Neuroblastoma therapy: what is in the pipeline?

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Abstract

Despite the expansion of knowledge about neuroblastoma (NB) in recent years, the therapeutic outcome for children with a high-risk NB has not significantly improved. Therefore, more effective therapies are needed. This might be achieved by aiming future efforts at recently proposed but not yet developed targets for NB therapy. In this review, we discuss the recently proposed molecular targets that are in clinical trials and, in particular, those that are not yet explored in the clinic. We focus on the selection of these molecular targets for which promising in vitro and in vivo results have been obtained by silencing/inhibiting them. In addition, these selected targets are involved at least in one of the NB tumorigenic processes: proliferation, anti-apoptosis, angiogenesis and/or metastasis. In particular, we will review a recently proposed target, the microtubule-associated protein (MAP) encoded by doublecortin-like kinase gene (DCLK1). DCLK1-derived MAPs are crucial for proliferation and survival of neuroblasts and are highly expressed not only in NB but also in other tumours such as gliomas. Additionally, we will discuss neuropeptide Y, its Y2 receptor and cathepsin L as examples of targets to decrease angiogenesis and metastasis of NB. Furthermore, we will review the micro-RNAs that have been proposed as therapeutic targets for NB. Detailed investigation of these not yet developed targets as well as exploration of multi-target approaches might be the key to a more effective NB therapy, i.e. increasing specificity, reducing toxicity and avoiding long-term side effects.

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Introduction

The most common solid extracranial neoplasm in children is neuroblastoma (NB). It is also the most common childhood cancer diagnosed in children before the age of 1 year (Maris et al. 2007). NB is characterised by a broad range of clinical behaviour. The International Neuroblastoma Staging System (INSS) merges some characteristics of the previously used Pediatric Oncology Group and Children’s Cancer Group systems and has identified distinct prognostic stages (1, 2A, 2B, 3, 4 and 4S; Brodeur et al. 1993). Based on the INSS stage, group age and tumour biology, patients can be assigned to a low-, intermediate- or high-risk group (Brodeur et al. 1993, Haase et al. 1999). The biological features of the tumour for the assignment to one of these three groups include MYCN status (Brodeur et al. 1984), International Neuroblastoma Pathologic Classification score (Shimada et al. 1999) and tumour DNA index (Look et al. 1991), which describes the number of chromosomes in the tumour cells compared to normal cells. The therapy applied to NB patients depends on the risk category (Haase et al. 1999). Low-risk patients can be cured with surgery or just observed without receiving treatment (Park et al. 2008). Intermediate-risk patients are usually treated with surgery and chemotherapy (Modak & Cheung 2010). High-risk NB is treated with surgery, intensive chemotherapy, radiation therapy, bone marrow or haematopoietic stem cell transplantation and targeted biologic therapies with 13-cis-retinoic acid and immunotherapy. The immunotherapy involves the administration of cytokines
such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL2) and/or administration of monoclonal antibodies that target GD2, an NB surface antigen (Johnson et al. 2007, Park et al. 2008, Castel et al. 2010, Modak & Cheung 2010). Despite advances in treatment, significant complications remain, particularly in patients with high-risk NB. First, a majority of the patients suffers remission relapse in bone/bone marrow or, less commonly, in soft tissue. Secondly, isolated relapses in the central nervous system are also being detected in some patients (Modak & Cheung 2010).

Clearly, improved therapeutic approaches are needed to increase specificity, reduce toxicity and avoid the long-term side effects. Therapeutics that selectively inhibits the activity of a single molecule has been proposed. Ideally, the targeted molecule plays an essential role in the genesis and/or maintenance of the tumour of interest such that its partial or complete inhibition is cytotoxic to tumour cells resulting in tumour regression in the absence of any secondary effects. Very few molecules with such ideal characteristics have been identified and drugged in cancers in general, and especially in paediatric cancers such as NB. Several molecule-targeted therapeutics are under investigation in the pre-clinical or clinical phase of drug development and promising results have been obtained (Fong & Park 2009, Wagner & Danks 2009, George et al. 2010, Modak & Cheung 2010). Here, we provide an overview of some of the targets that are presently being studied in the clinic (Table 1) and particular attention is paid to the most recently proposed molecular targets for NB therapy that have not yet reached the clinic (Tables 2 and 3), which might be crucial for the origin and progression of NB and possibly also for other cancer types. In addition, we will discuss multi-target therapeutic approaches for NB and provide future perspectives in this field for the coming years.

**Origin and progression of NB**

NB derives from multi-potent neural crest (NC) cells. NB tumours are formed as a result of genetic mutation and/or changes in epigenetic factors responsible for the correct programming of the NC cells (Fig. 1; Gershon et al. 2005). NC cells migrate from the neural tube to generate the primordial of the sympathetic chain along the abdominal aorta (Nakagawara 2005). During this process, several abnormalities might occur that contribute to tumorigenesis, including loss of control of cell proliferation, differentiation or apoptosis. The NB cell transcriptome reflects its origin in neuronal crest-derived tissues (Nakagawara 2005). Other neuroectodermal tumours have similar origin and are also pluripotent, proliferative and migratory, like NC cells. These are neurofibroma, schwannoma, malignant nerve sheath tumour, melanoma, medulloblastoma, supratentorial primitive neuroectodermal tumour and Ewing’s sarcoma (Pomeroy et al. 2002, Gershon et al. 2005).

Several genes are regulated in an orchestrated manner to drive the correct differentiation of NC cells into sympathetic neurons (Fig. 1). Some of these genes, such as PHOX2A, PHOX2B, NTRK2 (TrkB) (Hallbook et al. 1995, Nakagawara 2001) and MYCN (Zimmerman et al. 1986), are up-regulated in high-risk NBs as well as in medulloblastoma (Nakagawara 2004, Nakagawara & Ohira 2004, Harel et al. 2010, Swartling et al. 2010). MYCN promotes proliferation and prevents the differentiation of neuronal progenitors during embryonic development (Alam et al. 2009). The Notch pathway also seems to play a role in keeping the sympathetic precursors in a proliferative state and to inhibit neuronal differentiation (Tsarovina et al. 2008). Moreover, several of these genes are highly specific for NB, enhancing the possibility for accurate diagnosis and identification of therapeutic targets (Cheung 2005). Understanding the processes involved in NB progression and maintenance is of great importance for an effective NB therapy. These processes include cell proliferation, survival, angiogenesis, invasion and metastasis.

**Targeting NB**

Identifying and validating new therapeutic targets for enhanced treatment of children with high-risk NB is of main priority. Understanding the mechanisms underlying high-risk NB may allow the discovery of novel potential targets. Exploring the pathophysiological and mechanistic action of existing therapeutic agents are two other routes that have been followed for finding new targets. However, the progress in those research fields can be slow. Therefore, there is strong incentive to seek shortcuts based on the use of novel technologies to select and validate new targets, such as microarrays, next-generation sequencing technologies, phosphoproteomics and transcriptome sequencing, among others.

One strategy for target identification in NB is investigating the mechanisms of origin and maintenance of NB and the genes, micro-RNAs (miRNAs) and proteins that play a key role in those processes. Here, we provide an overview of several coding genes, miRNAs and proteins that might play a role in NB...
Molecular targets under investigation in the clinic

The field of NB therapy is progressing and several of the proposed targets have reached pre-clinical and clinical studies in the last years (Table 1). Those molecular targets include tyrosine kinase receptors that have been implicated in NB pathology, such as anaplastic lymphoma kinase (ALK), the insulin-like growth factor 1 receptor (IGF1R) and tropomyosin receptor kinase (TRK). Inhibition of Aurora A kinase (AURKA) and mechanistic target of rapamycin (mTOR) pathway are other examples of approaches that are under investigation in the clinic and that we will review here.

ALK was originally identified as an oncogene in lymphoma (Shiota et al. 1994) but is now known to have a role in NB. The anaplastic lymphoma kinase (ALK) is a transmembrane receptor protein tyrosine kinase that is involved in the development of the brain. Inhibition of ALK has shown promising results in pre-clinical studies, with compounds such as PF-2341066 and NVP-AEW541 being investigated for their potential therapeutic effects.

Table 1 Proposed target genes for neuroblastoma therapy that are under investigation in the clinic

<table>
<thead>
<tr>
<th>Gene symbols</th>
<th>Names</th>
<th>Function/processes</th>
<th>Compound(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT1</td>
<td>v-akt murine thymoma viral oncogene homologue 1</td>
<td>Cell growth/proliferation, apoptosis</td>
<td>A-443654, perifosine (KRX-0401)</td>
<td>Opel et al. (2007), LoPiccolo et al. (2008), Li et al. (2011)</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma receptor tyrosine kinase</td>
<td>Transmembrane receptor protein tyrosine kinase activity, development of the brain</td>
<td>PF-2341066</td>
<td>George et al. (2008), Mosse et al. (2008), Ogawa et al. (2011)</td>
</tr>
<tr>
<td>AURKA</td>
<td>Aurora kinase A</td>
<td>Cell cycle regulation</td>
<td>MLN8237</td>
<td>Wagner &amp; Danks (2009), George et al. (2010), Carol et al. (2011)</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
<td>Anti-apoptosis</td>
<td>Obatoclax</td>
<td>Dole et al. (1994), Rheingold et al. (2007)</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
<td>Cell proliferation, cell–cell adhesion, apoptosis</td>
<td>Gefitinib (ZD1839)</td>
<td>Modak &amp; Cheung (2010), Furman et al. (2011)</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
<td>Transcriptional regulation, cell cycle progression and development</td>
<td>Valproic acid, Vorinostat</td>
<td>Coffey et al. (2001), George et al. (2010)</td>
</tr>
<tr>
<td>HSP90AA1</td>
<td>Heat-shock protein 90 kDa alpha (cytosolic), class A member 1</td>
<td>Signal transduction, protein folding, protein degradation cell growth</td>
<td>17-AAG</td>
<td>Kang et al. (2006), Fuchert et al. (2007), George et al. (2010)</td>
</tr>
<tr>
<td>IGF1R</td>
<td>IGF1 receptor</td>
<td>Anti-apoptotic, tyrosine kinase activity</td>
<td>NVP-AEW541, EM164, SCH71745, IMC-A12</td>
<td>Liu et al. (1998), Wagner &amp; Danks (2009) Segerstrom et al. (2006), Sims et al. (2008), Morton et al. (2011)</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase insert domain receptor (a type III receptor tyrosine kinase)</td>
<td>Growth factor, endothelial proliferation, survival, migration, tubular morphogenesis and sprouting</td>
<td>Bevacizumab, sunitinib, cediranib (AZD2171)</td>
<td>LoPiccolo et al. (2008), Fulda (2009), Wagner &amp; Danks (2009) Evans et al. (1999, 2001)</td>
</tr>
<tr>
<td>NTRK2</td>
<td>Neurotrophic tyrosine kinase, receptor, type 2</td>
<td>Cell signalling, cell differentiation</td>
<td>CEP-701 (KT-6587)</td>
<td>Fulda (2009)</td>
</tr>
<tr>
<td>PDK1</td>
<td>Pyruvate dehydrogenase kinase, isozyme 1</td>
<td>Carbohydrate and pyruvate metabolic processes</td>
<td>OSU-03012</td>
<td>Fulda (2009)</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Phosphoinositide-3-kinase, catalytic, alpha polypeptide</td>
<td>Anti-apoptosis, glucose metabolism process, signal transduction</td>
<td>GDC-0941, NVP-BEZ235</td>
<td>LoPiccolo et al. (2008)</td>
</tr>
<tr>
<td>TNFRSF10B</td>
<td>Tumour necrosis factor receptor superfamily, member 10b</td>
<td>Apoptosis</td>
<td>Lexatumumab (ETR2-ST01)</td>
<td>Zhang et al. (2007b), Modak &amp; Cheung (2010)</td>
</tr>
</tbody>
</table>

Gene symbols and names are in agreement with HUGO Gene Nomenclature Committee even when the nomenclature used in the references is different.
Table 2 Proposed target genes for neuroblastoma therapy

<table>
<thead>
<tr>
<th>Gene symbols</th>
<th>Names</th>
<th>Function/processes</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL6</td>
<td>B-cell CLL/lymphoma 6</td>
<td>Cell proliferation, differentiation, apoptosis</td>
<td>Chamdin et al. (2009)</td>
</tr>
<tr>
<td>BIRC5</td>
<td>Baculoviral IAP repeat-containing 5</td>
<td>Apoptosis, cell cycle</td>
<td>Islam et al. (2000b), Duffy et al. (2007)</td>
</tr>
<tr>
<td>CASP8</td>
<td>Caspase 8, apoptosis-related cysteine peptidase</td>
<td>Apoptosis, cell adhesion and metastasis</td>
<td>McKee &amp; Thiele (2006)</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
<td>Cell cycle, differentiation</td>
<td>Molenaar et al. (2008, 2010)</td>
</tr>
<tr>
<td>CD44</td>
<td>CD44 molecule (Indian blood group)</td>
<td>Cell adhesion and metastases</td>
<td>Yoon &amp; Danks (2009)</td>
</tr>
<tr>
<td>CDK2</td>
<td>Cyclin-dependent kinase 2</td>
<td>Cell cycle, DNA replication</td>
<td>Molenaar et al. (2009)</td>
</tr>
<tr>
<td>CENPE</td>
<td>Centromere protein E</td>
<td>Cell cycle</td>
<td>Balamuth et al. (2010)</td>
</tr>
<tr>
<td>CRABP2</td>
<td>Cellular retinoic acid binding protein 2</td>
<td>Epidermis development, signal transduction, retinoic acid metabolic process</td>
<td>Itoh et al. (2010)</td>
</tr>
<tr>
<td>CTSL1</td>
<td>Cathepsin L1</td>
<td>Proliferation, apoptosis, angiogenesis, invasion and metastasis</td>
<td>Zheng et al. (2009), Colella et al. (2010), Lankelma et al. (2010)</td>
</tr>
<tr>
<td>DCLK1</td>
<td>Doublecortin-like kinase 1</td>
<td>Cell proliferation, survival, neuronal cell migration, neurogenesis</td>
<td>Shu et al. (2006), Vreugdenhil et al. (2007), Verissimo et al. (2010)</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Diablo, IAP-binding mitochondrial protein</td>
<td>Apoptosis, neuroblastoma progression</td>
<td>Shang et al. (2010)</td>
</tr>
<tr>
<td>DUSP26</td>
<td>Dual specificity phosphatase 26 (putative)</td>
<td>Protein dephosphorylation</td>
<td>de Tudela et al. (2010)</td>
</tr>
<tr>
<td>EPAS1</td>
<td>Endothelial PAS domain protein 1</td>
<td>Keeps tumour-initiating cells in a undifferentiated state</td>
<td>Pietras et al. (2009), Qing et al. (2010), Nakagawara &amp; Ohira (2004)</td>
</tr>
<tr>
<td>GCLC</td>
<td>Glutamate–cysteine ligase, catalytic subunit</td>
<td>Glutamate–cysteine ligase, apoptosis</td>
<td>de Tudela et al. (2010)</td>
</tr>
<tr>
<td>GSK3B</td>
<td>Glycogen synthase kinase 3 beta</td>
<td>Neuronal cell development, hippocampus development, glycogen metabolic process</td>
<td>Li et al. (2010), Dickey et al. (2011), Yeo et al. (2003)</td>
</tr>
<tr>
<td>HIF1A</td>
<td>Hypoxia inducible factor 1 alpha subunit (basic helix-loop-helix transcription factor)</td>
<td>Differentiation of neural crest cells, modulation of energy metabolism in cancer</td>
<td>Nakagawara &amp; Ohira (2004)</td>
</tr>
<tr>
<td>Id2</td>
<td>Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein</td>
<td>Differentiation of neural crest cells, transcription factor</td>
<td>Lasorella et al. (2002), Nakagawara (2004)</td>
</tr>
<tr>
<td>IGFBP5</td>
<td>IGF binding protein 5</td>
<td>Regulation of cell growth, signal transduction, apoptosis, cell migration</td>
<td>Tanno et al. (2005)</td>
</tr>
<tr>
<td>LDHA</td>
<td>Lactate dehydrogenase A</td>
<td>Anaerobic glycolysis, oxidation–reduction process</td>
<td>Qing et al. (2010)</td>
</tr>
<tr>
<td>LGALS1</td>
<td>Lectin, galactoside binding, soluble, 1</td>
<td>Cell proliferation, migration, differentiation, apoptosis</td>
<td>Cimmino et al. (2009)</td>
</tr>
<tr>
<td>MCL1</td>
<td>Myeloid cell leukaemia sequence 1 (BCL2-related)</td>
<td>Apoptosis, differentiation</td>
<td>Lestini et al. (2009)</td>
</tr>
<tr>
<td>METAP2</td>
<td>Methionine aminopeptidase 2</td>
<td>Angiogenesis</td>
<td>Shusterman &amp; Maris (2005)</td>
</tr>
<tr>
<td>MIF</td>
<td>Macrophage migration inhibitory factor (glycosylation-inhibiting factor)</td>
<td>Cell proliferation, negative regulator of apoptosis, negative regulator of cell cycle arrest</td>
<td>Ren et al. (2006)</td>
</tr>
<tr>
<td>NME1</td>
<td>Non-metastatic cells 1, protein (NM23A) expressed in</td>
<td>Cell adhesion, metastasis, cell differentiation, negative regulation of apoptosis</td>
<td>van Noesel &amp; Versteeg (2004), van Noesel &amp; Versteeg (2004)</td>
</tr>
<tr>
<td>NME2</td>
<td>Non-metastatic cells 2, protein (NM23A) expressed in</td>
<td>Cell adhesion, negative regulation of apoptosis</td>
<td>van Noesel &amp; Versteeg (2004)</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
<td>Tumour cell proliferation, angiogenesis</td>
<td>Lu et al. (2010)</td>
</tr>
<tr>
<td>NPY2R</td>
<td>Neuropeptide Y receptor Y2</td>
<td>Tumour cell proliferation, angiogenesis</td>
<td>Lu et al. (2010)</td>
</tr>
<tr>
<td>PAX3</td>
<td>Paired box 3</td>
<td>Apoptosis, regulation of transcription, multi-cellular organism development</td>
<td>Gershon et al. (2005)</td>
</tr>
<tr>
<td>PAX7</td>
<td>Paired box 7</td>
<td>Anti-apoptosis, differentiation, neuronal fate</td>
<td>Gershon et al. (2005)</td>
</tr>
<tr>
<td>PKC1</td>
<td>Polo-like kinase 1</td>
<td>Cell cycle, cell proliferation, G2/M transition DNA damage checkpoint</td>
<td>Hu et al. (2009)</td>
</tr>
<tr>
<td>PRAF2</td>
<td>PRA1 domain family, member 2</td>
<td>Protein transport, L-glutamate transport, apoptosis</td>
<td>Geerts et al. (2007)</td>
</tr>
</tbody>
</table>
be preferentially expressed in neuronal cells at late embryonic stages. Activation of ALK induces cell growth and neurite outgrowth that is mediated by the MAPK pathway (Motegi et al. 2004). Mutated forms of ALK have been identified in high-risk NB (Wagner & Danks 2009, George et al. 2010). In fact, around 10% of NB tumours are estimated to have an ALK mutation (Fong & Park 2009). Gain or amplification of ALK has been associated with aggressive clinic phenotype (George et al. 2010) and specific block of ALK results in growth inhibition and increases apoptosis (Fong & Park 2009). Therefore, recent interests in targeting ALK for NB therapy have arisen and several ALK inhibitors have been developed and are under investigation (Ardini et al. 2010). First clinical trials with ALK inhibitors show promising results for the treatment of non-small cell lung cancer (http://clinicaltrials.gov #NCT00585195) in which ALK signalling is deranged (Soda et al. 2007). Also, a phase I/II study, using PF-02341066 as an ALK inhibitor (Table 1), is presently ongoing for NB (http://clinicaltrials.gov #NCT00939770).

TRK, originally identified as an oncogene (Martin-Zanca et al. 1986), is now known as the high-affinity receptor for nerve growth factor and as such is crucially involved in the growth, differentiation and apoptosis of neuronal cells in both the central and the peripheral nervous system (for review, see Nakagawara et al. 2001). High expression levels of TRK have been correlated with poor NB outcome (Nakagawara et al. 1993) and chemotherapy resistance (Ho et al. 2002). Since its discovery in 1986 (Martin-Zanca et al. 1986), TRK has been a focus of intense pharmaceutical experimentation and several TRK-blocking small compounds, such as CEP-701, have been developed. It has been shown that blocking TRK using CEP-701 results in induction of apoptosis (Evans et al. 1999) and growth inhibition of human NB xenografts in nude mice (Evans et al. 2001). Presently, a phase I trial is ongoing in patients with recurrent or refractory high-risk NB (http://clinicaltrials.gov #NCT00084422).

IGF1R is involved in the regulation of cell proliferation, survival, differentiation and transformation (Bahr & Groner 2005). IGF1R is highly expressed in NB (El-Badry et al. 1989) and activation of IGF1R induces the expression of MYCN (Misawa et al. 2000). The expression level of IGF1R has been correlated with tumorigenicity and metastasis.
Blocking IGF1R with anti-IGF1R antibodies resulted in the inhibition of NB cells growth and tumour regression in NB xenograft mouse models (Geoerger et al. 2010). The anti-IGF1R monoclonal antibody (IMC-A12) is presently under investigation in phase II trial (http://clinicaltrials.gov #NCT00831844).

AURKA is a serine/threonine kinase, which stabilises the microtubule at the spindle pole during chromosome segregation. Therefore, AURKA is essential for G2-M progression and its inhibition results in cell cycle arrest and apoptosis (Hirota et al. 2003, George et al. 2010). AURKA is overexpressed in multiple tumours, including NB, and amplification of AURKA gene has also been observed in NB cells (Otto et al. 2009). In phase I trials, promising results have also been obtained with AURKA inhibitor MLN8237 (Wagner & Danks 2009, George et al. 2010, Carol et al. 2011). A phase II trial is ongoing (http://clinicaltrials.gov #NCT01154816).

Inhibition of mTOR pathway, targeting phosphatidylinositol 3-kinases, IGF1R, mTOR and/or vascular endothelial growth factor (VEGF), is under investigation as well (Kang et al. 2008, George et al. 2010). mTOR pathway is involved in the regulation of cell growth and proliferation (Sarbassov et al. 2005). Notably, the simultaneous inhibition of different proteins (e.g. mTOR and IGF1R) seems to be a more effective therapeutic approach than targeting them individually (Coulter et al. 2008). For instances, in phase I trial (http://clinicaltrials.gov #NCT01204450), mTOR is targeted using temsirolimus in combination with valproic acid which targets histone deacetylase.

In Table 1, we provide a general overview of the target genes/proteins that are under investigation in the clinic. For further reading on these promising molecular targets for NB therapy, we refer to excellent reviews (Fong & Park 2009, Wagner and Danks 2009, George et al. 2010, Modak & Cheung 2010).

**Proposed molecular targets not yet explored in the clinic**

Several interesting targets (genes/proteins) have recently been proposed for NB therapy but have not reached the clinic yet (Table 2). However, they might be the targets to consider for future successful NB therapy. They include, for example, cyclin-dependent kinase 2 (CDK2), which is involved in DNA replication and cell cycle. CDK2 is a regulator of S-phase progression (Shapiro 2006). Inactivation of CDK2 has been shown to be synthetically lethal to MYCN-amplified NB cells and is therefore an interesting molecular target (Molenaar et al. 2009). The anti-apoptotic regulatory protein survivin

**Table 3** Potential micro-RNAs as targets for neuroblastoma therapy

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Validated target(s)</th>
<th>Function/processes</th>
<th>Types</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-17-5p-92</td>
<td>TGFβ-signalling, CDKN1A (p21); BCL2L11 (Bim)</td>
<td>Cell proliferation, cell adhesion</td>
<td>Oncogene</td>
<td>Fontana et al. (2008), Mestdaghi et al. (2010)</td>
</tr>
<tr>
<td>miR-34a</td>
<td>NMYC, BCL-2, E2F3</td>
<td>Cell cycle progression, apoptosis, DNA repair and angiogenesis</td>
<td>Tumour suppressor</td>
<td>Welch et al. (2007), Cole et al. (2008), Wei et al. (2008)</td>
</tr>
<tr>
<td>miR-184</td>
<td>AKT2</td>
<td>Neural differentiation and/or apoptosis</td>
<td>Tumour suppressor</td>
<td>Foley et al. (2010), Tivnan et al. (2010)</td>
</tr>
<tr>
<td>miR-380-5p</td>
<td>p53</td>
<td>Apoptosis</td>
<td>Oncogene</td>
<td>Swarbrick et al. (2010), Laneve et al. (2007), Ma et al. (2010), Khe-Goodall &amp; Goodall (2010)</td>
</tr>
<tr>
<td>miR-9</td>
<td>E-cadherin, tropomyosin-related kinase C</td>
<td>Angiogenesis, metastasis</td>
<td>Oncogene</td>
<td>Foley et al. (2010), Tivnan et al. (2010)</td>
</tr>
<tr>
<td>miR-125a</td>
<td>Bmf, tropomyosin-related kinase C</td>
<td>Cell proliferation, apoptosis</td>
<td>Oncogene</td>
<td>Laneve et al. (2007)</td>
</tr>
<tr>
<td>miR-125b</td>
<td>Bmf, tropomyosin-related kinase C</td>
<td>Cell proliferation, apoptosis</td>
<td>Oncogene</td>
<td>Laneve et al. (2007)</td>
</tr>
<tr>
<td>miR-152</td>
<td>CHUK, CUL5 and GADD45A</td>
<td>Neuroblast differentiation, migration/invasion and apoptosis</td>
<td>Oncogene</td>
<td>Ragusa et al. (2010)</td>
</tr>
<tr>
<td>miR-338</td>
<td>PTPRT</td>
<td>Neuroblast differentiation and apoptosis</td>
<td>Oncogene</td>
<td>Ragusa et al. (2010)</td>
</tr>
<tr>
<td>miR-200B</td>
<td>ZEB1</td>
<td>Neuroblast differentiation, migration/invasion and apoptosis</td>
<td>Tumour suppressor</td>
<td>Ragusa et al. (2010)</td>
</tr>
</tbody>
</table>
(baculoviral IAP repeat-containing 5 (BIRC5)), which is selectively expressed in the most common human cancers but not in normal adult tissues, has been shown to be overexpressed in NB (Islam et al. 2000a,b). Hence, survivin has been proposed as NB target as well (Duffy et al. 2007). Cyclin D1 (CCND1) is up-regulated in NB compared with other types of tumours and normal tissue (Molenaar et al. 2010). GATA3 was found to be implicated in cyclin D1 overexpression in NB (Molenaar et al. 2010). Silencing of the proposed therapeutic target cyclin D1 causes the differentiation of NB cells (Molenaar et al. 2008). Another proposed target, lactase dehydrogenase A, plays a role in anaerobic glycolysis, which is known to be a crucial process in providing energy for NB tumours (Qing et al. 2010). Some examples of other cancer types that also expressed the proposed targets for NB therapy are shown in Supplementary Table 1, see section on supplementary data given at the end of this article.

For further detailed information, we selected some proposed genes/proteins that have been shown to be involved in at least one of the different NB tumorigenic processes: proliferation, anti-apoptosis, angiogenesis and/or metastasis. In addition, for these genes/proteins, promising in vitro and in vivo results were obtained by silencing/inhibiting them. We recently proposed to target doublecortin-like kinase (DCLK1) to inhibit NB proliferation and induce apoptosis (Verissimo et al. 2010). In addition, neuropeptide Y (NPY) and its Y2 receptor (NY2R) were selected as examples of anti-angiogenesis targets (Lu et al. 2010). The high expression levels of the sympathetic neurotransmitter NPY correlates with MYCN amplification and with poor clinic outcome (Dötsch et al. 1998). Furthermore,
for NB invasion and metastasis, cathepsin L was chosen for further reviewing (Lankelma et al. 2010). Cathepsin L is also involved in the development of drug resistance (Zheng et al. 2004).

**Inhibiting NB proliferation and survival: DCLK1**

Several proteins have been shown to play a crucial role in NB proliferation and survival, MYCN being probably the best known and characterised. In addition, there are a number of genes that have been reported as regulators in the neuronal system, such as small GTPase RAB6B, cell recognition molecule Caspr2 (CNTNAP2), neuroexophilin (Nxph1) and DCLK1 that are also expressed in NB (Nakagawara & Ohira 2004). Of particular interest are members of the DCX gene family like DCLK1 (Coquelle et al. 2006, Reiner et al. 2006). By virtue of alternative splicing, the DCLK1 gene encodes for several microtubule-binding protein (MAP). MAPs have been considered as potential targets for cancer therapy. However, since most MAPs are not specifically expressed in cancer cells, high toxicity due to the treatment has been reported. In contrast, DCLK1-containing microtubule-binding domains are particularly highly expressed in neuroblasts and NBs but not in other cell types, suggesting that targeting these MAPs is a highly interesting potential therapeutic approach with low cytotoxic side effects.

The DCLK1 gene encodes numerous splice variants. The main splice variants are DCL, DCLK-long, DCLK-short and calcium/calmodulin-dependent protein kinase (CaMK)-related peptide (CARP; Vreugdenhil et al. 2001, Burgess & Reiner 2002, Dijkmans et al. 2010). DCLK-long and DCL contain two microtubule-binding domains, also called DCX domains (Gleeson et al. 1999, Burgess & Reiner 2000, Vreugdenhil et al. 2007), whereas DCLK-long and DCLK-short contain a CaMK-like domain (Schenk et al. 2007). Both DCX and CaMK-like domains are not present in CARP (Vreugdenhil et al. 1999).

*In vivo* studies have shown that DCLK1 gene-derived MAPs regulate neurogenesis by being involved in the mitotic spindle formation in neuroblasts (Shu et al. 2006, Vreugdenhil et al. 2007). Both loss and gain of function of the DCLK1 MAPs result in an impairment of proliferation of neuroblasts in *in vivo* (Shu et al. 2006, Vreugdenhil et al. 2007). However, these MAPs are not only involved in the regulation of the cell cycle and determination of cell fate but also in neuronal migration and retrograde transport of glucocorticoid receptors (GR; Koizumi et al. 2006, Fitzsimons et al. 2008). The stabilisation of the microtubules by DCLK1 MAPs seems similar to the stabilisation provided by the highly homologous DCX (Shu et al. 2006).

Recently, we showed that DCL and DCLK-long are highly expressed in human NBs and gliomas (Verissimo et al. 2010). DCX is also expressed in NBs, being considered as diagnostic marker to detect minimal residual disease in NB patients (Oltra et al. 2005). We demonstrated that DCL and DCLK-long are essential for the proliferation and survival of NB cells (Verissimo et al. 2010). Moreover, the knockdown of these proteins induced apoptosis in mouse and human NB cells (Verissimo et al. 2010). Figure 2 schematically shows the consequences of silencing of DCLK1 MAPs. Gene expression profiling of NB cells after DCL and DCLK-long knockdown showed that several pathways related to the cell cycle and apoptosis were affected. Oxidative phosphorylation and oxidative stress were identified to be among the most over-expressed biological pathways and mitochondria were the most affected cell components. Hence, these studies indicate a pro-apoptotic effect of DCL/DCLK-long knockdown that may be induced by oxidative stress mechanisms that involve changes in mitochondrial activity, as reported previously (Green & Reed 1998, Nazarewicz et al. 2007). The results also suggest that induction of apoptosis might be related to the level of disruption of mitotic spindles, which would be in agreement with the observations obtained by inhibiting proteins that stabilise mitotic spindles, such as AURKA. As explained above, AURKA inhibition leads to mitotic spindle defects and apoptosis (Hirot A et al. 2003). Several studies have shown that silencing or overexpression of MAPs of the DCX family results in inhibition of cell proliferation by mitotic spindle disruption (Santra et al. 2006, 2009, Shu et al. 2006, Vreugdenhil et al. 2007). Indeed, the fact that BIRC5 is down-regulated in NB cells with DCL and DCLK-long knockdown gives indication of mitotic spindle catastrophe (Bhalla 2003, Verissimo et al. 2010). Moreover, the pro-apoptotic gene Bax was detected to be up-regulated (Verissimo et al. 2010) and has been shown to be involved in induction of apoptosis, possibly resulting from disruption of mitotic spindles and mitotic arrest (Bhalla 2003). An alternative explanation for the induction of apoptosis is the disruption of the intracellular transport of signalling proteins due to the silencing of DCL and DCLK-long. As demonstrated previously, DCL plays a crucial role in regulating retrograde translocation of signalling proteins like the GR in neuronal progenitor cells (Fitzsimons et al. 2008).

Another interesting finding was a significant correlation between DCL expression and the
expression of genes related to mitochondrial activity in human NBs. Therefore, the connection between MAP derived from the DCLK1 gene and mitochondria deserves further study. One possibility is that this connection is related to the fact that mitochondria are transported along microtubules (Morris & Hollenbeck 1995).

In summary, DCLK1-derived MAPs are highly expressed in neuroblasts and perform crucial functions related to neuroblast proliferation, migration and differentiation (Shu et al. 2006, Vreugdenhil et al. 2007). These proteins showed to be highly expressed in human NBs and their silencing induces profound apoptosis of NB cells (Verissimo et al. 2010). The apoptotic process seems to be dependent on mitochondria and may result from disruption of the mitotic spindles and arrest of the cells at prometaphase (Shu et al. 2006, Vreugdenhil et al. 2007). Therefore, we propose DCLK1 as a potential molecular target for NB treatment with the promises of high specificity and low toxicity.

**Targeting angiogenesis: NPY and its NY2R**

High-risk NB tumours present an increased angiogenesis with high vascular index and are correlated with poor prognosis (Shusterman & Maris 2005). This finding indicates a relation between the active angiogenesis and the growth of aggressive tumours. Hence, the inhibition of angiogenesis may represent a therapeutic approach or a powerful adjunct to other therapies for NB. The pro-angiogenic phenotype is promoted by growth factors such as VEGF and methionine aminopeptidase 2 (Shusterman & Maris 2005, Modak & Cheung 2010).

NPY has also been shown to stimulate angiogenesis and NB proliferation (Cohen et al. 1990). NPY is a sympathetic neurotransmitter, which acts through G-protein-coupled receptors (Y1–Y5; Lu et al. 2010). NPY is a growth factor for various cells including endothelial cells and neuronal precursors (Movafagh et al. 2006, Lu et al. 2010). NBs produce and release NPY neurotransmitter. Hence, NB patients present high levels of NPY in their plasma. These high NPY levels correlate with MYCN amplification and poor clinical outcome (Dötsch et al. 1998). NPY induces NB tumour growth and angiogenesis (Kitlinska et al. 2005). NY2R is the most common NPY receptor expressed in NB cells and blocking the binding of NPY to NY2R has been proposed as an approach to inhibit NB growth (Lu et al. 2010). It has been shown that blocking NY2R results in a decrease in NB proliferation rate, it induces apoptosis and in vivo studies show an impairment of the tumour vascularisation as well (Lu et al. 2010). Therefore, there are strong indications that targeting angiogenesis is a promising approach, being NPY, NY2R and/or other proteins involved in this tumorigenic process (Table 2) targets to consider for NB therapy.

**Targeting NB invasion and metastasis: cathepsin L**

Invasion and migration of NB cells may lead to metastasis, which is the major cause of death in NB
patients. Thus, inhibition of the invasive potential of NB cells could have major positive impact on the clinical outcome of patients that present metastatic disease.

Loss of cell adhesion and digestion of the extracellular matrix are processes that allow the invasion and migration of cancer cells (Cairns et al. 2003, Gocheva et al. 2006). Cathepsin L seems to be involved in these processes (Lankelma et al. 2010). Indeed, the active isoforms of cathepsin L can be found, not only intracellularly (liposomes, cytoplasm and nucleus) but also in the extracellular matrix (Zheng et al. 2009). Owing to an increase in anaerobic glycolysis, the tumour cells are in an acidic environment (Lankelma et al. 2010). In these acidic conditions, cathepsin L is active and digests components of the extracellular matrix, such as collagen types I and IV (Skrzydlewska et al. 2005). This indicates that inhibition of cathepsin L might lead to a reduction of the degradation of the extracellular matrix and, consequently, to reduction of invasion and migration of the cancer cells through the basal lamina (Lankelma et al. 2010).

Moreover, the inhibition of cathepsin L might contribute to a better action of the chemotherapeutic agents and, therefore, reduce the need for these toxic compounds. This is due to the fact that cathepsin L is involved in the sequestration of therapeutic drugs (Zheng et al. 2004). It has been shown that a combination of doxorubicin and cathepsin L inhibition is able to induce senescence in NB cells (Zheng et al. 2004). Doxorubicin is a chemotherapeutic drug used to treat cancer, such as NB. It intercalates the DNA of the cells, blocking proliferation and inducing apoptosis (Zheng et al. 2004). In vitro and in vivo studies have shown that the inhibition of cathepsin L not only reversed but also prevents the development of drug resistance (Zheng et al. 2009). It has been suggested that the inhibition of cathepsin L allows the stabilisation and increase in availability of the drug target (Zheng et al. 2009).

Altogether, there are substantial indications that cathepsin L plays a key role in the metastasis process and in the development of drug resistance. Therefore, cathepsin L is a promising molecular target for NB therapy.

miRNAs as targets for NB therapy

miRNAs are non-coding RNAs that repress translation and promote mRNA degradation by sequence-specific interaction with mRNA. Hence, miRNAs are important modulators of gene expression and are involved in homeostatic processes such as development, differentiation, proliferation and apoptosis (Lynam-Lennon et al. 2009, Wu 2010). Because of these properties, it has been proposed that some miRNAs may be involved in tumour initiation and progression, functioning as oncogenes or tumour suppressors (Zhang et al. 2007a,b). Therefore, modulation of miRNAs for potential cancer therapy is of great promise (Calin & Croce 2007, Wang & Wu 2009, Wu 2011). For tumour suppressor miRNAs, restoring suppressor miRNAs by forcing their expression may be a strategy (Li et al. 2009). miRNAs with oncogene capabilities can be effectively targeted by oligonucleotides that are complementary to them, termed anti-miRNA oligonucleotides (AMOs), antagonirs or anti-miRs (Calin & Croce 2007, Stenvang et al. 2008). Antagonirs or AMOs are 2-O-methyl oligoribonucleotides and anti-miRs are locked nucleic acid (LNA) nucleotides containing oligodeoxyribonucleotides (Dalmary 2008). For example, Stenvang et al. (2008) have shown in vitro and in vivo that LNA-anti-miR allows sequence-specific inhibition of miRNAs function. The hybridisation of LNA-modified oligonucleotides with their target RNA results in high thermal stability. In addition, they show good aqueous solubility, good transfection efficiency and low toxicity, and LNA-anti-miR compounds are in clinical trials for cancer therapy (Stenvang et al. 2008).

Despite the advances in the development of miRNA-mediated therapy, some challenges still remain (Li et al. 2009). The first one is to sustain target specificity, which is particularly challenging because silencing only requires partial complementary between miRNA and mRNA. A second challenge is to achieve high therapeutic efficiency. Hence, the promising miRNA-mediated therapy needs further investigation to improve the target selection, the molecule design and the delivery approach (Li et al. 2009).

The expression levels of miRNAs and their role in proliferation, differentiation and apoptosis have also been studied in NB (Table 3). There are indications that miRNA expression levels are predictive of clinical outcome. This suggests that miRNAs might be used as diagnostic markers and targets for NB therapy (Bray et al. 2009). An overview of differently expressed miRNAs that have been proposed as NB therapeutic targets is provided in Table 3 and their expression in other tumours is given in Supplementary Table 2, see section on supplementary data given at the end of this article.

Results show that the widespread deregulation of miRNAs expression in NB tumours is due to the chromosomal imbalances and overexpression of the MYCN (Bray et al. 2009). MYCN might regulate
the expression of miRNAs that play a role in NB proliferation (Chen & Stallings 2007). For example, it has been demonstrated that the MYCN oncogene in high-risk NB induces the expression of the miR-17-92 cluster (Schulte et al. 2008). This cluster consists of six individual miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) from a polycistronic transcript on human chromosome 13 (van Haafoten & Agami 2010). miRNAs that are targeted by miRNAs derived from the miR-17-92 cluster have key roles in cell cycle control and cell death. In particular, miR-17 and miR-20a target the CDK inhibitor CDKN1A (p21), a negative regulator of the G1-S transition and miR-17 targets the pro-apoptotic BCL2L11 (Bim) (Fontana et al. 2008). Recently, it was also found that miR-17-92 targets several effectors of transforming growth factor beta (TGFB) signalling cascade in NB cells (Mestdagh et al. 2010). Therefore, the miR-17-92 cluster is of particular interest as target for NB therapy.

MiR-34a may be one of the miRNAs regulated by MYCN as well. In fact, MYCN and also the anti-apoptotic BCL2 have been identified as miR-34a targets (Cole et al. 2008, Wei et al. 2008). A number of other targets for miR-34a, including BIRC3, and decoy receptor 3, are related to regulation of cell cycle progression, apoptosis, DNA repair and angiogenesis (Chang et al. 2007). MiR-34a has tumour suppressor functions and is expressed at low levels in not favourable NB tumours (Welch et al. 2007, Cole et al. 2008). In addition, low expression levels of miR34a have been associated with NB resistance towards p53-activating chemotherapeutic agents. There are indications that miR34a is transactivated by p53 (Chang et al. 2007, Hermeking 2010).

An miRNA that is highly expressed in MYCN-amplified NB is MiR-380-5p. It modulates p53 expression, controlling the proliferation of NB cells and is associated with poor therapeutic outcome (Swarbrick et al. 2010). The overexpression of MiR-380-5p leads to the formation of NB tumours in mice and the inhibition of this miRNA induces apoptosis via p53 (Swarbrick et al. 2010). In vivo delivery of a miR-380-5p antagonist by i.p. injection of modified anti-sense oligonucleotides resulted in a reduction of tumour size in an NB orthotopic mouse model (Swarbrick et al. 2010). Other miRNAs suggested as targets for NB therapy are indicated in Table 3.

Clearly, expression of specific miRNAs is of critical importance for different tumorigenic processes in NB, such as tumour proliferation and metastasis. Therefore, miRNAs are certainly valuable diagnostic markers for NB and the development of novel therapeutic approaches based on NB-associated miRNAs is of great promise.

### Future prospects

Targeting specific molecules represents a promising therapeutic approach for cancer, including NB (Segerstrom et al. 2006, Petrelli & Giordano 2008, George et al. 2010). In contrast to conventional chemotherapy, targeted drugs give the possibility to specifically hit subpopulations of cells, thereby reducing the toxic effects (Petrelli & Giordano 2008). Therefore, the high-affinity/high-specificity compounds have been of high interest (Schattenholz et al. 2010). However, despite the initial enthusiasm for the efficacy of these treatments, some disappointing results have been obtained (Modak & Cheung 2010). Patients have been confronted with relapse and developed drug resistance, which might be due to the activation of alternative pathways (Petrelli & Giordano 2008, Modak & Cheung 2010).

At the moment, there is a general agreement that multi-target approaches could be more effective than single-target agents (Espinoza-Fonseca 2006, Petrelli & Giordano 2008). Some models indeed indicate that partial inhibition of a few targets is more effective than full inhibition of a single target (Csermely et al. 2005). Targeting NB cells and tumours as a system instead of targeting single molecules might allow the discovery of a novel class of multi-target drugs, which would have fewer adverse effects and less toxicity. This ‘systemic’ drug approach represents a new challenge in the coming decade but is of great promise (Espinoza-Fonseca 2006, Schattenholz & Noskić 2008).

Novel drugs or small molecules directed at specific targets or pathways will certainly be identified. Nevertheless, as explained above, a combination of a reduced number of these novel agents could be more effective in NB therapy than single-target approaches. A combination of low doses of agents already on the market and novel single-target agents that target, for example, ALK or DCLK1-derived MAPs is definitely a multi-target approach to consider. One would expect, for instance, a synergetic effect in the disruption of microtubules by targeting DCLK1-derived MAPs, which are crucial for microtubule stabilisation, in combination with microtubule-disrupting agents, such as vinca alkaloids. Of consideration is also targeting the different processes involved in NB origin and maintenance by multitargeting proteins and/or miRNAs that play a role in those processes.

In summary, in the next 5–10 years, we predict that research in the NB therapy field will focus not only on the individual promising molecular targets but also on the different multi-target approaches.
Particular emphasis on the understanding of the interaction between NB, its micro-environment and pharmacogenomics will be crucial. Furthermore, selection of patients that are likely to respond to the treatment will be a challenge to overcome as well. Molecular analysis of patient-specific tumours at the diagnostic might allow a precise prognosis and determination of the most effective treatment.

Concluding remarks
A new and better therapy for NB is needed, particularly for high-risk NB. Many molecular targets have been proposed in recent years but most of them have not yet been investigated in the clinic. Thus, the key for more effective therapeutic strategies might be in the novel and unexplored targets. Here, we have reviewed the existing literature on some of the targets that are under investigation in the clinic and on the proposed targets that are not yet explored in the clinic. These targets play crucial roles in the transformation of progenitor cells into NB and in the processes involved in NB progression, such as proliferation, survival, angiogenesis, invasion, migration and metastasis. Some of these not yet developed targets are undoubtedly of main interest for further investigation. We have discussed some of the targets that are not yet under investigation in the clinic in more detail. For example, we have shown that the microtubule-binding protein DCLK1 is a promising target due to its crucial role in NB proliferation and cell survival. DCLK1-derived MAPs are overexpressed in NB cells compared to normal tissues and other tumour types. miRNAs have been shown to be involved in NB tumorigenic processes and therefore proposed as therapeutic targets as well. There are several ongoing projects for modulating miRNAs expression in NBs.

Aiming at just one target involved in one of the processes of NB origin and progression has been shown to result in the activation of alternative and compensatory pathways, leading in some cases to drug resistance. Therefore, future success including reduction of long-term toxic effects may depend on rational studies of novel molecular patient-specific multi-target approaches.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0251.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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