					
GD2 CAR Tri-virus CTL	Phase I	Completed	NB	2011	NCT01460901
iC9-GD2 T	Phase I	Active	NB	2013	NCT01822652
iC9-GD2-CAR-VZV T	Phase I	Active	NB and OS	2013	NCT01953900
GD2 – CAR T	Phase I	Completed	NB, melanoma, OS, and sarcoma	2014	NCT02107963
CD171-CAR T	Phase I	Active	NB and ganglioneuroblastoma	2014	NCT02311621
GD 2 CAR T NK T cells	Phase I	Withdrawn	NB	2015	NCT02439788
GD2-CAR T	Not applicable	Unknown	Relapsed or refractory NB	2016	NCT02919046
4SCAR-GD2 CAR T	Phase I	Suspended	NB	2016	NCT02765243
GD2-CAR T	Phase I	Completed	Relapsed or refractory NB	2016	NCT02761915 [144]
GD2-CAR T 01	Phase I and II	Recruiting	NB, recurrent NB and GD2 positive tumors	2017	NCT03373097
GD 2 CAR-NK T	Phase I	Recruiting	NB	2017	NCT03294954
iC9-GD2-CAR-IL-15-T	Phase I	Recruiting	NB and OS	2018	NCT03721068
C7R-GD2-CAR T	Phase I	Recruiting	Refractory NB; relapsed NB and OS; and other solid tumors	2018	NCT03635632
4SCAR T	Phase I and II	Recruiting	NB	2020	NCT04637503
-GD2-PSMA-CD276					
GD2 CAR T	Phase I	Recruiting	Recurrent and refractory NB and OS	2020	NCT04539366
B7-H3 CAR T cells					
B7H3 CAR T	Phase I	Recruiting	NB, retinoblastoma and other pediatric solid tumors	2020	NCT04483778
CD276(B7-H3) CAR T	Phase I	Not yet recruiting	NB, OS, gastric and lung cancer	2021	NCT04864821
B7-H3 CAR T	Phase I	Not yet recruiting	NB and other pediatric solid tumors	2021	NCT04897321
Other CAR T cells					
EGFR806 CAR T	Phase I	Recruiting	NB, retinoblastoma and other pediatric solid tumors	2018	NCT03618381
MEDULLOBLATOMA					
EGFR806 CAR T	Phase I	Recruiting	MB, glioma, primitive neuroectodermal tumor and other CNS pediatric tumors	2018	NCT03638167
HER2-CAR T	Phase I	Recruiting	MB, glioma, primitive neuroectodermal tumor and other CNS pediatric tumors	2018	NCT03500991
B7H3-CAR T	Phase I	Recruiting	MB, glioma, primitive neuroectodermal tumor and other CNS pediatric tumors	2019	NCT04185038
IL13Ralpha2-CAR T	Phase I	Recruiting	Leptomeningeal MB, Glioblastoma, Ependymoma	2020	NCT04661384
NKG2D-CAR T	Phase I	Withdrawn*	MB, glioblastoma, colon, and hepatic cancers	2020	NCT04270461
NKG2D-CAR T	Phase I	Recruiting	MB, glioblastoma, colon, and hepatic cancers	2021	NCT05131763

CNS, central nervous system; MB, medulloblastoma; NB, neuroblastoma; OS, osteosarcoma

* Withdrawn for administrative reasons

of NB. These studies highlight the potential concomitant use of nanotechnology to improve the antitumor efficacy of cell-based therapies in the treatment of NB.

3.2. Nanomedicines and cell-based therapies for medulloblastoma treatment

In addition to the complexity of MB pathology, treatments must be able to reach the cerebellum to be effective. In patients with WNT MB subtype, the BBB is altered, which facilitate the entry of drugs to the brain due to the lack of a functional BBB. This explain that these patients have the best prognoses among subtypes and respond well to treatment. In the other 3 MB subtypes, treatments must be able to cross the BBB because this barrier is intact [145,146]. Moreover, in all subtypes the concentration of the therapeutic compound at the target cerebellum should be high enough to be efficacious. Recently, the application of nanomedicine to MB treatment has attracted significant attention due to its potential to improve the pharmacological properties, BBB permeability and cell-specific delivery of current drugs. In particular, the ability of NPs to cross the BBB and selectively target the cerebellum makes this technology promising. Nevertheless, one needs to bear in mind that most of time the amount of NP that cross the BBB is not enough to result in therapeutic effects [147,148]. Additionally, the majority of nanomedicines currently investigated for MB treatment are non-targeted formulations. The necessity to develop better tumor-targeted therapies has also motivated the investigation of cell-based therapies. In this section, we will highlight studies that have shown

that nanotechnology and cell-therapies enhance the biopharmaceutical properties and anticancer efficacy of therapies for MB.

3.2.1. Non-targeted nanomedicines

Most of the nanomedicines investigated for MB focused on the treatment of the SHH subtype, in which the BBB is functional. One of the most commonly investigated approaches for the treatment of this subtype of MB is the use of SMO antagonists. However, as previously mentioned, their limited bioavailability, toxicity and low BBB permeability have questioned their utility. These issues can be overcome using nanotechnology. In a recent study, vismodegib was formulated in polyoxazoline block copolymer nanomicelles (POx-vismo) and their efficacy was evaluated in transgenic mice engineered to develop endogenous SHH-MB [149]. Notably, POx-vismo improved CNS pharmacokinetics and reduced bone toxicity in MB-bearing mice while also prolonging survival compared to control groups. From a mechanistic point of view, the NPs acted within the vascular compartment to improve drug delivery without entering the CNS. Based on this study, the authors later designed a novel POx block copolymer for palbociclib encapsulation and demonstrated their efficacy in SHH-driven MB that developed spontaneously in Smo-mutant mice [150]. Two main issues hinder palbociclib potential in MB, poor brain drug delivery and mechanisms of resistance. To solve these issues, pabociclib was nano-encapsulated and administered in combination with the mammalian target of rapamycin (mTOR) inhibitor sapanisertib. This inhibitor was selected based on single-cell transcriptomic analysis of MB tumors in POx-Palbo-treated mice that showed specific transcriptional changes in

the tumor cells that remained proliferative during palbociclib treatment. The current paper raises the possibility of combining the potential of nanotechnology to improve CNS delivery and of transcriptomics with single-cell resolution to reduce recurrence.

The delivery of some GLI1 inhibitors has also been enhanced through nanotechnology. For instance, glabrescione B is a potent GLI1 inhibitor whose systemic administration is very challenging owing to its low aqueous solubility and poor bioavailability. To improve these aspects, glabrescione B was loaded in self-assembled nanomicelles prepared from mPEG5kDa-cholesterol and its efficacy was investigated in an HH-dependent MB orthotopic model [151]. Notably, glabrescione B nanotherapeutics crossed the BBB upon intravenous administration and reduced tumor growth, being more effective than the non-formulated drug.

SHH-Driven Cerebellar Progenitor Cells, the MB precursors, show increased levels of reactive oxygen species and enhanced expression of NADPH oxidase 4, which suggests that these cells can be treated with NADPH oxidase (NOX) inhibitors. Imipramine Blue (IB), a molecule that inhibits NOX, has been loaded within liposomal NPs and its efficacy has been evaluated in the Smoothed A1 (SMOA1) transgenic mouse model of SHH-MB [152]. The nanomedicine prolonged SMOA1 mouse survival, induced tumor regression and delayed tumor progression. Lipo-IB nanoformulation showed a remarkable ability to cross the BBB and target SHH MB tumor cells, making it an attractive candidate for SHH MB treatment.

Another strategy for MB treatment involves the use of disulfiram, which is a potent anticancer drug with rapid degradation in the gastric and plasma environment. A nanoformulation containing disulfiram was successfully prepared using mPEG-PLGA polymer [153]. Notably, disulfiram-loaded NPs showed higher efficacy in intracranial xenografts of MB as compared to the free drug. The nanoformulation selectively accumulated in the brain and subcutaneous xenografted tumors via the EPR effect.

NPs have also been investigated for gene therapy in pediatric MB, as non-viral vectors for the delivery of nucleic acids such as plasmid DNA. In recent work, the efficacy of polymeric poly(beta-amino ester) (PBAE) NPs containing plasmid DNA encoding the suicide gene herpes simplex virus I thymidine kinase (HSVtk) was reported. [154]. The intratumor infusion of the nanoformulation using convection-enhanced delivery enhanced survival in a mouse orthotopic xenograft model of group 3 MB. This proof-of-concept study demonstrates that PBAE NPs can be used to deliver genetic material to MB cells with therapeutic effects.

NPs have been explored for siRNA delivery as well. In one approach, Kievit *et al.* develop SPIONs loaded with siRNA for knocking down the expression of apurinic endonuclease 1 (Ape1), an enzyme associated with radiation resistance in cancer [155]. The NPs were coated with biocompatible, biodegradable chitosan, PEG, and polyethyleneimine (PEI) to bind and protect siRNA from degradation. These NPs decreased Ape1 expression by 75% in cell lines derived from pediatric MB and reduced Ape1 activity by 80%. Accordingly, decreased cell survival was found in clonogenic assays and enhanced sensitivity of MB cells to low doses of radiation was also reported demonstrating the efficacy of this approach. Now, the clinical potential of this approach has to be demonstrated in animal models of MB. A recent study by Guo *et al.* examines the delivery of SMO-siRNA loaded in cationic lipid-polymer hybrid NPs (LPHs:siRNA) in combination with microbubble-enhanced focused ultrasound (MB-FUS)[156]. This technology provides a physical method to transiently open the BBB in targeted areas, improving the delivery of anticancer drugs into the brain [157]. MB-FUS increased more than 10 times LPHs:siRNA penetration into the brain tumor environment and reduced tumor SMO protein expression in an SHH-activated MB model in mice. This work, while preliminary, illustrates the potential of MB-FUS to improve NP penetration and cancer cell uptake for the effective delivery of nucleic acids, among other drugs against brain tumors.

3.2.2. Targeted nanomedicines

Targeted nanomedicines that enable MB-targeted delivery are sorely needed. However, up to now, only a few studies have explored this possibility and the vast majority of nanomedicines under development are non-targeted approaches. In this section, we provide an overview of targeted nanomedicines that have shown efficacy in preclinical models of MB.

In one attempt to facilitate drug transport to the brain and target MB cells, JQ1, a highly hydrophobic GLI1 inhibitor was encapsulated into polymeric NPs [158] (Fig. 3A). These particles were decorated with an apolipoprotein (ApoE) mimetic peptide that facilitates drug transport to the brain and targets MB cells, demonstrating increased JQ1 concentration in the tumor after systemic administration into orthotopic MB tumor-bearing mice compared to non-targeted JQ1 loaded NPs.

A subset of MBs expresses the antigen CD15, and therefore, the development of approaches to target them has become a goal. Towards this end, Kim *et al.* have demonstrated that high-density lipoprotein (HDL) mimetic NPs decorated with the targeting antibody anti-CD 15 and loaded with sonidegib cross the BBB *in vivo* and deliver the payload to the cancer stem-like cell population in a highly targeted manner in an SHH MB mouse model [159] (Fig. 3B). To facilitate NPs transport through the BBB, NPs contained Apolipoprotein A1, which interacts with receptors of brain endothelial cells and enables BBB transport through receptor-mediated transcytosis.

3.2.3. Cell-based therapies

Cell therapy has emerged as a promising approach for MB treatment, neural stem cells (NSCs) and CAR T cells being the most relevant examples for therapeutic applications. NSCs have demonstrated significant migratory behavior and selective tropism for primary brain tumors and metastasis after intravenous and intracerebral administration in experimental models of MB. Initial studies evidenced the therapeutic effect of enzyme/prodrug therapy mediated by NSCs in models of intracerebral and disseminated metastatic MB [160,161]. More recently, effective targeting of the residual MB foci was demonstrated using thymidine kinase/ganciclovir-expressing NSCs administered in the post-operative cavity of a novel image-guided orthotopic model of MB resection and recurrence generated using tumors with different phenotypes (Fig. 3C) [173]. Remarkably, the ability of cytotoxic NSCs to limit tumor regrowth and prolong survival was demonstrated in two genetically distinct MB resection/recurrence models, showing the potential of these cells in the management of different molecular subgroups of MB.

In the last few years, novel treatments such as CAR T cell therapy have been investigated. Some of them are beginning to be tested in phase I clinical trials (Table 4) [162–167]. Early studies showed promise by demonstrating that the first generation of HER2-specific T cells have antitumor activity in an orthotopic, xenogenic SCID mouse model (Fig. 3D) [164]. Then, durable regression was reported by Nellan *et al.* after regional and intravenous administration of the second generation of anti-HER2 CAR T cells [165]. Since PRAME expression is found in a high percentage of MB tumors, immunotherapy using PRAME-specific T cells has also been explored with positive outcomes [167]. As a further example, B7-H3 CAR T cells demonstrate significant *in vivo* activity against xenograft models of MB. More recently, Donovan *et al.* have validated the locoregional cerebrospinal fluid delivery of CAR T cells in group 3 MB orthotopic xenograft models, the locoregional administration being more effective than *i.v.* administration [163]. The above preclinical studies have highlighted the difficulty of defining the best mode of delivery or the ideal antigenic target for MB. Overall, these preclinical studies have achieved encouraging results that have laid the foundation for strategies based on CAR T cells to fight MB that are being clinically investigated in phase I clinical trials (Table 4). At the time of writing, 5 CAR T candidates had entered phase I clinical trials and are recruiting volunteers (Table 4).

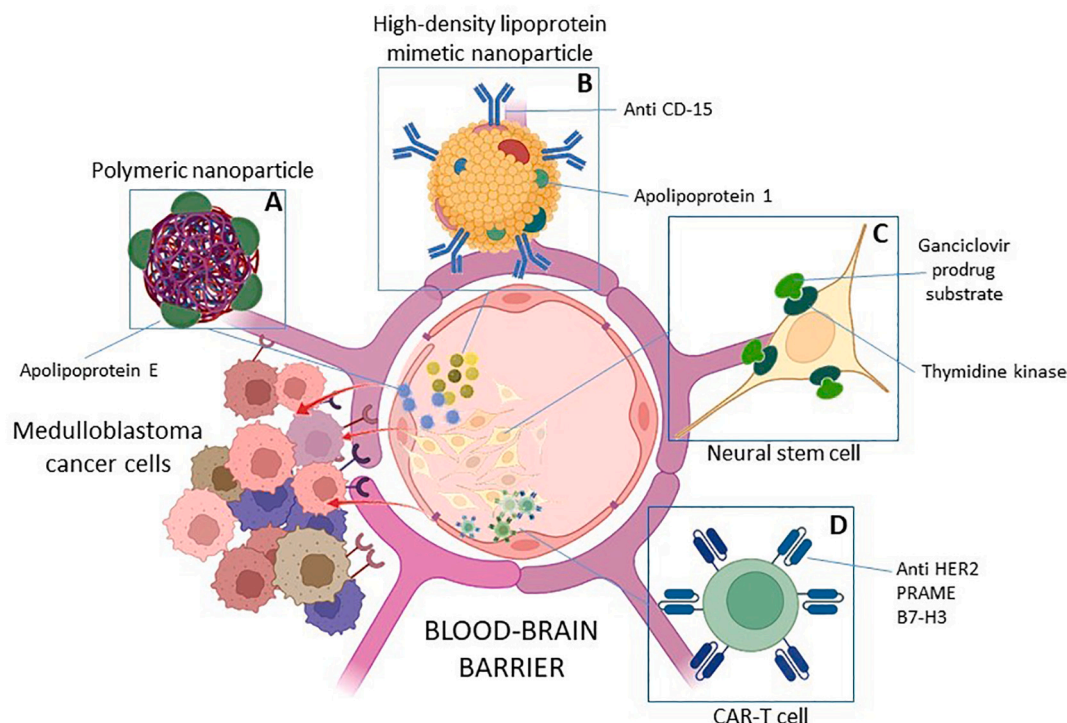


Fig. 3. Experimental therapies to bypass the blood-brain barrier and target medulloblastoma cancer cells. These novel approaches entail the use of polymeric (A) or lipoprotein mimetic (B) nanoparticles decorated with apolipoproteins and/or antibodies. Some effective cell therapies involve engineering neural stem cells to express a pro-drug/enzyme therapy (C) but also genetically modified CAR T cells expressing chimeric MB antigen receptors.

4. Overcoming the hurdles of preclinical and clinical research in embryonal neural tumors

In this review, different strategies based on nanomedicine and cell therapy for the treatment of ENTs have been depicted. As seen, some CAR T therapies are being translated from preclinical to clinical studies. Indeed, although more than 200 articles on NPs for NB and around 30 for MB have been published since 2016, none of these strategies has reached the clinical stage yet. To change this situation, some directions should be followed [168]. This section will try to elucidate how to ameliorate preclinical but also clinical studies for both nanomedicines and cell therapies to accelerate translation to clinical practice.

First, *in vitro* studies could be improved by switching from 2D to 3D models for both nanomedicines and cell-based therapies. Currently, an increasing number of researchers are employing 3D models for better emulation of uptake, diffusion, and efficacy studies *in vitro*. Indeed, 3D models emulate the tumor microenvironment and the possible stiffness found in these types of tumors [169]. 3D scaffold composition is variable but often presents components that can be found physiologically in the extracellular matrix such as collagen, hyaluronic acid or glycosaminoglycans [170]. For instance, standardized 3D *in vitro* models of the BBB could enhance the translational power of preclinical studies although no specific 3D MB model has been developed so far [171]. Ideally, a good 3D *in vitro* model should include multiple cell lines as well as extracellular matrix components to correctly emulate the functional and morphological properties of the BBB. As an example, the use of a microfluidic to study permeability has shown very reproducible results in comparison to 2D models [172].

Another important hurdle is that the uptake of NPs within tumors *in vivo* can be heterogeneous. Solid tumors are heterogeneous and participate in the variability of the response to treatments, including the variability of the EPR effect [173]. However pre-clinical models of tumors are frequently considered to be uniform. A study of Doxil® uptake using CT imaging in two orthotopic mice models of NB: NGP (MYCN amplified) and SH-SY5Y (MYCN non-amplified) showed that NGP

tumors grew much faster than SH-SY5Y and confirmed that MYCN amplified tumors can be more aggressive [174]. According to this, NP uptake relied on tumor age post-inoculation, especially for MYCN amplified cells. On the contrary, tumor volume affected the uptake only for SH-SY5Y cells but not for NGP cells. *In fine*, there was more variability of NP uptake for fast-growing NGP cells when compared to slow-growing SH-SY5Y cells. This study unravels the importance of addressing the uptake of nanomedicines in different sets of NB models to have an overview of the disease heterogeneity. Braekeveldt *et al.* present three options to assess intratumor heterogeneity in NB for improving robustness in drug testing [175]. First multiple patient-derived xenografts from different regions of the same tumor could be established, although this comes as a non-reproducible and complicated option. Second, a patient-derived xenograft with intratumor heterogeneity could be created by using heterogeneous patient-derived cell cultures from different tumor regions. Additionally, the creation of patient-derived xenografts with blood-borne tumor cells derived from liquid biopsies could be another option.

Clinically, there are fundamental differences between adults and children in terms of pharmacokinetics and drug efficacy/toxicity that limit the design and development of clinical trials [176]. Added to this is the difficulty to justify a clinical trial in pediatric patients with only preclinical data. Moreover, clinical trial participants are difficult to recruit as they represent a very small group of patients and in the case of NB, already 44% of patients with NB are enrolled in up-front clinical trials with conventional treatment [177]. At least four challenges could be considered for current and future clinical trials for HR NB. First, there is the definition of the best induction chemotherapy that achieves the best control of metastatic disease and allows surgical resection [32]. The survival advantages of sequential double of autologous stem cell transplantation over a single transplant [32] and the possible benefit of increasing the dose of radiotherapy to improve local control of the disease in patients with macroscopic rest tumor after surgery should also be analyzed. And finally, an exhaustive biological characterization of HR and ultra-HR tumors is necessary to better explain and understand

different biological behaviors and assess possible therapeutic targets [27,32,36,178]. Regarding MB, future preclinical studies must be oriented to three fundamental lines: first, improving knowledge of MB cell of origin and molecular subtype. Second, to ameliorate the understanding of the mechanisms that determine relapse and resistance to treatment. And finally, the design of targeted therapies for specific molecular alterations of metastatic nodules. Future clinical trials should necessarily include not only information from the MB subgroup but also key genetic, cytogenetic and epigenetic markers that can guide prognosis or a particular targeted therapy [179].

Methodologies in clinical trials should also be improved specifically for nanomedicines development. Most of the time, NPs can reduce toxicities in comparison to free treatments in clinical trials, but there is little evidence regarding improvements in efficacy. Protocols for patient stratification will help to maximize the chances of success of cancer nanomedicine. Depending on their location, grade and stage, cancers are treated with a combination of surgery, radiotherapy, chemotherapy and/ or immunotherapy. Strikingly, however, clinical trials involving nanomedicines are generally designed for evaluation in monotherapy settings. This makes it difficult to compare nanodrugs to the standard of care in terms of efficacy and, more importantly, it does not optimally exploit some of the intrinsic capabilities of nanomedicine formulation [180]. Additionally, there is hardly any clinical data available on the safety of nanodevices in pediatric populations [14], although some very recent cohort studies have been published regarding the use of lipid NPs COVID-19 vaccines in pediatric populations [181]. In parallel, further improvements should be made in areas such as better dosing schedules, the correct management of nanomedicines manufacturing or their commercial and clinical feasibility (Fig. 4). All the above directions would help to improve the translation of cancer nanomedicines and cell-

based therapies to the clinics.

5. Concluding remarks

Progress in medical research has improved the prognosis of pediatric malignancies, and currently around 70 % of children diagnosed with an ENT will survive. The increasing number of childhood cancer survivors has led researchers to focus on the long-term consequences of cancer treatment in children. Studies converge in the conclusion that cancer treatment in pediatric populations leads to the development of chronic severe diseases (such as second neoplasms, spinal cord disorders, pulmonary and cardiac diseases) and increased years of life lost. Therefore, one of the major points to consider for the future of pediatric cancer research is to avoid the appearance of long-term side effects that will only be visible later. For this reason, improving the therapeutic index of classic but also novel anticancer drugs such as RNA therapeutics is crucial. Nanomedicine has proven to be a good alternative strategy as it provides a safer way to carry anticancer drugs specifically to the tumor tissue. Among them, non-targeted and stimuli-responsive nanomedicines seem to hold the brightest prospects for both MB and NB as they are the most extensively studied and likely to be translatable to the clinics. Regarding targeted nanomedicines, using GD2 analogues attached to nanocarriers seem to be the most promising approach for NB treatment. While for MB, although the available information is limited, surface decoration with ApoE mimetic peptide and anti-CD15 seem to help to reach the CNS. An optimal nanoparticulate system should be composed of biodegradable materials such as biological lipids or polymers, the formulation being as simple as possible. Moreover, the manufacturing process should be simple and reproducible. On the other hand, cell-based therapies are revolutionizing cancer treatment as they

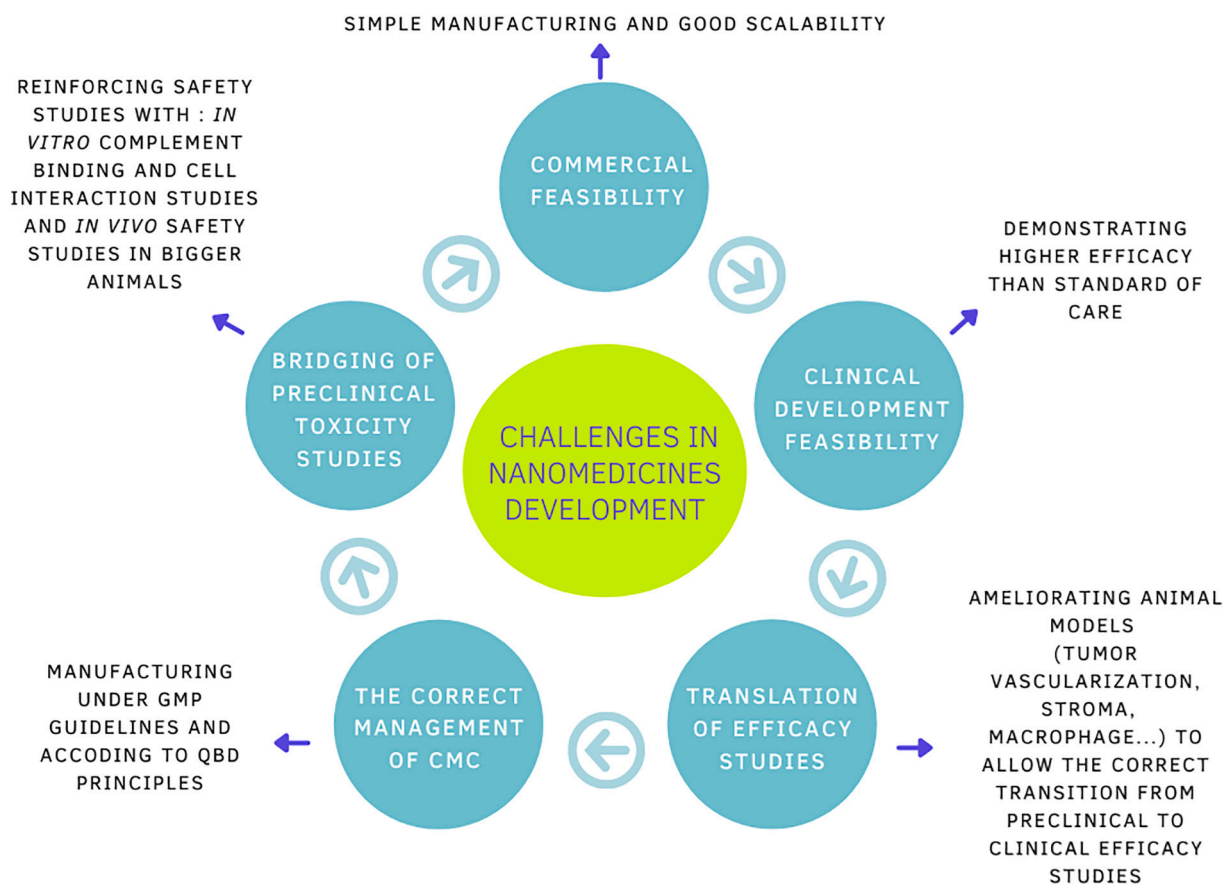


Fig. 4. Schematic illustration summarizing the major key points identified by Matselaar and Lammers to overcome the challenges faced in the development of nanomedicines [168]. CMC, Chemistry manufacturing and controls; QBD, Quality by design.

enable practitioners to deliver precise action towards specific tumor features. CAR T cells are particularly promising as they provide a targeted and durable antitumor response and can interact with the immune response. Beyond the strategies discussed above, they represent the most advanced approach. Although the success of these therapies is not comparable yet to that of blood malignancies, some CAR T cell therapies for NB and MB have already reached clinical trials and it that they could certainly play a major role in the treatment of ENTs. Key aspects such as the efficient and effective CAR-T cell production for the global patient population and regulatory challenges still need to be improved. Notably, the combination of nanomedicines with cell therapies can be highly advantageous in terms of efficacy and represent a new therapeutic opportunity for the treatment of embryonal tumors. In particular, the possibility of using nanotechnology to boost CAR T cells by facilitating a less expensive and more effective therapy remains very attractive. NPs can simplify CAR T cell manufacturing process by avoiding viral gene vectors for stable CAR expression, which require expensive biosafety testing. These therapeutic approaches have greatly contributed to precision medicine, which is gradually becoming the gold standard for cancer treatment. Indeed, precision medicine moves towards the correct characterization of tumors and the specific targeting of molecular tumor features to achieve the best treatment for each patient. However, although many of the results depicted in this review are promising, poor clinical translation is also a concern. The effective preclinical evaluation of cancer nanomedicine and cell-based therapies as well as the improved design of clinical trials with patient stratification will contribute towards a faster translation of these strategies to clinical practice to make a significant impact on the survival and quality of life of these pediatric patients.

Credit author statement

S. El Moukhtari, E. Garbayo and C. Rodriguez-Nogales, A. Fernández-Teijeiro: Writing- Original draft preparation.

P. Couvreur : Reviewing

María J Blanco-Prieto: Conceptualization, Supervision and Writing- Reviewing and Editing.

Declaration of Competing Interest

The authors declare no conflicts of interests.

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