The handle http://hdl.handle.net/1887/20248 holds various files of this Leiden University dissertation.

**Author:** Leiria Verissimo, Carla Sofia  
**Title:** Doublecortin-like kinase : a potential therapeutic target for neuroblastoma  
**Date:** 2012-12-06
1

General introduction
1.1 Neuroblastoma and doublecortin-like kinase

1.1.1 Neuroblastoma

Neuroblastoma (NB) is the most common solid neoplasm in children under five years of age (Maris et al., 2007; van Noesel and Versteeg, 2004). It is a neuroendocrine tumor arising from the immature sympathetic neuroblast cells (Maris and Matthay, 1999). The most common sites of origin are the adrenal glands, but it can also develop from the nerve tissue in the abdomen, pelvis, chest and neck (Maris and Matthay, 1999). NB is the most aggressive neuroblastic tumor being composed almost entirely of neuroblasts. Ganglioneuroma and ganglioneuroblastoma are other types of neuroblastic tumors that present more differentiated cells (Shimada et al., 1999). Based on the prognostic stage (International Neuroblastoma Staging System: 1, 2A, 2B 3, 4 or 4S), patient age, status of MYCN oncogene (not amplified or amplified) and deletion of chromosome 1p; patients can be assigned to low-, intermediate- and high-risk groups (Brodeur et al., 1993; Haase et al., 1999). About half of the patients are diagnosed with high-risk NB (Brodeur et al., 1993; Haase et al., 1999). The overall survival rate for patients with high-risk NB is lower than 50%, despite the intensive multimodal therapy usually applied (Maris et al., 2007).

1.1.1.1 Neuroblastoma treatment

Low-risk NB patients have a good prognosis and can be treated with surgery alone. Nevertheless, patients with high-risk NB are treated with surgery, radiation therapy, intensive chemotherapy, bone marrow or haematopoietic stem cell transplantation, and with targeted biologic therapies, immunotherapy and 13-cis-retinoic acid (Modak and Cheung, 2010; Verissimo et al., 2011). These diverse therapeutic approaches aim to stop the tumorigenic processes by inhibiting tumor proliferation, angiogenesis, metastasis, and by inducing differentiation and cell death (Verissimo et al., 2011). NB apoptosis is programmed cell death induced by treatment and correlates with a reduction of tumor volume (Kim, 2005). The induced apoptotic process might be mediated through caspase-dependent or -independent pathways. Autophagy and necrosis are cell death processes that might result from the therapy as well. In fact, apoptosis may induce autophagy via crosstalk between these two processes (Kim, 2005). The drugs used in NB therapy are directed to the cellular microtubule structure, mitochondria activity and several other molecular targets that play a crucial role in the tumorigenic process (George et al., 2010; Verissimo et al., 2011; Wagner and Danks, 2009).

Microtubules as NB targets

Microtubules are crucial for cell development and shape, and play an important role in the intracellular transport of mitochondria, vesicles, signaling molecules and other components. Microtubules are also important in cell signaling, mitosis and cell division (Jordan and Wilson, 2004). The rapid dynamic of the microtubules in mitotic spindles is crucial for successful mitosis and cell proliferation. Therefore, microtubules have been important targets for cancer therapy: anti-mitotic agents suppress the dynamic of the spindle-microtubules, resulting in cell cycle arrest and apoptotic cell death (Jordan and Wilson, 2004).
Vinca alkaloids, such as vinblastine and vincristine, are microtubule-destabilizing agents used in high-risk NB therapy (George et al., 2010). These compounds were the first tubulin-binding agents to be discovered and are since the 1970s used in cancer treatment (Kavallaris, 2010). Vinca-alkaloids depolymerize microtubules, disrupt mitotic spindles, and induce mitotic arrest and cell death (Jordan et al., 1991). Unfortunately, these anti-mitotic drugs have severe side effects leading to toxicity, bone marrow suppression and development of resistance in NB patients (Don et al., 2004). Therefore these agents are now used at low doses, in combination with other drugs (Kavallaris, 2010). Moreover, several combination approaches that involve vinca alkaloids are currently under investigation, including the combination of vinblastine and rapamycin (sirolimus), an immunosuppressant (http://clinicaltrials.gov # NCT01135563; Marimpietri et al., 2007); the combination of vincristine together with topotecan (topoisomerase I inhibitor) and doxorubicin (http://clinicaltrials.gov # NCT00392340); and vinorelbine combined with tariquidar, a p-Glycoprotein inhibitor (http://clinicaltrials.gov # NCT00011414), among others.

Mitochondria as targets
Mitochondria are vital for maintaining cell life, but also for regulating cell death (Fulda et al., 2010). Mitochondria are the main energy producers of the cell and are also crucial regulators of the apoptotic process. These organelles regulate the translocation of pro-apoptotic proteins from the mitochondrial intermembrane space to the cytosol, which occurs upon permeabilization of their membranes (Armstrong, 2006). Factors that regulate mitochondrial membrane permeabilization include the mobilization of members of the Bcl-2 family to the mitochondria and changes on the redox status, such as alterations on reactive oxygen species (ROS) levels (Armstrong, 2006).

Cancer cells present a reprogrammed mitochondria metabolism (Fulda et al., 2010). In standard oxygen tension conditions, non-malignant cells rely on oxidative phosphorylation for ATP production. However, malignant cells have an overall increase in anabolism. The rapid cell proliferation and tumor growth lead to a shift from high availability of nutrients and oxygen, to competition, starvation and hypoxia (Ralph and Neuzil, 2009). Thus, malignant cells present an altered metabolism allowing their survival and proliferation in such micro-environment (Ralph and Neuzil, 2009). Several tumor cells, including NB, switch to an aerobic glycolytic metabolism (Smith et al., 2008). This process is rarely observed in non-cancer cells and is known as the Warburg effect (Racker and Spector, 1981; Warburg, 1956). The metabolic changes that distinguish cancer cells from normal cells have been of high interest for the identification of therapeutic targets (Fulda et al., 2010; Pelicano et al., 2006; Ralph and Neuzil, 2009).

Since mitochondria are key regulators of cell death, and their functions are altered in cancer cells, targeting mitochondria is a promising approach to eradicate cancer cells (Fulda et al., 2010). Several anticancer agents have been identified that act via mitochondria. These compounds, termed mitocans, disrupt mitochondrial function in cancer cells, particularly at the oxidative phosphorylation (OXPHOS) system and the electron transport chain, causing ROS production, altering key redox proteins and inhibiting pro-survival functions (Ralph and Neuzil, 2009). Compounds used in NB therapy that target mitochondria include Fenretinide.
(G3139), which induces the production of ROS and inhibits complex IV of electron transport chain (Cuperus et al., 2010; Garaventa et al., 2003; George et al., 2010), and Doxorubicin, which also inhibits the electron transport chain (Modak and Cheung, 2010; Rohlena et al., 2011; Tokarska-Schlattner et al., 2006).

Despite the advances in NB treatment, significant complications and side effects remain present in the current therapy and hence there is a clear need for an improved therapeutic approach. Aiming for a more specific, less toxic and more effective therapy, several molecular targets have been investigated in recent years (George et al., 2010; Verissimo et al., 2011; Wagner and Danks, 2009). These targets are involved in at least one of the processes involved in tumorigenesis, such as proliferation, angiogenesis, invasion and/or metastasis. Here, we propose Doublecortin-like kinase (DCLK1) as a novel molecular target for NB therapy.

1.1.2 Doublecortin-like kinase

DCLK1 is a member of doublecortin (DCX) family (Coquelle et al., 2006; Reiner et al., 2006). The main splice variants encoded by DCLK1 gene are doublecortin-like (DCL), DCLK-long, DCLK-short, and calcium/calmodulin-dependent protein kinase (CaMK)-related protein (CARP) (Burgess and Reiner, 2002; Dijkmans et al., 2010; Vreugdenhil et al., 2007). DCL and DCLK-long contain two microtubule domains (DCX domains) and are microtubule-associated proteins (MAPs) crucial for correct proliferation of neuroprogenitor cells (Vreugdenhil et al., 2007). In addition to neurogenesis, DCL and DCLK-long are known to regulate neural migration and transport along microtubules (Fitzsimons et al., 2008; Koizumi et al., 2006; Shu et al., 2006; Vreugdenhil et al., 2007). These MAPs are mainly expressed in areas of high neuroblast proliferation (Vreugdenhil et al., 2007). In vivo studies have shown that both loss- and gain-of-function of DCL and DCLK-long result in impairment of neuroblast proliferation (Vreugdenhil et al., 2007). DCL/DCLK-long knockdown by RNA interference in NB cells results in disruption of mitotic spindles and cell cycle arrest at prometaphase (Shu et al., 2006; Vreugdenhil et al., 2007). Hence, there are indications that DCL and DCLK-long might be suitable targets for NB therapy. The investigation and validation of DCL and DCLK-long as therapeutic targets for NB are presented in this thesis.

Validation of molecular targets

The relevance of a potential target for cancer therapy needs to be evaluated. The study of the modulation of the activity of a given target might be assessed using available experimental models. These models can be subdivided in generic, functional and animal validation models (Benson et al., 2006).

Identification of DNA amplification or somatic mutations of the target gene in the tumor tissue allows the evaluation of the target. Other genetic assessment approaches include the measurement of the RNA and protein expression in malignant cells compared to normal cells (Benson et al., 2006). The functional validations in cell-based systems may include the knock-down or knock-in of the target under investigation using short interference RNA (siRNA) or short hairpin RNA (shRNA) and studies in tissue culture. Knock-outs or exogenous gene expression are other approaches that have been used (Benson et al., 2006).
Animal models include transgenic mouse models and orthotopic or heterotopic tumor xenograft models in immunocompromised mice, e.g. athymic \textit{nu/nu} or \textit{SCID} (severe combined immunodeficiency) mice (Ke et al., 2006). Such xenograft tumors might use a reversible target knockdown by inducing shRNA expression using doxycycline (Ke et al., 2006; Zhang et al., 2007).

Since each target validation method might present some limitations, it is of main importance to combine different approaches for the evaluation of the target under investigation. In this study we looked at the expression of DCL and DCLK-long in human tumors, normal tissue and cell lines. We performed several functional studies and also used NB xenograft mouse models, covering a large rage of techniques used in anticancer target validation.

The targets that might emerge from these evaluation/validation studies might be termed “validated target” and further pre-clinical and clinical research is of high interest.

1.2 Scope and outline of the thesis

1.2.1 Rationale

There is a need for improving the therapeutic outcome for children with high-risk NB. This might be initiated by identifying and validating new therapeutic targets. \textit{DCLK1} gene encodes microtubule-associated proteins (MAPs), DCL and DCLK-long, crucial for correct proliferation of neuroblasts and NB cells. Suppression of DCL/DCLK-long in NB cells results in disruption of the mitotic spindles and cell cycle arrest. To investigate whether DCL and DCLK-long are potential molecular therapeutic targets for NB, we performed several \textit{in vitro} and \textit{in vivo} studies.

1.2.2 Objectives

The general aim of the research described in this thesis is to explore DCLK1-derived MAPs (DCL and DCLK-long) as target for NB therapy. This main objective can be specified in six sub-objectives:

1) To provide a general overview on the different molecular targets for NB therapy

2) To study the expression levels of DCL and DCLK-long in human NBs

3) To investigate the effect of DCL/DCLK-long knockdown in NB cells

4) To explore the combination of DCL/DCLK-long knockdown and microtubule-destabilization as an effective approach to induce apoptosis in NB cells

5) To study the link between DCL and mitochondria activity in NB cells

6) To verify the anti-tumor effect of DCL silencing in NB xenograft mouse model

1.2.3 Outline of the thesis

In chapter 2 we provide information about the different types of NB, its origin, the therapies currently applied and present a general overview on the different molecular targets proposed for NB therapy. We focus particularly on molecular targets that are not yet explored in the clinic. Those targets are involved in tumorigenic processes, such as proliferation, anti-apoptosis, angiogenesis and/or
metastasis. DCL and DCLK-long are the proposed molecular targets that we describe here in detail.

ii. In chapter 3 we describe very high expression of the microtubule-associated proteins DCL and DCLK-long in human NBs. Using gene expression profiling we reveal that silencing of DCL/DCLK-long results in the induction of apoptotic pathways. By time-lapse imaging of phosphatidylserine translocation, caspase-3 activation, live/death double staining and fluorescence-activated cell sorting we demonstrate that DCL and DCLK-long knockdown induces apoptosis in NB cells.

iii. In chapter 4 we tested the hypothesis that silencing of DCL/DCLK-long potentiates the action of the microtubule-destabilizing vinca alkaloids. Our results show that the combined treatment results in a synergistic apoptotic effect in NB cells.

iv. In chapter 5 we show that silencing of DCL results in a delay in NB tumor growth. Using N1E-115 mouse NB cells we demonstrate that DCL silencing induces a decrease in mitochondria activity, ATP synthesis and cell proliferation. In a primary structure/function characterization, the use of DCL mutants indicates that the presence of Ser/Pro-rich domain and the second microtubule-binding domain are required for cytochrome c oxidase activity and ATP synthesis.

v. In chapter 6 we discuss the main findings and we present a general overview of the processes that might be involved in the induction of apoptosis in NB cells with DCL/DCLK-long MAPs knockdown. We provide the final conclusions and describe the future perspectives of the present study.

1.3 References


Chapter 1


