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

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

Gene symbols	Names	Function/processes	Compound(s)	Reference(s)
<i>AKT1</i>	v-akt murine thymoma viral oncogene homologue 1	Cell growth/proliferation, apoptosis	A-443654, perifosine (KRX-0401)	Opel <i>et al.</i> (2007), LoPiccolo <i>et al.</i> (2008), Li <i>et al.</i> (2011)
<i>ALK</i>	Anaplastic lymphoma receptor tyrosine kinase	Transmembrane receptor protein tyrosine kinase activity, development of the brain	PF-2341066	George <i>et al.</i> (2008), Mosse <i>et al.</i> (2008), Ogawa <i>et al.</i> (2011)
<i>AURKA</i>	Aurora kinase A	Cell cycle regulation	MLN8237	Wagner & Danks (2009), George <i>et al.</i> (2010), Carol <i>et al.</i> (2011)
<i>BCL2</i>	B-cell CLL/lymphoma 2	Anti-apoptosis	Obatoclox	Dole <i>et al.</i> (1994), Rheingold <i>et al.</i> (2007)
<i>CTLA4</i>	Cytotoxic T-lymphocyte-associated protein 4	Immune response, apoptosis	Ipilimumab (MDX-010)	Contardi <i>et al.</i> (2005), Modak & Cheung (2010)
<i>EGFR</i>	Epidermal growth factor receptor	Cell proliferation, cell–cell adhesion, apoptosis	Gefitinib (ZD1839)	Modak & Cheung (2010), Furman <i>et al.</i> (2011)
<i>HDAC</i>	Histone deacetylase	Transcriptional regulation, cell cycle progression and development	Valproic acid, Vorinostat	Coffey <i>et al.</i> (2001), George <i>et al.</i> (2010)
<i>HSP90AA1</i>	Heat-shock protein 90 kDa alpha (cytosolic), class A member 1	Signal transduction, protein folding, protein degradation	17-AAG	Kang <i>et al.</i> (2006), Furchert <i>et al.</i> (2007), George <i>et al.</i> (2010)
<i>IGF1R</i>	IGF1 receptor	Anti-apoptotic, tyrosine kinase activity	NVP-AEW541, EM164, SCH71745, IMC-A12	Liu <i>et al.</i> (1998), Wagner & Danks (2009)
<i>KDR</i>	Kinase insert domain receptor (a type III receptor tyrosine kinase)	Growth factor, endothelial proliferation, survival, migration, tubular morphogenesis and sprouting	Bevacizumab, sunitinib, cediranib (AZD2171)	Segerstrom <i>et al.</i> (2006), Sims <i>et al.</i> (2008), Morton <i>et al.</i> (2011)
<i>mTOR</i>	Mechanistic target of rapamycin (serine/threonine kinase)	Cell cycle, proliferation, cellular responses to stresses	Rapamycin, everolimus, temsirolimus, AP23573	LoPiccolo <i>et al.</i> (2008), Fulda (2009), Wagner & Danks (2009)
<i>NTRK2</i>	Neurotrophic tyrosine kinase, receptor, type 2	Cell signalling, cell differentiation	CEP-701 (KT-6587)	Evans <i>et al.</i> (1999, 2001)
<i>PDK1</i>	Pyruvate dehydrogenase kinase, isozyme 1	Carbohydrate and pyruvate metabolic processes	OSU-03012	Fulda (2009)
<i>PIK3CA</i>	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	Anti-apoptosis, glucose metabolism process, signal transduction	GDC-0941, NVP-BEZ235	LoPiccolo <i>et al.</i> (2008)
<i>TNFRSF10B</i>	Tumour necrosis factor receptor superfamily, member 10b	Apoptosis	Lexatumumab (ETR2-ST01)	Zhang <i>et al.</i> (2007b), Modak & Cheung (2010)

Gene symbols and names are in agreement with HUGO Gene Nomenclature Committee even when the nomenclature used in the references is different.

origin and progression and that have been proposed as therapeutic targets. This overview is subdivided into three separate sections: 1) molecular targets under investigation in the clinic, 2) proposed molecular targets not yet explored in the clinic and 3) miRNAs as targets for NB therapy.

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

Table 2 Proposed target genes for neuroblastoma therapy

Gene symbols	Names	Function/processes	Reference(s)
<i>BCL6</i>	B-cell CLL/lymphoma 6	Cell proliferation, differentiation, apoptosis	Chamdin <i>et al.</i> (2009)
<i>BIRC5</i>	Baculoviral IAP repeat-containing 5	Apoptosis, cell cycle	Islam <i>et al.</i> (2000b), Duffy <i>et al.</i> (2007)
<i>CASP8</i>	Caspase 8, apoptosis-related cysteine peptidase	Apoptosis, cell adhesion and metastasis	McKee & Thiele (2006)
<i>CCND1</i>	Cyclin D1	Cell cycle, differentiation	Molenaar <i>et al.</i> (2008, 2010)
<i>CD44</i>	CD44 molecule (Indian blood group)	Cell adhesion and metastases	Yoon & Danks (2009)
<i>CDK2</i>	Cyclin-dependent kinase 2	Cell cycle, DNA replication	Molenaar <i>et al.</i> (2009)
<i>CENPE</i>	Centromere protein E	Cell cycle	Balamuth <i>et al.</i> (2010)
<i>CRABP2</i>	Cellular retinoic acid binding protein 2	Epidermis development, signal transduction, retinoic acid metabolic process	Itoh <i>et al.</i> (2010)
<i>CTSL1</i>	Cathepsin L1	Proliferation, apoptosis, angiogenesis, invasion and metastasis	Zheng <i>et al.</i> (2009), Colella <i>et al.</i> (2010), Lankelma <i>et al.</i> (2010)
<i>DCLK1</i>	Doublecortin-like kinase 1	Cell proliferation, survival, neuronal cell migration, neurogenesis	Shu <i>et al.</i> (2006), Vreugdenhil <i>et al.</i> (2007), Verissimo <i>et al.</i> (2010)
<i>DIABLO</i>	Diablo, IAP-binding mitochondrial protein	Apoptosis, neuroblastoma progression	Wolf <i>et al.</i> (2010)
<i>DUSP26</i>	Dual specificity phosphatase 26 (putative)	Protein dephosphorylation	Shang <i>et al.</i> (2010)
<i>EPAS1</i>	Endothelial PAS domain protein 1	Keeps tumour-initiating cells in a undifferentiated state	Pietras <i>et al.</i> (2009), Qing <i>et al.</i> (2010)
<i>GCLC</i>	Glutamate–cysteine ligase, catalytic subunit	Glutamate–cysteine ligase, apoptosis	de Tudela <i>et al.</i> (2010)
<i>GSK3B</i>	Glycogen synthase kinase 3 beta	Neuronal cell development, hippocampus development, glycogen metabolic process	Li <i>et al.</i> (2010), Dickey <i>et al.</i> (2011)
<i>HIF1A</i>	Hypoxia inducible factor 1 alpha subunit (basic helix-loop-helix transcription factor)	Differentiation of neural crest cells, modulation of energy metabolism in cancer	Yeo <i>et al.</i> (2003), Nakagawara & Ohira (2004)
<i>Id2</i>	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	Differentiation of neural crest cells, transcription factor	Lasorella <i>et al.</i> (2002), Nakagawara (2004)
<i>IGFBP5</i>	IGF binding protein 5	Regulation of cell growth, signal transduction, apoptosis, cell migration	Tanno <i>et al.</i> (2005)
<i>LDHA</i>	Lactate dehydrogenase A	Anaerobic glycolysis, oxidation–reduction process	Qing <i>et al.</i> (2010)
<i>LGALS1</i>	Lectin, galactoside binding, soluble, 1	Cell proliferation, migration, differentiation, apoptosis	Cimmino <i>et al.</i> (2009)
<i>MCL1</i>	Myeloid cell leukaemia sequence 1 (BCL2-related)	Apoptosis, differentiation	Lestini <i>et al.</i> (2009)
<i>METAP2</i>	Methionine aminopeptidase 2	Angiogenesis	Shusterman & Maris (2005)
<i>MIF</i>	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	Cell proliferation, negative regulator of apoptosis, negative regulator of cell cycle arrest	Ren <i>et al.</i> (2006)
<i>NME1</i>	Non-metastatic cells 1, protein (NM23A) expressed in	Cell adhesion, metastasis, cell differentiation, negative regulation of apoptosis	van Noesel & Versteeg (2004), Tee <i>et al.</i> (2006)
<i>NME2</i>	Non-metastatic cells 2, protein (NM23A) expressed in	Cell adhesion, negative regulation of apoptosis	van Noesel & Versteeg (2004)
<i>NPY</i>	Neuropeptide Y	Tumour cell proliferation, angiogenesis	Lu <i>et al.</i> (2010)
<i>NPY2R</i>	Neuropeptide Y receptor Y2	Tumour cell proliferation, angiogenesis	Lu <i>et al.</i> (2010)
<i>PAX3</i>	Paired box 3	Apoptosis, regulation of transcription, multi-cellular organism development	Gershon <i>et al.</i> (2005)
<i>PAX7</i>	Paired box 7	Anti-apoptosis, differentiation, neuronal fate	Gershon <i>et al.</i> (2005)
<i>pLK1</i>	Polo-like kinase 1	Cell cycle, cell proliferation, G2/M transition DNA damage checkpoint	Hu <i>et al.</i> (2009)
<i>PRAF2</i>	PRA1 domain family, member 2	Protein transport, L-glutamate transport, apoptosis	Geerts <i>et al.</i> (2007)

Table 2 continued

Gene symbols	Names	Function/processes	Reference(s)
<i>PTGS2</i>	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	Cell cycle, apoptosis, cell migration, angiogenesis	Kaneko <i>et al.</i> (2009)
<i>PTK2</i>	PTK2 protein tyrosine kinase 2	Regulates both cellular adhesion and apoptosis	Beierle <i>et al.</i> (2010)
<i>RAC1</i>	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP-binding protein Rac1)	Cytoskeleton organisation, cell proliferation, migration, cell survival	Lazer & Katzav (2011)
<i>RAN</i>	RAN, member RAS oncogene family	Regulator in the nervous system, cell cycle	Tietze <i>et al.</i> (2008)
<i>SOX10</i>	SRY (sex determining region Y)-box 10	Embryonic development and cell fate, neural crest and peripheral nervous system development	Gershon <i>et al.</i> (2005)
<i>TFAP2A</i>	Transcription factor AP-2 alpha	Ectoderm development, skeletal system morphogenesis	Gershon <i>et al.</i> (2005)
<i>TLR9</i>	Toll-like receptor 9	Positive regulation of JNK cascade and JUN kinase activity, positive regulation of inflammatory response	Brignole <i>et al.</i> (2010)
<i>TOP2A</i>	Topoisomerase (DNA) II alpha 170 kDa	Apoptosis, DNA repair and replication	Glynn <i>et al.</i> (2010)
<i>TP73</i>	Tumour protein p73	Differentiation of neural crest cells, apoptosis, migration	Moll & Slade (2004), Wolter <i>et al.</i> (2010)
<i>UGCG</i>	UDP-glucose ceramide glucosyltransferase	Lipid and glucosylceramide biosynthetic process, keratinocyte differentiation	Barth <i>et al.</i> (2010)
<i>YBX1</i>	Y box binding protein 1	Cell proliferation, regulator of transcription, metastasis	Wachowiak <i>et al.</i> (2010)

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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

Table 3 Potential micro-RNAs as targets for neuroblastoma therapy

miRNA	Validated target(s)	Function/processes	Types	Reference(s)
miR-17-5p-92 cluster	TGFβ-signalling, CDKN1A (p21); BCL2L11 (Bim)	Cell proliferation, cell adhesion	Oncogene	Fontana <i>et al.</i> (2008), Mestdagh <i>et al.</i> (2010)
miR-34a	NMYC, BCL-2, E2F3	Cell cycle progression, apoptosis, DNA repair and angiogenesis	Tumour suppressor	Welch <i>et al.</i> (2007), Cole <i>et al.</i> (2008), Wei <i>et al.</i> (2008)
miR-184	AKT2	Neural differentiation and/or apoptosis	Tumour suppressor	Foley <i>et al.</i> (2010), Tivnan <i>et al.</i> (2010)
miR-380-5p	p53	Apoptosis	Oncogene	Swarbrick <i>et al.</i> (2010)
miR-9	E-cadherin, tropomyosin-related kinase C	Angiogenesis, metastasis	Oncogene	Laneve <i>et al.</i> (2007), Ma <i>et al.</i> (2010), Khew-Goodall & Goodall (2010)
miR-125a	Bmf, tropomyosin-related kinase C	Cell proliferation, apoptosis	Oncogene	Laneve <i>et al.</i> (2007)
miR-125b	Bmf, tropomyosin-related kinase C	Cell proliferation, apoptosis	Oncogene	Laneve <i>et al.</i> (2007)
miR-152	CHUK, CUL5 and GADD45A	Neuroblast differentiation, migration/invasion and apoptosis	Oncogene	Ragusa <i>et al.</i> (2010)
miR-338	PTPRT	Neuroblast differentiation and apoptosis	Oncogene	Ragusa <i>et al.</i> (2010)
miR-200B	ZEB1	Neuroblast differentiation, migration/invasion and apoptosis	Tumour suppressor	Ragusa <i>et al.</i> (2010)

(George *et al.* 2010). 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 (Sarbasov *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

Figure 1 Development of the lineages derived from neural crest cells and the origin of the different stages of neuroblastoma (NB). This figure is based on the information provided by Maris & Matthay (1999), Mora & Gerald (2004), Nakagawara & Ohira (2004), and Huber (2006). Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-11-0251>.

(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, lactate 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*), neurexophilin (*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 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 (Hirota *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

Figure 2 Representation of the consequences of targeting *DCLK1*-derived MAPs (DCLK). This figure is based on the results shown in Shu *et al.* (2006), Vreugdenhil *et al.* (2007), Fitzsimons *et al.* (2008) and Verissimo *et al.* (2010). Full colour version of this figure available via <http://dx.doi.org/10.1530/ERC-11-0251>.

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 (Skrzydewska *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), antagomirs or anti-miRs (Calin & Croce 2007, Stenvang *et al.* 2008). Antagomirs or AMOs are 2'-O-methyl oligoribonucleotides and anti-miRs are locked nucleic acid (LNA) nucleotides containing oligodeoxyribonucleotides (Dalmay 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 Haaften & Agami 2010). mRNAs 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 BCL2L1 (Bim) (Fontana *et al.* 2008). Recently, it was also found that miR-17-92 targets several effectors of transforming growth factor beta (TGF β) 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 (Schrattenholz *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, Schrattenholz & Soskić 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 multi-targeting 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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