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Therapeutic opportunities in neuroblastoma using nanotechnology

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List of non-standard abbreviations:

ALK, anaplastic lymphoma kinase;

GD2, disialoganglioside 2;

DOX, doxorubicin;

HDL, High density lipoprotein;

MIBG, metaiodobenzylguanidine;

Nab-PTX, albumin-bound paclitaxel nanoparticles;

NB, Neuroblastoma;

NP, nanoparticle;

PEG, poly(ethylene glycol);

PLGA, poly(lactic-co-glycolic) acid;

PTX, paclitaxel;

siRNA, small interference RNA;

ABSTRACT

Neuroblastoma is the most common extracranial solid tumor that preferentially occurs in preschoolers. Its characteristic aggressiveness and heterogeneous clinical behavior is especially visible in relapsed or refractory cases and hampers the therapeutic success. Although the introduction of novel antitumor agents such as dinutuximab, isotretinoin, irinotecan or I-131-MIBG has increased survival, the situation in high-risk neuroblastoma is still dismal. Moreover, treatment is particularly aggressive in these patients, leading to short- and long-term toxicities. The extensive research performed using nanotechnology in recent decades has prompted its application as a therapeutic alternative to overcome some of the common limitations of conventional chemotherapy. Nevertheless, the therapeutic role of nanomedicine in pediatric tumors like NB is not fully elucidated and to this date, only albumin-bound paclitaxel nanoparticles have reached clinic stages. In this review we summarize the neuroblastoma current therapeutic strategies with special attention to the use of nanomedicine. We also highlight the pre-clinical studies on passive and active targeting nanodelivery of therapeutics in experimental NB models.

Introduction

Neuroblastoma (NB) is the term that commonly refers to a family of peripheral neuroblastic or neurogenic tumors diagnosed almost exclusively in childhood, which are classified histologically as ganglioneuroblastoma, ganglioneuroma and NB (Matthay *et al.*, 2016). This embryonal tumor of the sympathetic nervous system arises from the primitive neural crest cells and normally originates in the adrenal glands or the retroperitoneal paravertebral ganglia (Brodeur and Bagatell, 2014; Luksch *et al.*, 2016). Thus, the most common location of the primary tumor is the abdomen, normally the adrenal medulla, but patients can present tumors also along the paraspinal or other sympathetic ganglia from the neck to the pelvis (Brodeur, 2003). NB cells are prone to spread and invade other tissues, and metastatic sites are numerous, including bone marrow, bones, lymph nodes, skin, liver, lung and sometimes central nervous system (Abbasi *et al.*, 2017). Clinical presentation and symptomatology are directly related to the location of the primary tumor and the metastatic sites: from an asymptomatic palpable mass to an abdominal distension, compression of nearby organs and systemic manifestations.

NB represents 7 % of total childhood cancers, and is the third most common cancer and the most frequent extracranial solid malignancy in childhood (Steliarova-Foucher *et al.*, 2017). It is the most frequently diagnosed cancer during the first year of life and approximately 90% of cases are diagnosed before the age of 5, while its incidence is minimum in children over 10 years old (Ward *et al.*, 2014). NB is responsible for 15% of childhood cancer deaths. Although prognosis in infants is favorable, 5-years survival rates remain below 70%, especially for children with high-risk disease (Esiashvili *et al.*, 2009; Trama *et al.*, 2016). The modest progress in survival has been associated with its

clinical behavior diversity due to its biological complexity and heterogeneity, leading to spontaneous recurrences and relapses.

Regarding the management of NB, diagnosis and staging represent a key point in the selection of the best treatment option. Exploratory surgery followed by histologic confirmation and ultrasonography are the mainstay for a primary diagnosis whereas computed tomography and magnetic resonance imaging have become essential especially to detect tumor spread. Although the complete detection of metastases remains a challenge in aggressive NBs, the application of nuclear medicine has shown high specificity and improved the accuracy of tumor localization (Swift *et al.*, 2018).

Therapeutic modalities include surgery, radiotherapy and chemotherapy. Sometimes surgery is the only treatment needed, but chemotherapy and radiotherapy are mandatory in metastatic and locally advanced disease. Current chemotherapeutic protocols are occasionally insufficient to effect cures and they may endanger survivors´ health as they grow up (Robison and Hudson, 2014; Elzembely *et al.*, 2018). This denotes the need to search for novel therapeutic strategies with the aim of beating the disease, as well as minimizing chemotherapy- and radiotherapy-associated toxicity. In this context, drug delivery systems have demonstrated many benefits by improving drug bioavailability and biodistribution (Sieswerda *et al.*, 2011; Ravi Kumar *et al.*, 2013). In particular, cancer nanomedicine emerged two decades ago to offer solid therapeutic alternatives to common chemotherapy, as illustrated by the market launch of liposomal doxorubicin (DOX) (Doxil) in 1995 (Shi *et al.*, 2016).

Nanomedicines include several types of colloid systems at the nanoscale size (1-1000 nm) (Fig. 1). They are constructed on polymers, lipids or metals, among other materials, that allow for encapsulation of multiple therapeutic agents (*e.g.* small molecules, peptides or nucleic acids) (Smith *et al.*, 2017; McClements, 2018). A wide range of

nanoformulations is available based on liposomes, micelles, solid/porous nanoparticles (NPs), dendrimers, carbon nanotubes and many other nanocarriers, incl. prodrug squalene based nanoassemblies (Fig.1B), that have shown encouraging outcomes in the therapeutic index improvement of several antitumor compounds (Peer *et al.*, 2007). An added reason for success has been associated in some occasions with an enhanced permeability and retention (EPR) effect in tumor tissues (Maeda *et al.*, 2000). Furthermore, nanocarriers can be functionalized at their surface with monoclonal antibodies in order to deliver drugs selectively towards tumor tissues (targeted nanomedicines) or with hydrophilic molecules (*i.e.* poly(ethylene glycol) (PEG)) to increase their blood circulation time in the body (Fig. 1C). Nanotechnology has been also proposed as a promising tool for cancer detection due to the physico-chemical properties of NPs, in which the superparamagnetism of metallic NPs for magnetic resonance imaging is noteworthy (Chen *et al.*, 2018b).

This framework fosters the introduction of nanomedicines in current NB protocols to improve therapeutics, especially in high-risk or relapsed NB patients where common treatments are still inefficient. The goals of this review are to describe the current state-of-the-art in NB therapeutics with special attention on the incoming role of nanomedicines for the management of this childhood cancer. The outcomes concerning the research and development of nanoformulations that have been tested in *in vitro* and *in vivo* models of NB in the last 5 years will be discussed in depth.

(Figure 1)

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Therapeutic Strategies in Neuroblastoma: Targeting High-Risk Disease

At diagnosis, patients with NB can be classified according to clinical and biological characteristics into three different risk groups: low, intermediate or high (Cohn *et al.*, 2009). The mainstay of treatment for low-risk group patients involves a complete surgical resection if possible, but in some cases the tumor undergoes a spontaneous regression (Brodeur and Bagatell, 2014). Intermediate-risk group patients receive from two to eight cycles of chemotherapy together with surgical resection. Low and intermediate risk group patients present an overall survival of greater than 90%; however, survival of high-risk NB patients is still below 40% (Pinto *et al.*, 2015). They frequently present an early inadequate treatment response so that therapy has to be intensified. Sadly, treatment is in many cases limited and unable to completely eradicate the metastatic cells (Speleman *et al.*, 2016). In light of this dismal situation, the search for novel therapeutic strategies (included cancer nanomedicine) should draw our attention.

Conventional Chemotherapy. NB protocols include vincristine, cyclophosphamide, cisplatin, DOX, ifosfamide, carboplatin, topotecan, irinotecan and taxoids among many other cytostatic drugs (George *et al.*, 2010). The regime selection varies according to risk stratification and phase of treatment, which is defined as induction, consolidation and maintenance therapy or post-consolidation (Fig. 2). The induction stage entails multiple cycles of anthracyclines and alkylating agents, aimed to reduce the tumor area prior to surgical resection and also to arrest metastatic spread. Afterwards, a myeloablative chemotherapy regimen is given in the consolidation phase followed by

autologous stem cell rescue, bone marrow transplantation and granulocytic cell stimulating factor administration (Sung *et al.*, 2013). The establishment of this strategy has considerably improved the outcome, and holds the key to achieving tumor remission in high-risk NB patients. Thus, the optimal conditioning of this regime is currently under investigation to lower toxicities and to prolong survival. These upgrades include a strategic choice of the stem cell collection stage and the anticancer drug cocktail, based on a combination of busulfan/melphalan or carboplatin/etoposide/melphalan or carboplatin/etoposide/cyclophosphamide (Smith and Foster, 2018).

In the maintenance phase, treatment is supplemented with isotretinoin together with immunotherapy (section 2.4). Salvage or experimental protocols have recently introduced topotecan, irinotecan, temozolomide and fenretinide in clinical settings in order to prolong survival (Villablanca *et al.*, 2011; Di Giannatale *et al.*, 2014; Mody *et al.*, 2017). Interestingly, the synthetic retinoid derivative fenretinide, currently in phase II clinical trials (NCT00053326), has been reported to trigger not only an antitumor effect similar to isotretinoin but also an immune-modulatory effect (Mark A Applebaum *et al.*, 2017).

Radiotherapy. NB is considered to be a radiosensitive tumor and radiation is given mainly in the consolidation phase, although it can be restricted due to its adverse effects (Li *et al.*, 2017). On the other hand, functional imaging with nuclear scintigraphy (computed tomography or magnetic resonance imaging) is fundamental particularly for accurate staging, treatment planning and detection of metastatic disease (Chen *et al.*, 2018). The most widely used radioisotope for this purpose is the I-123 metaiodobenzylguanidine (MIBG), with a sensitivity of approximately 90% (Sharp *et al.*, 2016). Bearing this in mind, the I-131 MIBG, a radio-metabolic epinephrine analogue, has been used alone or combined with chemotherapeutics as an alternative

radiotherapeutic approach in refractory cases and salvage therapy (Kayano and Kinuya, 2018). With the same localizing properties of I-123 MIBG but a longer half-life, it delivers a focal dose of radiation to all the tumor sites with promising response rates in refractory NB (Matthay *et al.*, 2012). A Phase III study has now been launched for newly diagnosed high-risk NB patients to test if the addition of I-131 MIBG at induction chemotherapy improves survival (NCT03126916).

Immunotherapy. The high heterogeneity of NB derives from its transient embryonic structure, leading to a wide range of cellular phenotypes, sometimes present even in a single tumor (Aygun, 2018). The phenotypic analysis has uncovered miscellaneous types of highly expressed surface markers that can also act in tumorigenesis or contribute to the progression of the oncologic process (*e.g.* CD117, CD133, CD114, CD57, CD171 or GD2) (Malone and Stegmaier, 2017; Kholodenko *et al.*, 2018).

At present, tumor associated gangliosides have become the most important targetable epitopes in the clinical setting (Suzuki and Cheung, 2015; Sait and Modak, 2017). These molecules participate in cellular communication, adhesion, growth, and differentiation. In fact, the FDA (Food and Drug Administration) approved Unituxin (dinutuximab) in 2015, the first approved anti-disialoganglioside 2 (GD2) specific monoclonal antibody, for the treatment of high-risk NB patients (Ploessl *et al.*, 2016). This immunotherapeutic approach is in many cases administered along with interleukin-2 (IL-2) and isotretinoin in the maintenance therapy. Clinicians have reported that the combination of this anti-GD2 antibody with other therapies increased the 5-year survival rates of NB patients by 20% (Kholodenko *et al.*, 2018). An incoming Children's Oncology Group (COG) trial is planning to include immunotherapy in the induction stage to evaluate the benefits when combined with chemotherapy.

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Some cell therapy and genome editing approaches have recently reached clinical trials. For instance, the infusion of haploidentical natural killer (NK) cells in combination with other therapies is currently being investigated in Phase II clinical trials (NCT00698009). One goal of this approach is to evaluate if the cytolytic action of NK cells against NB cells could lower the doses of the concomitant treatments required and thus, reduce their toxicity (Nguyen *et al.*, 2018b). In addition, the next generation of chimeric antigen receptor (CAR)-T cells for adoptive cell therapy will be directed against GD2-positive tumors (Tesfaye and Savoldo, 2018). Another strategy currently in Phase I/II studies is based on gene modification via virus of autologous NB cells separated from the patient to secrete lymphotactin and IL-2 (NCT00062855). Once re-injected into the patient, these gene-modified NB cells are able to attract the immune system, helping the body to kill the malignant cells. The results of these studies will determine if this immune response is sufficient to overcome active recurrent NB.

Next Generation of Antitumor Therapies. In recent years, extra efforts have been made in order to define the molecular landscapes of NB (Matthay *et al.*, 2012; Cheung and Dyer, 2013). The use of next-generation sequencing approaches led to the identification of new molecular targets such as *ALK, PHOX2B, BARD1, TERT, ATRX* and *PTPRD* genes, in addition to *MYCN* gene (Peifer *et al.*, 2015; Schulte and Eggert, 2015; Cao *et al.*, 2017). Among them, *MYCN* amplification is reported to be present in around 25% of cases and is associated with aggressive NBs (Tonini *et al.*, 1997).

NB relapse is reported to present an increased mutational burden and hence, a higher pool of targetable aberrations that translate into novel therapeutic opportunities (Fletcher *et al.*, 2018). For instance, serine/threonine kinases Aurora A and Aurora B inhibitors such as alisertib are crucial regulators of the cell cycle and have also shown to be efficient blockers of *MYCN/MAX* interactions (Mossé *et al.*, 2012). Noteworthy, the

only druggable target clearly validated in this tumor to date is the anaplastic lymphoma kinase (*ALK*) mutation. In favor of the proposed ALK inhibitors, crizotinib is yielding results that hold promise for treatment of patients with deregulated *ALK* function (Esposito *et al.*, 2017). Epigenetic regulation has been also reported to be a decisive factor in tumor development (Gröbner *et al.*, 2018). Recently, the histone deacetylase inhibitor vorinostat reached phase II clinical trials to treat high-risk NB patients (NCT02559778). Within the multiple signaling pathways several AKT/PI3K/mTOR inhibitors have been postulated. Among them the AKT inhibitor perifosine is noteworthy, administered in combination with the mTOR inhibitor temsirolimus (Becher et al., 2017).

Improvement of conventional chemotherapy protocols is reaching its limit and some of the novel compounds investigated display a low half-life, bad penetration or solubility issues that hinder their use in the clinic. Also, cancer cells develop resistance to chemotherapy though many different mechanisms. Multidrug resistance (MDR) is one of the major events that limits the use of cytostatic agents. The most important associated mechanism is the increased efflux rate of drugs from cancer cells through the ATP-binding cassette transporters. In this sense, cancer stem cells are reported to be the master regulators of chemoresistance (Alisi *et al.*, 2013; Fruci *et al.*, 2016). Moreover, the inclusion and promotion of novel therapeutic candidates in clinical trials is hampered due to the small and heterogeneous patient population (Fletcher *et al.*, 2018). Having this in mind, cancer nanomedicine represents an alternative therapeutic approach that can provide novel opportunities for the treatment of pediatric cancers (Rodríguez-Nogales *et al.*, 2018).

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Nanomedicines for Neuroblastoma

Current therapeutic failure reported especially in aggressive disease has encouraged researchers to design novel nanoformulations for treating NB patients. Encouragingly, albumin-bound paclitaxel NPs (nab-PTX) (Fig. 2) reached phase I clinical trials for refractory NB and other pediatric solid tumors (Moreno *et al.*, 2018). These studies have reported a manageable toxicity profile and a suitable preliminary clinical activity of Abraxane (nab-PTX) in monotherapy, which have allowed for promotion to phase II trials (NCT01962103). One possible reason for success is that nab-PTX has shown enhanced cell transport, better penetration and lower elimination of PTX in comparison with non-encapsulated PTX (Chen *et al.*, 2014). The next generation of non-targeted and targeted nanomedicines for NB will hence be reviewed and discussed in the following sections.

(Figure 2)

Non-targeted Nanomedicines for Neuroblastoma

Anthracyclines and Alkylating Agents Delivery. Chemotherapeutic associated toxicity is reported to provoke not only abandonment of treatment but also late sequelae and chronic complications, making long-term monitoring necessary (von der Weid, 2008). In this sense, endocrine deficits, as well as secondary malignant neoplasms or chronic renal failure have been frequently described (Mark A. Applebaum *et al.*, 2017). The aimless cytotoxicity that leads to these issues is often derived from the use of potent alkylating agents and anthracyclines (Fulbright *et al.*, 2010). For example, cardiac dysfunction induced by the use of DOX contributes to early morbidity and mortality among childhood cancer survivors. In the same way, it has been reported that NB

survivors have an increased risk of developing congestive heart failure, myocardial infarction, pericardial disease or valvular abnormalities (Mulrooney *et al.*, 2009; Friedman and Henderson, 2018). Nano graphene oxidated NPs were coated with gelatin for carboplatin and DOX delivery (Makharza *et al.*, 2015; Vittorio *et al.*, 2018). *In vitro* studies demonstrated that the effect of gelatin coating was essential for the biocompatibility of graphene NPs, making possible its future administration in animals. DOX-Nano graphene oxide was coated with a dextran-catechin conjugate to form nanohybrids. This combinatory approach between DOX and the anticancer polyphenol catechin reported both a reversion of the DOX resistance mechanism (via P-gp downregulation) and a synergistic effect *in vitro*. Although the surface modification of graphene improved the biocompatibility of this nanomaterial, the safety of the nanoformulation is questionable. Given that graphene material is not strictly biodegradable, more experiments *in vivo* are required to confirm the feasibility of this type of nanocarrier. Conversely, Zhen et al. (2013) proposed a safer approach by using milk-derived proteins as scaffolds to formulate casein NPs cross-linked by transglutaminase to encapsulate cisplatin. The *in vitro* experiments assayed on the SH-SY5Y cell line did not show conclusive differences between bulk cisplatin and cisplatin-NP treatments. Nonetheless, the *in vivo* experiments determined a significant efficacy and toxicity improvement with encapsulated cisplatin. These results denote that the clinical success of nanomedicines relies on complex biological interactions with the tumor tissue. Thus, more data about the NPs' interaction with the tumor microenvironment is needed for nanomedicines to progress in clinical practice. Interestingly, a pre-clinical *in vivo* study designed in 2014 provided a kidney capsule model of NB to evaluate the cell uptake and behavior of liposomes and their interaction with vascular leaks in the tumor area (Ghaghada *et al.*, 2016). Using a liposomal

contrast agent as a nanoprobe, high-resolution computed tomography imaging results indicated that the liposome cell uptake in NB was not governed by tumor volume or area. It is important to bear in mind that NB is a solid tumor and potential therapeutic benefit of nanomedicines could rely on a hypothetic EPR effect. Even though the EPR effect of nanomedicines has been well-established and documented, these results have been postulated based on pre-clinical models. The outcomes in clinical settings are still modest or unknown and deserve further attention (Taurin *et al.*, 2012).

Camptothecins and Synthetic Retinoid Derivative Delivery. SN-38, the active metabolite of irinotecan, is unable to reach the market due to its poor solubility and high toxicity (Hahn *et al.*, 2018). Given that it is considerably more active than irinotecan, SN-38 was conjugated with tocopherol succinate to form a pro-drug and was afterwards encapsulated in biodegradable poly(lactide)-PEG based NPs (Alferiev *et al.*, 2015). Administered NPs in a xenograft model of NB provided rapid tumor regression and a prolonged animal survival, confirming the feasibility and therapeutic efficacy improvement of the formulation. In a subsequent pre-clinical study, this formulation was tested in a mouse model of NB using an irinotecan treatment regimen as control (Iyer *et al.*, 2015). SN-38 prodrug-NPs regime was reported to be more effective at restraining NB tumor growth and recurrence. According to this, the nanoformulation increased exposure of tumor tissue to the cytotoxic effects but with no evidence of chemotherapeutic toxicity. More recently a similar strategy was proposed by linking tocopherol oxyacetate to SN-38 to enhance NP retention and to improve drug delivery (Nguyen *et al.*, 2018a). Afresh, compared to irinotecan treatment polymeric NPs showed a greater tumor response and prolonged event-free survival in SH-SY5Y and IMR-32 NB xenografts. While the irinotecan group controlled tumor for 45 days, the nanoparticulated SN38-progrug achieved a tumor remission and control for at least 180

days. Therefore, nanotechnology might be crucial for the rescue of abandoned drugs that were not able to reach the market because of their troublesome formulation.

In the case of topotecan, another camptothecin inhibitor of topoisomerase I, the main limitation is its low half-life (<30 min). Topotecan undergoes a pH-dependent reversible hydrolysis into a non-active carboxylate at physiological pH (Devriese *et al.*, 2015). With this in mind, a liposomal topotecan formulation was prepared in order to protect the cytostatic drug from this pH- induced hydrolysis (Chernov *et al.*, 2017; Gilabert-Oriol *et al.*, 2017). Pharmacokinetic studies showed that the nanoformulation exhibited a 10- fold increase in plasma half- life values in comparison with equivalent doses of Hycamtin (commercial topotecan). However, encapsulated topotecan increased the life span of NB mice only modestly. This reminds us that the ability of nanomedicines to effect cures is sometimes controversial and that a further optimization is sometimes needed to demonstrate a strong benefit over conventional treatments. Nevertheless, the evidence concerning avoiding toxicities is more solid and well-documented. For example, fenretinide was incorporated in reconstituted high density lipoprotein (HDL)-based NPs prior to their administration to NB cell lines SMS-KCNR and SK-N-SH (Sabnis *et al.*, 2013). Although *in vitro* assays only showed a slight improvement in efficacy, the novel formulation proved to be non-toxic in non-malignant cells, unlike free fenretinide administration. Synthetic and reconstituted HDL based-NPs have been suggested to be an ideal carrier for the delivery of drugs in NB because tumor cells and their cancer stem cells overexpress the scavenger receptor class B type 1 receptor that can bind to HDL. (Basha *et al.*, 2014; Subramanian *et al.*, 2018).

Non-viral gene delivery. The use of gene therapy was proposed with the aim of downregulating *MYCN* expression by means of small interference RNA (siRNA). The use of drug delivery systems is a suitable non-viral alternative approach to overcome the

poor stability in biological fluids and the low intracellular penetration of siRNAs (Ravi Kumar *et al.*, 2004). The MYC-associated factor X dimerization (MYC-MAX) protein 3 transcription factor siRNA was nanocomplexed in superparamagnetic iron oxide NPs (Duong *et al.*, 2017). This group´s purpose was to treat NB cell lines with the developed formulation either as a single therapeutic agent or in combination with common cytotoxic drugs used in NB treatment. Cells exposed to siRNA nanocomplexes underwent apoptosis caused by the oncogene knockdown. Moreover, the authors reported an additive effect of these siRNA-NPs with other cytostatic drugs that supports a hypothetical combinatory strategy.

Other strategies. Less-aggressive modalities are also gaining interest as anticancer therapies in NB. They are characterized by drug activation after a given external stimulus, and combination with nanotechnology may reinforce their potential (Abrahamse and Hamblin, 2016). For instance, a sonodynamic strategy was designed by incorporating a porphyrin into polymeric NPs (Serpe *et al.*, 2013). In this study, SH-SY5Y cells were exposed to poly-methyl methacrylate core-shell NPs loaded with meso-tetrakis (4-sulfonatophenyl) porphyrins. The formulation was reported to ameliorate the sonosentitized activation of porphyrin, generating reactive oxygen species and leading to NB cell death *in vitro*.

Cano-Mejia *et al.* (2017) described a photothermal immunotherapy approach using prussian-blue NPs. NPs injected in the tumor area triggered a cancer cell death response via photothermal activation. Moreover, in order to boost the immune response elicited by the prussian NPs tumor ablation, anti-CTLA-4 antibodies were administered as an immune checkpoint, unleashing the immune response of infiltrated T-lymphocytes against the remaining tumor cells. Treated mice exhibited long-term survival and the authors underlined the utility of this strategy to ameliorate the outlook in high-risk NB.

Of note, the same group recently established a thermal therapeutic window for immunogenic cancer cell death triggered by these NPs that resulted in a long-term survival *in vivo* (Sweeney *et al.*, 2018).

The above-highlighted approaches are part of a longer list of non-targeted nanomedicines that have been recently proposed for the treatment of NB patients (table 1). Some of them have demonstrated solid evidence *in vivo*, which supports their future implementation in clinical practice to high-risk NB patients. Special attention should be given to the best-in-class biodegradable and biocompatible nanocarriers that have exhibited a great capacity in the minimization of associated anthracycline and alkylating agent toxicity. Furthermore, some of these NP-based strategies investigated have been shown to overcome efflux-mediated resistances that often hamper the use of common chemotherapeutic agents in the clinic. In that sense, the inclusion of Abraxane (nab-PTX) in clinical trials endorses the implementation of similar nanosystems. With respect to irinotecan or topotecan, their nano-encapsulation has been shown to overcome bioavailability issues of classical formulations that frequently hinder their therapeutic success. In the case of nanomedicines for gene therapy delivery, the current data is encouraging but limited. Bearing in mind the high genetic burden involved especially in high-risk NB, more experiments are warranted to elucidate their therapeutic feasibility. On the other hand, we believe that it is too early to estimate the real impact of NPs used in alternative therapies such as sonodynamic therapy or photothermal immunotherapy. Future experiments will assess their clinical utility at least as adjuvant or complementary treatments.

(Table 1)

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Targeted Nanomedicines for Neuroblastoma

In order to avoid systemic toxicities, oncologists demand strategies to increase cytostatic drug exposure in the tumor area. NP-surface engineering has broken new ground in novel therapeutic opportunities with the objective of carrying drugs selectively towards tumor tissue (Bazak *et al.*, 2015). Moreover, the latest findings in the biology and epitope recognition of NB cells have supplied improved knowledge of substance to technologists, prompting the construction of several targeted nanomedicines against NB. In the attempt to overcome tumor relapses, surface-modified nanomedicines are also intended to detain distant metastases or circulating cancer stem cells.

Antibody Targeting. Children with high-risk NB are at present under anti-GD2 mAb therapy (dinutuximab) (Ploessl *et al.*, 2016). Targeting nanomedicines against cell-surface gangliosides has attracted researchers' attention also as an immune-targeted strategy. As can be seen in table 2, several experiments have been performed by linking anti-GD2 antibodies or their fractions to NPs. In the case of iron (Xu *et al.*, 2014; Baiu *et al.*, 2015) and gold anti-GD2 NPs (Jiao *et al.*, 2016), the authors concurred with high tumor specificity targeting and suggested the use of anti-GD2 NPs not only as anticancer nanomedicines *per se* but also as suitable diagnostic nanodevices in NB therapy. Theragnosis (simultaneous diagnosis and treatment) is nowadays gaining relevance in nanotechnology (Lee *et al.*, 2015). Given that metallic NPs can be monitored by magnetic resonance imaging (MRI), they might be very useful in NB where diagnosis and staging are crucial (Chen *et al.*, 2018a). Recently, SN-38 was loaded into anti-GD2 poly(lactic-co-glycolic) acid (PLGA)- PEG NPs (Monterrubio *et al.*, 2017). When this targeted nano-drug delivery system was administered to mice bearing GD2-high expression NB, the extent of tumor penetration by SN-38 was

considerably higher in comparison to non-targeted NPs or just the free drug. This group performed a microdialysis technique to confirm a sustained exposure to the targeted drug in the interstitial fluid of the tumors without any of the interference from the unreleased drug that can be found in common pharmacokinetic studies. Thus, the authors suggested that their antigen specific delivery system using monoclonal antibodies could be administered in NB GD2 positive patients in order to enhance the anti-tumor activity while reducing toxicity of SN-38.

NB expresses common tumor epitopes, receptors or moieties that have also been investigated for targeting nanomedicines with antibodies. Such is the case of VEGF (Vascular Endothelial Growth Factor), involved in the angiogenesis process and cancer progression. Bevacizumab selectively binds to this protein, so it was conjugated to SiO_2 layered double hydroxide DOX loaded nano-composites with the goals of: (i) improving the cellular uptake and the targeting effect of DOX; and (ii) inhibiting angiogenesis (Zhu *et al.*, 2017). *In vitro* and *in vivo* assays demonstrated that this combinatory approach considerably alleviated DOX side effects and enhanced the anti-NB and anti-angiogenesis efficiency mediated both by the targeted DOX delivery and the inhibition of the VEGF related signaling pathways. These promising approaches based on antibody-NPs suggest that they may well be applied in the near future; furthermore, the fact that dinutuximab or bevacizumab are already commercialized reinforces their use in clinical practice. However, again safety might be a matter of concern since anti-GD2 therapy is reported to provoke acute toxicities, including severe neuropathies that gravely affect these patients (Anghelescu *et al.*, 2015). Bearing this in mind, antibody-NPs will have to show considerable benefits over common treatments and non-targeted nanomedicines in future pre-clinical evaluations.

Peptide Targeting. Peptide-based ligands engineered to NPs have been designed from the neuronal signaling pathways of the peripheral nervous system. The decoration of PLGA NPs with rabies virus glycoproteins, which are able to target the nicotinic acetylcholine receptor of NB cells, has been described (Lee *et al.*, 2016). NPs were loaded with calcium carbonate and administered intravenously to tumor-bearing mice. Once the NPs reached the tumor, facilitated by specific-tumor targeting, calcium carbonate converted into microbubbles of CO_2 triggered by the acidic tumor environment. *In vivo* results determined that gas generated from NPs was a safe and tumor-specific contrast agent for ultrasound imaging, allowing the authors to propose this formulation as another interesting tool for NB theragnosis. Next, the same group took a step forward and loaded DOX in a similar nanoformulation previous to its administration in a NB-bearing mouse model (Jang *et al.*, 2018). Interestingly, the CO_2 gas generated in the tumor microenvironment accelerated the release of DOX, attaining an enhanced therapeutic efficacy *in vivo* as compared with free DOX. Very interestingly, this approach combines active NP tumor targeting with a stimuli-responsive strategy that facilitates the delivery of drugs in a spatial-, temporal- and dosage-controlled manner (Mura *et al.*, 2013).

Other targeting ligands are currently being explored. Loi *et al.* (2013) performed a combined *in vitro/ex-vivo* phage display screening to validate NB-targeting moieties and were able to recognize and isolate novel peptide ligands. Selected peptide ligands were coupled to DOX-loaded liposomes and their administration in pre-clinical NB animal models led to a significant decrease in tumor volume and survival enhancement. Then, other multi-target approaches, including the endothelial cell marker aminopeptidase A, the perivascular cell marker aminopeptidase N and the above-mentioned GD2, were investigated (Cossu *et al.*, 2015). The authors were able to validate a novel peptide

ligand called HSYWLRS. The conjugation of this peptide to DOX-liposomes increased their tumor vascular permeability and perfusion *in vivo* and thus boosted the therapeutic response of DOX-targeted liposomes. In another study, synthetized proteasome inhibitor bortezomib-loaded liposomes were decorated with targeting-ligand peptides for the tumor endothelial cell marker (Zuccari *et al.*, 2015). These tumor vascular targeted liposomes were evaluated *in vivo,* exhibiting lower toxicity and a better therapeutic index than free bortezomib.

Other Targeting Moieties. NP functionalization with drugs, aptamers or surfactants is also frequently described in the literature. Instead of using peptides, CDC20siRNA and PTX were co-encapsulated in a cationic amphiphilic liposomal formulation decorated with nipecotic acid-derived moieties. Bhunia *et al.* (2017) postulated that nipecotic acid-derived molecules attached to NPs may competitively inhibit the GABA transporter, overexpressed in NB cells, attaining a liposome targeted delivery. Intravenously administered targeted liposomes considerably inhibited tumor growth of xenografted human NB mice. This is a striking example of a combinatorial approach that harnesses cancer nanomedicine to merge targeting and gene/cytostatic drug delivery. Beyond doubt, the use of peptides or small molecules represents an interesting alternative to antibodies. Of note, some of these nanoformulations probably display less specificity towards tumor tissue than antibody-decorated NPs. Nonetheless, it may be taken as an advantage rather than a hurdle to progress in the clinic. Unlike antibodies, these molecules do not show an inherent immunotoxicity, so further investigations are needed to elucidate the best strategies.

The studies described have shed light on the possibilities of targeted nanomedicines in the field (table 2). Some of them have been reported to lower toxicities and to enhance

the antitumor efficacy in pre-clinical models, which may be optimal in relapsed NB. However, its extreme variability and our poor understanding of the disease oblige us to be cautious until a suitable candidate is validated. Among these, the experience gained in clinical practice with immunotherapy supports the option of anti-GD2 targeted nanomedicines loaded with cytostatic drugs to progress in clinical settings in the future. Sadly, there are more challenges than opportunities to effect total cures in NB children with poor prognosis to this date. The effectiveness of targeted nanomedicines in detaining metastases is nowadays a matter of debate. The metastatic process is not merely a cellular-centered phenomenon but a response to supra-cellular alterations linked to tissue disorganization. The complexity of tumor biology is not only determined by cell-cell interaction but also by cell-matrix interplay (Tadeo *et al.*, 2016a). Nonetheless, the demonstration that the components of the tumor microenvironment decisively mediate in clinical aggressiveness, prognosis (Tadeo *et al.*, 2016b; Tadeo *et al.*, 2018) and response to treatment (Johnsen *et al.*, 2018), opens up a new field of knowledge and clinical application. This is especially visible in new and promising nanomedicines that are much more respectful towards the bodily integrity of NB children.

(Table 2)

Conclusions and Future Directions

Current NB protocols range from conventional chemotherapy administration to up-to-date immunotherapy regimes. Even if the constant optimization of these therapies has ameliorated the perspectives and prognosis of diseased children, high-risk NB is still incurable in approximately 50% of cases. In addition, some of the novel targets proposed, such as actionable genetic aberrations, often meet with insufficient patients to validate therapies. This quandary has prompted the advent of cancer nanomedicine for improving therapeutics in NB.

Efforts have been made in the last few years to attain the best nanoformulations to replace current treatments. We have highlighted some pre-clinical studies on nanomedicines that have proven to alleviate chemotherapeutic-associated toxicity, suggesting that their use would avoid treatment abandonment and late sequelae. Abraxane (nab-PTX), currently in phase I-II for NB treatment, is at the head of these outlined approaches and leads the pathway for the implementation of analogous nanomedicines. Likewise, we anticipate that other biocompatible and biodegradable nanocarriers carrying anthracyclines, alkylating agents or taxoids might reach the clinic in the near future. The outcomes gathered concerning targeted nanomedicines are very valuable. Surface-decorated nanomedicines with anti-GD2 antibodies have demonstrated an enhanced antitumor efficacy over non-targeted therapies that establish pre-clinical consolidation for designing future experiments.

Cancer nanomedicine is gradually gaining relevance in adult cancer. To extend this approach to a pediatric cancer such NB, proposed nanoformulations have to show strong benefits over current treatments like the reduction of the cardiotoxicity associated with DOX treatment.

Some aspects, such as the safety profile, drug cargo capacity or biodistribution of drug delivery systems, are crucial. Very importantly, a suitable validation of candidates in standardized orthothopic models of NB that correctly reproduce the disease is mandatory. The complex NB biology still represents the main obstacle, so collaboration between pediatric oncologists, biologists and technologists is essential for achieving therapeutic success. We are confident that the knowledge acquired will uncover the real potential of nanomedicines for the management of NB.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Rodríguez-Nogales, Noguera, Couvreur and Blanco-Prieto.

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Footnotes

Footnote to title

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Figure legends

Fig.1. General features of nanomedicines. (A) Non-targeted nanoparticle (NP) for cytostatic drug delivery. (B) Image of cryo-transmission electronic microscopy (Cryo-TEM) showing a squalenoyl-gemcitabine nanoassembly, under investigation for pediatric cancer treatment by our group. (C) Targeted NP for cytostatic drug delivery. (D) Components and moieties of nanomedicines.

Fig. 2. Management of neuroblastoma. Current therapies in clinic (chemotherapy, radiotherapy, immunotherapy, molecular therapy and nanomedicine) *vs* treatment phases (induction, consolidation, maintenance, salvage/experimental).

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Tables

TABLE 1

Non-targeted nanomedicines for neuroblastoma

Formulation	Type of NP	Drug	Size (nm)	Assays	Ref.
Cisplatin-casein NPs	Protein-based	Cisplatin	257	*In vitro*/*In vivo*	(Zhen *et al.*, 2013)
Fenretinide rHDL NPs	Lipoprotein	Fenretinide	40	*In vitro*	(Sabnis *et al.*, 2013)
TTPS poly-methyl methacrylate NPs	Polymeric	TTPS	72	*In vitro*	(Serpe *et al.*, 2013)
Carboplatin-gel-NGO	Graphenic	Carboplatin	100	*In vitro*	(Makharza *et al.*, 2015)
SN38-TS-HO-PLA-mPEG NPs-	Polymeric	SN38	<100	*In vitro*/*In vivo*	(Alferiev *et al.*, 2015; Iyer *et al.*, 2015)
Iodixanol-liposomes	Lipid	Iodixanol	135	*In vivo*	(Ghaghada *et al.*, 2016)
PAMAM Anthocianin dendrimer NPs	Dendrimer	Anthocyanin	134	*In vitro*	(Yesil-Celiktas *et al.*, 2017)
MXD3 siRNA iron oxide NPs	Metallic	MXD3 siRNA	56.	*In vitro*	(Duong *et al.*, 2017)
Prussian blue NPs	Inorganic	-	10-100	*In vitro*/*In vivo*	(Cano-Mejia *et al.*, 2017; Sweeney *et al.*, 2018)
Liposomal topotecan	Lipid	Topotecan	100	*In vitro*/*In vivo*	(Chernov *et al.*, 2017; Gilabert-Oriol *et al.*, 2017)
Pyrazolo[3,4-d] pyrimidine HAS NPs	Protein-based	Pyrazolo[3,4-d] pyrimidine	100-200	*In vitro*	(Fallacara *et al.*, 2017)

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Dextran-nanoceria curcumin NPs	Metallic	Curcumin	14	*In vitro*	(Kalashnikova *et al.*, 2017)
GO-AgNPs	Inorganic	Silver	15	*In vitro*	(Yuan *et al.*, 2017)
Fe_3O_4 Prussian blue NPs	Metallic	-	164	*In vitro/In vivo*	(Kale *et al.*, 2017)
Dex-CT-rGO DOX nanohybrids	Inorganic	DOX	300	*In vitro*	(Vittorio *et al.*, 2018)
PL-block-PEG SN-38 TOA NPs	Polymeric	SN-38	50-70	*In vitro/In vivo*	(Nguyen *et al.*, 2018)
sHDL WGA-TA NPs	Lipoprotein	WGA-TA	8-12	*In vitro/In vivo*	(Subramanian *et al.*, 2018)
C@HSA-MNPs@rGO-DOX	Inorganic	DOX	100	*In vitro*	(Lerra *et al.*, 2018)

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TABLE 2

Targeted nanomedicines for neuroblastoma

Targeting moiety/ Formulation	NB cell target	Type of NP	Drug	Size (nm)	Assays	Ref.
Anti-GD2/ MicroRNA-34a- -silica NPs	GD2	Organic-inorganic	MicroRNA-34a	74	*In vitro/In vivo*	(Tivnan *et al.*, 2012)
HSYWLRS Peptide/ DOX-liposomes	Neuropilin-1	Lipid	DOX	130	*In vitro/In vivo*	(Loi *et al.*, 2013; Cossu *et al.*, 2015)
Anti-GD2/ iron oxide NPs	GD2	Metallic	-	99	*In vitro/In vivo*	(Xu *et al.*, 2014; Baiu *et al.*, 2015)
AntiGD2/ ALK siRNA liposomes	GD2	Lipid	ALK siRNA	135-165	*In vitro/In vivo*	(Di Paolo *et al.*, 2015)
NGR peptide/ bortezomib liposomes	aminopeptidase N	Lipid	Bortezomib	165	*In vitro*	(Zuccari *et al.*, 2015)
Anti-GD2/ gold NPs	GD2	Metallic	-	50	*In vitro*	(Jiao *et al.*, 2016)
RVG/ PLGA-$CaCO_3$ NPs	nicotinic acetylcholine receptor	Polymeric	DOX	200-220	*In vitro/In vivo*	(Lee *et al.*, 2016; Jang *et al.*, 2018)
NACA/ CDC20 siRNA -PTX-liposomes	Gaba receptor	Lipid	CDC20 siRNA + PTX	130-150	*In vitro/In vivo*	(Bhunia *et al.*, 2017)

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PGA/ PTX NPs	Neural cell adhesion molecule	Polymeric	PTX	9	*In vitro/In vivo*	(Markovsky *et al.*, 2017)
Anti-GD2/ PLGA-PEG SN-38 NPs	GD2	Polymeric	SN-38	272	*In vitro/In vivo*	(Monterrubio *et al.*, 2017)
Bevacizumab/ SiO_2 LDH DOX NPs	VEGF	Inorganic	DOX	253	*In vitro/In vivo*	(Zhu *et al.*, 2017)
Dendritic PG/ NTP-PTX-PEG NPs	Neural cell adhesion molecule	Polymeric	PTX	70	*In vitro/In vivo*	(Vossen *et al.*, 2018)
Anti-GD2/ YM155 liposomes	GD2	Lipid	Sepantronium bromide	170	*In vitro/In vivo*	(Gholizadeh *et al.*, 2018)

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Figures

Figure 1

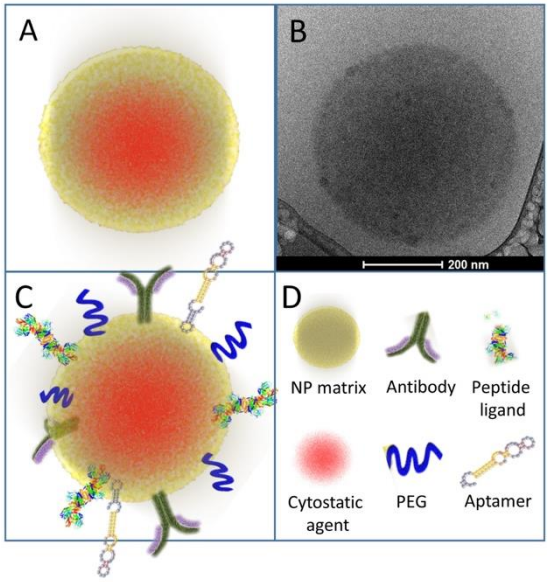

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Figure 2