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Small Molecule Drugs and Targeted Therapies for Neuroblastoma

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1. Introduction

Neuroblastoma currently accounts for approximately 15% of all childhood cancer related deaths despite intensive multimodal chemotherapy (Maris & Matthay, 1999). Innovative treatment approaches are therefore needed for this disease. Recent advancements in molecular genetics of neuroblastoma have enabled the identification of several prospective molecular targets that provide opportunities for the development of new therapeutic strategies. Targeted therapy is defined as a type of treatment that employs chemical small molecules or other substances, such as monoclonal antibodies, to specifically identify and attack cancer cells. This type of therapy contrasts with traditional chemotherapy that relies on the elimination of rapidly dividing cells, regardless of whether or not they are malignant. Thus, targeted therapies offer a number of potential advantages over conventional chemotherapy including: (1) an increased therapeutic index (i.e. effective cancer treatment with less side effects) due to the targeting of a unique characteristic within the tumour cells, which is usually absent in normal cells of the body (Oeffinger, Mertens et al., 2006); and (2) a decreased likelihood of the development of resistance to the targeted therapy due to the molecular target being essential for the viability of the cancer.

Targeted cancer therapies ultimately interfere with one or more biological pathways within the cancer cell that are critical to its growth or survival. Examples of these pathways include signal transduction, apoptosis, regulation of gene transcription, and tumour angiogenesis. Most targeted therapeutics are either chemical small molecules or monoclonal antibodies. The former can act on targets located inside the cell since they are typically able to diffuse across the lipid bilayer, whereas most monoclonal antibodies usually cannot penetrate the cell's plasma membrane and hence are directed against targets that are either outside cells or on the cell surface. This chapter will review some of the recent findings involving the development of potential small molecule drugs as targeted therapies for childhood neuroblastoma.

2. Cell-based small molecule drug screening

Small molecule drugs or therapeutics are simple organic chemicals with low molecular weights, usually under 1000 Daltons. Compared to larger molecular weight pharmaceuticals such as proteins and peptides, small molecule drugs can be delivered orally or intravenously, and in most instances, more easily penetrate cell membranes and the blood brain barrier. The process of discovery and development of small molecule drugs involves the identification of a target, the discovery of drug candidates that can block or activate the target, product development, pre-clinical research on animals, and finally human clinical trials. The screening of collections or libraries of small molecules has long been used in the pharmaceutical industry as a method of finding drug candidates. The screening process generally falls into one of the two categories: Virtual screening and real screening. Virtual screening is based on computationally inferred or simulated real screening. By using this methodology, huge numbers of chemicals can be screened, and it is also possible to design and investigate compounds that have yet to be synthesized. Real screening on the other hand, is conducted using a variety of readout systems involving either biochemical assays or cell-based assays.

Most *in vitro* screening assays utilize a surrogate readout system that relies on labelled molecules or some type of biochemical reaction, and is often done in so called high-throughput screening (HTS) facilities. HTS can experimentally test the activity of thousands of compounds against a target on a daily basis and provides real information for drug discovery. In addition to biochemical assays, various cell-based assays such as reporter gene assays, secondary messenger assays, cell-based enzyme-linked immunosorbent assay (ELISA), cell-based proximity assays, and pathway screening are also used in current HTS (Digan, Pou et al., 2005). The advantage of a cell-based readout is that one can use a functional assay, thus bringing selection conditions closer to the final application of the compounds. It also allows testing the desired activity of the compounds while filtering out cytotoxic ones in one assay. A cell-based assay normally includes a cell line or a primary cell population, a target molecule that records the cellular response, instruments for conducting and monitoring the assay, and methods of data analyses. The choice of cells for the readout is a compromise between those systems that most adequately reflect the “disease” properties *in vitro*, and the requirements of high throughput screening. For drug discovery screening, a cell-based assay, in certain aspects, is more useful than an *in vitro* assay, as potential liabilities can be assessed earlier, and structure-activity relationships can be explored at reasonable cost in a shorter timeframe. Although cell-based approaches are sometimes less robust due to certain problems with living cells, this technology is proving to be one of the most successful ways in identifying bioactive compounds (Gudkov & Komarova, 2003).

Chemical libraries are available from many vendors and differ in their origin, composition, complexity and purity. They consist of large sets of individual organic molecules usually dissolved in DMSO and formatted to facilitate their transfer to microtitre plates for screening purposes (Gudkov & Komarova, 2003). Following establishment of a read-out system, an initial library screening will yield a number of “hits”, with an acceptable hit rate usually below 0.5%. These hits are then subjected to a filtering process to remove those compounds representing false positives. Additional rounds of screening, including screening of focussed libraries generated around the best and most active compounds, is

performed with the aim of finding further compounds with increased activity and specificity. After completing the screening process, the chemical structure of particularly promising compounds can be used as a starting point for modifications in order to further improve potency and selectivity, ultimately leading to the selection of “lead compounds”. Use of cell-based readout systems, has led to the discovery of small molecule inhibitors, including p53 inhibitors such as pifithrin- α (Komarov, Komarova et al., 1999), P-glycoprotein modulators (Kondratov, Komarov et al., 2001) and the Multidrug Resistance-Associated Protein 1 (MRP1) inhibitor, Reversan (Burkhart, Watt et al., 2009). The methodology of drug screening in cell-based readout systems and its application in the discovery of p53 inhibitors have been comprehensively reviewed previously (Gudkov & Komarova, 2003).

3. MycN targeted inhibition

3.1 Rationale for targeting Myc oncoproteins

Target-based drug discovery begins with the identification of the function of a potential therapeutic drug target and an understanding its role in the disease process. In this regard, amplification of the *MYCN* oncogene in neuroblastoma tumours represents one of the most powerful prognostic markers yet identified for this disease (Maris & Matthay, 1999). *MYCN* belongs to the *MYC* family of proto-oncogenes (including *CMYC* and *MYCL*), and these genes encode a family of basic helix-loop-helix leucine zipper transcription factors. Myc proteins localize to the nucleus and form heterodimers with the basic helix-loop-helix molecule, Max. Myc/Max heterodimers bind to DNA at specific ‘E-box’ sequences (CAC(G/A)TG) to drive transcription of target genes that are important for proliferation, differentiation and apoptosis. Max also heterodimerizes with Mxd or Mnt proteins to influence the transcription of other downstream genes and often to antagonize the proliferative effects of Myc proteins (Meyer & Penn, 2008). Among Myc proteins, MycN and c-Myc share several regions of homology as well as similar cellular functions. Replacement of the *CMYC* gene with the *MYCN* gene in mice does not affect murine embryonic development, indicating MycN is capable of replacing most of the essential c-Myc functions required for embryonic development and for proliferation of differentiated cells (Malynn, de Alboran et al., 2000). The universal deregulation of *CMYC* gene expression in tumour cells and the high dependency of tumour growth on elevated c-Myc levels suggests that this oncoprotein represents an attractive target for cancer therapy (Prochownik & Vogt, 2010). The same concept applies to the *MYCN* gene in neuroblastoma as the genetic feature most consistently associated with treatment failure is amplification of the *MYCN* gene that strongly correlates with advanced disease (Brodeur, Seeger et al., 1984; Seeger, Brodeur et al., 1985). These notions are supported by numerous experimental data showing that inhibition of c-Myc or MycN significantly halts tumour cell growth and proliferation (Ponzielli, Katz et al., 2005; Gustafson & Weiss, 2010).

3.2 Challenges in targeting Myc by small molecule drugs

Although many efforts have been made to develop anti-Myc therapeutics, to date, no specific Myc modulators have approached clinical trials. The difficulty in identifying drugs that target Myc oncoproteins for the treatment of cancer is multifactorial. Myc is considered a challenging therapeutic target for a number of reasons (Prochownik & Vogt, 2010) that

includes (1) Despite its high level of expression in cancer, *MYC* genes are rarely mutated, with the exception of mutations found in primary Burkitt and AIDS-related lymphomas (~30%); (2) Difficulties exist in Myc inhibitor design. For instance, targeting the association between Myc and Max or other essential cofactors involves the disruption of protein-protein interactions. The surfaces at which these interactions occur tend to be large, flat, and relatively featureless, and often lack recognizable motifs or clefts. In addition, disruption of protein-protein interactions must overcome a large free energy of association from the interacting protein moieties.

Ubiquitous expression of c-Myc in normal proliferating cells also raises concerns about potential side effects of such therapeutic agents. Recent findings, however, demonstrated that whole-mouse genetic inhibition of c-Myc resulted in rapid regression of incipient and established tumours, whereas the side effects to normal tissues were well tolerated and completely reversible even over extended periods of time of Myc inhibition (Soucek, Whitfield et al., 2008). Thus, the inhibition of c-Myc, as well as MycN, appears to be a safe and efficient method to eliminate cancer although the actual effects in human context remain to be tested.

3.3 Targeting Myc oncoproteins

3.3.1 Identification of inhibitors of Myc-Max dimerization

Several *in vitro* and cell-based methods have been used in screening for inhibitors of Myc-Max dimerization (Prochownik & Vogt, 2010). The *in vitro* techniques are based on either fluorescence resonance energy transfer (FRET) or fluorescence polarization, while cell-based screening has employed the yeast 2-hybrid approach, in which the interaction between the bHLH-ZIP domains of c-Myc and Max has been used to isolate inhibitors of Myc-Max dimerization (Yin, Giap et al., 2003). Seven compounds were identified with specificity for Myc-Max inhibition. It was subsequently shown that these compounds work via common mechanisms involving their direct binding to the dimerization domain of the c-Myc monomer. However, their clinical application is likely to be limited due to their relatively low potencies, and rational design of compounds with greater potencies is underway using structure-based computational approaches (Wang, Hammoudeh et al., 2007). In general, an increasing amount of data suggests that inhibition of the protein-protein interactions between c-Myc and Max by small molecules is a feasible approach toward the inhibition of c-Myc functions. More recently, stabilization of Max homodimers to reduce the amount of Max available for activating c-Myc has also been demonstrated to counteract Myc activity (Berg, 2011). It is highly likely that any small molecule inhibitors of c-Myc will also work to inhibit MycN functions due to the high level of structural and functional homology between the two proteins.

3.3.2 Development of stabilizers of the Max homodimer

High levels of Myc protein in cancer cells require an abundance of Max to become functional. Stabilization of the Max-Max homodimer could therefore preferentially affect such overexpressed Myc and attenuate its oncogenic effects. Max-Max stabilizers have been identified by virtual ligand screening, although this type of screening is dependent on structural information being available for the target molecules (Prochownik & Vogt, 2010). One identified compound, NSC13728, which is an effective stabilizer of Max-Max, strongly

interferes with Myc-mediated oncogenic transformation in cell culture while not affecting transformation induced by Jun, Src, or PI3K. Transcriptional activity of Myc was also inhibited. Although virtual ligand screening suggests a binding site for this stabilizer, that site has not been experimentally verified.

3.3.3 Development of MycN inhibitors using cell-based screening

Although targeting MycN has been successfully achieved in the laboratory setting using RNA interference technologies, this approach suffers from the lack of an efficient *in vivo* delivery method. The development of small molecule inhibitors to MycN is needed and in this regard, Lu and colleagues have identified five potential MycN inhibitors in a pilot screen of 2800 compounds from the Cancer Research-UK collection (Lu, Pearson et al., 2003). To make this readout system, neuroblastoma cells were stably transfected with a luciferase gene construct under control of the *ornithine decarboxylase 1 (ODC1)* gene promoter. Resulting compounds are subject to further characterization. Similarly to this approach, we have created a cell-based MycN readout system (SHR6-17). This cell line was derived from human neuroblastoma SH-EP cells by stable transfection of a luciferase reporter gene under the control of a minimal heat shock protein promoter that contained 6 copies of the E-box sequence for MYC-specific transactivation. Because the SHR6-17 cells express low Myc proteins, the basal reporter activity is low. However, transduction with MYCN lentivirus resulted in the controlled induction of luciferase reporter activity (~5-10 fold). A small molecule library containing 34,000 individual compounds was used for primary screening and a number of promising compounds have been identified.

3.4 Targeting MycN upstream pathways

Available evidence suggests that targeting upstream pathways of MycN may also be an efficacious strategy for neuroblastoma therapy. This idea comes from studies showing that active Ras oncoprotein is needed to block MycN degradation, promoting cooperative Ras and MycN-dependent cell cycle progression in LAN-1 cells (Yaari, Jacob-Hirsch et al., 2005). Mutations in the *RAS* genes have rarely been found in neuroblastomas. Treating the MYCN-amplified neuroblastoma cell line LAN1 with the Ras inhibitor, farnesylthiosalicylic acid, or a dominant-negative Ras, led to growth inhibition and a decrease in MycN levels. The growth inhibition is attributed to attenuation of the Raf-MEK-ERK and phosphoinositide 3-kinase-Akt-glycogen synthase-3 (GSK-3) pathways, reduction in cyclin D1, phospho-retinoblastoma, and E2F, and an increase in p27Kip1 and retinoblastoma-binding protein-1. The down-regulation of MycN protein resulted from blocking of Akt-mediated inactivation of GSK-3, leading to GSK-3-dependent phosphorylation with consequent proteosomal degradation of MycN. More recent studies have confirmed that MycN phosphorylation and stability are controlled by the PI3K/Akt/mTOR pathway in neuroblastoma cells, and that activation of Akt predicts poor outcome in neuroblastoma patients (Fulda, 2009). Taken together, these data suggest that targeting pathways upstream of MycN may be a feasible strategy for MycN targeted therapy in neuroblastoma.

3.5 Targeting MYCN downstream pathways

Several potential downstream target genes of MycN have been identified as a result of expression profiling studies in neuroblastoma cell lines and tumours. Some of these genes have been confirmed to be direct or indirect transcriptional targets of MycN and their

therapeutic potential has been confirmed (Bell, Chen et al. 2010; Gustafson & Weiss, 2010). Representative genes include *ODC1*, *MDM2*, *Aurora Kinase A*, *DKK3* and *SKP2*, and for each of them, small molecule inhibitors are available or under development. Inhibition of *Odc1*, *Mdm2* and *Aurora Kinase A* in neuroblastoma will be reviewed below.

Apart from the above *MycN* downstream genes, we are particularly interested in another *Myc*-regulated gene *MRP4/ABCC4*. *MRP4/ABCC4* is a known *c-Myc* target gene found in Burkitt's lymphoma cells (Li, Van Calcar et al., 2003) and we have recently provided direct evidence that it is also a *MycN* target gene (Porro, Haber et al., 2010). In addition to *MRP1/ABCC1*, *MRP4/ABCC4* is also a powerful independent predictor of neuroblastoma outcome (Norris, Smith et al., 2005). While this study demonstrated that high levels of *MRP4* could protect neuroblastoma cells from the chemotherapeutic drug irinotecan *in vitro*, the patients in the study received neither irinotecan, nor any other drugs known to be *ABCC4* substrates, suggesting that the prognostic significance of this gene could also not be explained in terms of *MRP4*-mediated cytotoxic drug resistance. We are currently investigating in more detail precisely how *MRP4* contributes to highly malignant neuroblastoma using a range of experimental approaches. In particular, we have provided strong evidence using siRNA-mediated silencing of *MRP4* that this transporter contributes to neuroblastoma biology independently of its role in chemotherapeutic drug efflux (Henderson, Haber et al., 2011). This finding suggests that therapeutic targeting of *MRP4* has potential clinical utility for this disease, as well as for other cancers expressing high *MRP4* levels. Therefore we are using high-throughput screening of chemical libraries to generate novel inhibitors of *MRP4*. Several *MRP4* small molecule inhibitor "hits" have been identified by screening a 30,000 compound library, and further characterization of these compounds is underway.

4. *MRP1/ABCC1* targeted inhibition

4.1 Rationale for targeting *MRP1*

Intrinsic or acquired multidrug resistance (MDR) is one of the major causes of treatment failure in human malignancy, including childhood neuroblastoma (Maris & Matthay, 1999). In the laboratory, MDR mediated by multidrug transporters, such as P-glycoprotein and *MRP1*, results in resistance to a broad spectrum of structurally unrelated drugs. Amongst these transporters, there is strong evidence demonstrating the clinical relevance of *MRP1* in aggressive childhood neuroblastoma. *MRP1* is encoded by the *ABCC1* gene (ATP-binding cassette, sub-family C member 1) and acts as an ATP-dependent efflux pump for the transport of organic anions, glutathione-, glucuronate- or sulfate-conjugated drugs, or unconjugated drugs in concert with free glutathione (Hipfner, Deeley et al., 1999; Borst, Evers et al., 2000), including the chemotherapeutic agents vincristine, doxorubicin and etoposide. The down-regulation of *MRP1* activity in neuroblastoma cells by antisense mRNA (Kuss, Corbo et al., 2002) or by treatment with *MRP1* reversal agents (Norris, Gilbert et al., 2001) results in increased sensitivity to cytotoxic drugs. More importantly, high *MRP1* expression in primary neuroblastoma at diagnosis is strongly associated with poor patient outcome (Norris, Bordow et al., 1996; Haber, Smith et al., 2006). To determine the overall contribution of *MRP1* to drug resistance in neuroblastoma, we crossed mice lacking the *MRP1* gene (*MRP1*^{-/-}) with human *hMYCN* transgenic mice, which develop neuroblastoma characteristic of the human disease. This cross yielded murine neuroblastoma tumours that

were either wild-type ($MRP1^{+/+}$) or homozygous null ($MRP1^{-/-}$) for $MRP1$. Tumour cells of either $MRP1$ genotype were isolated and xenografted into nude mice and these mice were treated with clinically used drugs and monitored for tumour growth (Burkhart, Watt et al., 2009). Results showed that loss of $MRP1$ significantly increased the latency of tumour progression in response to vincristine and etoposide, both of which are known $MRP1$ substrates. In contrast, the lack of $MRP1$ had no effect on the efficacy of cisplatin or cyclophosphamide, which are not substrates for $MRP1$ (Burkhart, Watt et al., 2009). These data suggest that $MRP1$ is a major determinant of the response of neuroblastoma tumours to chemotherapy, further supporting the development of $MRP1$ small molecule inhibitors. Although a number of $MRP1$ inhibitors have been identified, the number of compounds close to or entered into clinical trials is limited (e.g. sulindac (O'Connor, O'Leary et al., 2007)).

4.2 Reversan modulates $MRP1$ function *in vitro*

Through our screening efforts (Burkhart, Watt et al., 2009), we have identified 6 structural scaffolds that can effectively inhibit $MRP1$ function with the most active compounds clustered within the pyrazolopyrimidine scaffold. Reversan, one of the most potent pyrazolopyrimidines identified to date, sensitized $MRP1$ overexpressing breast cancer cells (MCF7/VP) to a panel of drugs including vincristine (14.6 fold), etoposide (11.6 fold) and doxorubicin (3.8 fold), all of which are $MRP1$ substrates. In contrast, it did not increase sensitivity to two non- $MRP1$ substrates cisplatin and paclitaxel. The selectivity of Reversan for $MRP1$ in terms of modulating drug response was further examined by studying its effects on cell lines overexpressing one of several other multidrug transporters, including P-glycoprotein, $MRP2$, $MRP3$, $MRP4$, or $MRP5$. Reversan did not sensitize $MRP2$, $MRP3$, $MRP4$ or $MRP5$ overexpressing cell lines to known substrates of each of these transporters, namely vincristine ($MRP2$), etoposide ($MRP3$) or 6-mercaptopurine ($MRP4$ and $MRP5$). In contrast, this modulator significantly sensitized P-glycoprotein overexpressing neuroblastoma cells (BECHCb (Borst, Evers et al., 2000)) to vincristine, indicating that Reversan is not totally $MRP1$ specific.

In addition, effects of Reversan on cytotoxic drug response were examined in human neuroblastoma (BE(2)-C), renal cell carcinoma (SK-RC45) and colon (HCT116) tumour cell lines, which represent tumour types that are clinically refractory to cytotoxic drug treatment as well containing high levels of $MRP1$ protein. Reversan caused increased sensitivity of these cell lines to one or more cytotoxic drugs with the most dramatic effect observed in combination with vincristine (Burkhart, Watt et al., 2009). Furthermore, when compared to a panel of known drug transporter inhibitors, including verapamil, cyclosporin A, difloxacin, probenecid and PAK104P, for the effects on etoposide sensitivity of MCF7/VP cells, Reversan increased the sensitivity of MCF7/VP cells to etoposide to a level similar to the most potent of these modulators, PAK104P (25-fold). More importantly, Reversan was 6–8 times more potent than the rest of the panel of modulators, including the Phase I clinical trial drug, probenecid (Burkhart, Watt et al., 2009).

4.3 *In vivo* efficacy and toxicity of Reversan

The toxicity of Reversan has been tested in BALB/c mice and has been found to be safe and well tolerated (Burkhart, Watt et al., 2009). Furthermore, when used in combination with either vincristine or etoposide to treat neuroblastoma-prone *hMYCN* transgenic mice, 10 mg/kg Reversan increased tumour sensitivity to these conventional drugs with no

increased toxicity. The combination of Reversan with vincristine or etoposide significantly increased the survival time of mice compared to those treated with drug alone. While treatment with vincristine alone increased survival of tumour bearing *hMYCN* mice by approximately 10 days, the addition of Reversan to the vincristine treatment regimen increased survival by an additional 20 days (survival: 4.9 ± 0.49 days saline control, 16.2 ± 0.89 days vincristine alone, 36.5 ± 4.4 days vincristine plus Reversan). For treatment with etoposide alone, the duration of survival of tumour bearing *hMYCN* mice doubled compared to vehicle control and tripled when co-administered with Reversan (survival: 4.9 ± 0.49 days saline control, 11 ± 0.67 days etoposide alone, 16 ± 0.56 days etoposide plus Reversan). The combination of Reversan and cyclophosphamide, which is not an MRP1 substrate, had no effect on the duration of time between treatment and progression compared to cyclophosphamide alone. In addition, Reversan also significantly increased the efficacy of vincristine and etoposide against BE(2)-C human neuroblastoma xenografts. It should also be noted that oral administration of Reversan worked equally as well as ip administration for increasing the efficacy of etoposide administered to tumour-bearing *hMYCN* mice (Burkhart, Watt et al., 2009).

Past attempts at modulating MDR have failed mainly due to nonspecific side effects that became apparent when modulators have been combined with conventional drugs (Szakacs, Paterson et al., 2006). We found that Reversan did not significantly alter the toxicity profile of vincristine in BALB/c mice when treated with vincristine in the clinically relevant range for this drug (DeVita, Hellman et al., 1993) in the presence or absence of 10 mg/kg Reversan (Burkhart, Watt et al., 2009). In contrast, vincristine administered in combination with 10 mg/kg cyclosporin A, a first generation multidrug transporter inhibitor that underwent clinical trials in the 1990's, resulted in rapid weight loss and a dramatic shift in the toxicity profile of vincristine. Neither Reversan nor cyclosporin A were toxic when administered as individual drugs. Importantly, there was no toxicity associated with Reversan at clinically relevant doses of vincristine. Similar results for Reversan were obtained for both males and females in a second mouse strain, ICR, which demonstrates that lack of toxicity was not a gender-specific or strain-specific artefact. In addition, we tested BE(2)-C xenograft-bearing nude mice for signs of hematopoietic toxicity following treatment with etoposide alone or in combination with Reversan on Day 15. There was no effect of Reversan on the number of lymphocytes, monocytes, eosinophils, or basophils. In contrast, there was a significant increase (~ 2 fold, $P=0.027$) in the number of neutrophils in mice treated with the drug combination compared to mice treated with etoposide alone. Thus, Reversan did not enhance etoposide-induced neutropenia but rather appeared to have a protective effect on the neutrophil population. There was no significant effect of the etoposide/Reversan combination on the platelet population (Burkhart, Watt et al., 2009). Therefore, we have identified a safe small molecule inhibitor of MRP1 that may have clinical potential in the treatment of neuroblastoma and other cancers that overexpress MRP1.

4.4 Mechanism of action and novelty of Reversan

Classical inhibitors of multidrug transporters are substrates of these pumps themselves, reversing resistance by competitive inhibition. Such inhibitors compete with the conventional drugs for metabolism as well as for efflux (Schuetz, Schinkel et al., 1996; Wandel, Kim et al., 1999). Many of the first and second generation P-glycoprotein inhibitors were such classical inhibitors and it is believed that this mechanism of action contributed to

their failures (Thomas & Coley 2003). Currently, third generation P-glycoprotein inhibitors (e.g. tariquidar and zosuquidar) have reached various stages of clinical investigation (Thomas & Coley, 2003). These molecules are potent, highly specific Pgp inhibitors that are not themselves substrates of this transporter (Dantzig, Shepard et al., 1999; Roe, Folkes et al., 1999; Mistry, Stewart et al., 2001). Although, early clinical trials demonstrated that tariquidar could reverse drug efflux in patients (Stewart, Steiner et al., 2000; Agrawal, Abraham et al., 2003) and be co-administered with paclitaxel, vinorelbine or doxorubicin without the need for dose reduction of the chemotherapeutic agents (Thomas & Coley 2003). More recent clinical trials have failed to live up to the early promise and have delivered disappointing results (Pusztai L, 2005; Cripe, Uno et al., 2010; Libby & Hromas, 2010). However, in terms of MRP1, we have recently shown that this transporter has a more fundamental role in neuroblastoma, than that of simply effluxing cytotoxic drugs. Thus, using genetic and pharmacologic inhibition, we have provided the first direct evidence that MRP1 can contribute to neuroblastoma biology independently of chemotherapeutic drug efflux, thereby enhancing its potential as a target for therapeutic intervention (Henderson, Haber et al., 2011). Importantly, in this study, treatment of neuroblastoma-prone *hMYCN* transgenic mice with Reversan in the absence of chemotherapeutic agents, led to a significant delay in tumour progression

Although available evidence indicates that Reversan is not a substrate for MRP1, its exact mechanism of action as well as related pyrazolopyrimidines is currently unknown. Since the original P-glycoprotein modulators that were used to generate libraries in which Reversan was discovered, were found not to be ATPase inhibitors (Kondratov, Komarov et al., 2001), it is likely that the pyrazolopyrimidines are also not inhibitors of ATPase but this remains to be tested. It is possible that Reversan does not interact directly with MRP1 or P-glycoprotein but rather alters the physicochemical properties of the membrane surrounding the transporter, which could then alter the structure of the protein(s) within the membrane and affect its ability to transport. Indeed, it appears that the hydrophobic nature of Reversan and the other similarly active pyrazolopyrimidines may be important for their potency since more hydrophilic pyrazolopyrimidine analogs were found to be less effective in reversing drug resistance (Burkhart, Watt et al., 2009).

While some critics of this field have suggested that inhibitors of multidrug transporters should be specific for individual transporters, or toxicity could be increased due to off-target effects, the results of our own study suggest that this is not the case for Reversan. Despite the fact that Reversan inhibits the function of both MRP1 and P-glycoprotein equally well, it does not significantly alter the toxicity profile of conventional chemotherapeutic agents *in vivo* in contrast to cyclosporin A, which exemplifies the earlier generation of toxic multidrug transporter modulators. It is possible that the problem of off-target toxicities is not related to interactions with other transporters but more related to interplay between P-glycoprotein and CYP3A4 in terms of shared substrates (Schuetz, Schinkel et al., 1996; Wandel, B. Kim et al., 1999). The increase in the effectiveness of conventional chemotherapeutic agents observed with Reversan, as well as the ability of Reversan to inhibit neuroblastoma tumorigenesis in the absence of any antineoplastic agents, highlights the potential use of this compound in the clinical setting. In addition, Reversan has an excellent therapeutic index compared to multidrug inhibitors of the past. Therefore, it appears that Reversan represents a new class of "safe" multidrug transporter inhibitor that may be clinically useful in the

treatment of neuroblastoma and other cancers associated with aberrant MRP1/P-glycoprotein expression.

5. Polyamine targeted inhibition - targeting Odc1

Amplification of the *MYCN* oncogene is one of the most powerful predictors of poor clinical outcome in this disease. Although the mechanism by which *MYCN* amplification influences the prognosis of neuroblastoma remain largely unknown, it is widely accepted that identification of the requisite biopathways downstream of this oncogene may provide therapeutic opportunities. This concept has been evidenced by recent success in the treatment of neuroblastoma tumours of *hMYCN*-transgenic mice by targeting the polyamine biosynthesis enzyme ODC1 (Hogarty, Norris et al., 2008; Rounbehler, Li et al., 2009).

It has been shown that *MYCN*-amplified neuroblastomas have co-ordinately deregulated a range of polyamine enzymes (including Odc1, Srm, Sms, Amd1, Oaz2, and Smox) to enhance polyamine biosynthesis (Evageliou & Hogarty, 2009). Polyamines are organic cations that enhance transcription, translation, and replication (Pegg, Secrist et al., 1988) and support many cellular processes governed by *MYC* genes. Their maintenance is essential for cell survival as depletion activates growth arrest or apoptotic checkpoints (Bettuzzi, Davalli et al., 1999). Thus, intracellular polyamines are kept under tight control through posttranscriptional as well as transcriptional regulation, with the rate-limiting enzymes Odc1 and Amd1 having among the shortest half-lives of any mammalian enzyme as a result (Shirahata & Pegg, 1985). Odc1 activity is frequently elevated in cancer through deregulation of *MYC*, resulting in higher polyamine content to support rapid tumour cell proliferation (Pegg, Secrist et al., 1988). Overexpression of *ODC1* has been observed in a range of tumour cells, including neuroblastoma, and a number of studies have provided evidence for the oncogenic and transforming abilities of this protein (Gerner & Meyskens, 2004).

We have investigated the role of *ODC1* in neuroblastoma both in tumour samples and in preclinical models of this disease. In a large cohort of primary untreated neuroblastomas, we found that high levels of *ODC1* expression were strongly predictive of both event-free-survival and overall survival (Hogarty, Norris et al., 2008). These data suggest that inhibiting Odc1 activity should have a therapeutic advantage in treating neuroblastoma. To determine the effects of disabling Odc1 on both tumour initiation and progression, we treated neuroblastoma-prone *hMYCN* mice with α -difluoromethylornithine (DFMO), a known Odc1 inhibitor. DFMO treatment extended tumour latency and survival in homozygous mice and prevented oncogenesis in hemizygous mice. In the latter, transient Odc1 ablation permanently prevented tumour onset consistent with a time-limited window for embryonal tumour initiation. Similarly, an independent study showed DFMO treatment, but not Odc1 heterozygosity, impaired *MycN*-induced neuroblastoma in *hMYCN* mice (Rounbehler, Li et al., 2009). More importantly, our study demonstrated that combining DFMO with cisplatin, either concomitantly or afterward, prolonged tumour-free survival in these mice, compared with cisplatin alone. Similar results were seen when cyclophosphamide was combined with DFMO. There was no overt toxicity that could be attributed to DFMO in the treated mice (Hogarty, Norris et al., 2008). These data implicate polyamine biosynthesis as an arbiter of *MycN* oncogenesis and showed initial efficacy for polyamine depletion strategies in neuroblastoma. DFMO is considered a very promising drug due to its high specificity, low toxicity and water-soluble properties allowing oral

administration. It is currently undergoing Phase I clinical trial for testing its safety for refractory or relapsed neuroblastoma as there are limited data on its use in paediatric patients.

6. Targeting the p53 pathway

6.1 p53 pathway in neuroblastoma

p53 has been regarded as "the guardian of the genome" for it is a key mediator of cell response to a variety of stresses, inducing growth arrest or apoptosis, thereby eliminating damaged and potentially dangerous cells from the organism (Prives, 1999). This tumour suppressor is mutated in approximately 50% of human malignancies and is functionally inactivated in the majority of cancers that retain wild-type p53 by other members of the pathway (Arf) (Sherr, 1998), or by negative p53 regulators of cellular (Mdm2) (Momand, Jung et al., 1998) or viral origin (E6 of human papillomavirus) (Thomas, Pim et al., 1999). Loss of p53 provides tumour cells with a series of important selective advantages, including high tolerance to growth arrest and death-inducing stimuli and genomic instability that promotes tumour progression by rapid acquisition of mutations (Levine, 1997).

In contrast to adult cancer, p53 mutations are infrequent (2%) in neuroblastomas. However, Most of the few p53 mutations in neuroblastoma tumours reported are in relapsed or progressive tumours (Tweddle, Malcolm et al., 2001). Similarly, the majority of neuroblastoma cell lines with p53 mutations reported have been established from relapsed or progressive tumours, and in most cases the cell lines are more chemoresistant than wild-type cell lines (Tweddle, Pearson et al., 2003). Recently, a higher incidence of p53 mutations was found in a study on 84 neuroblastomas from 41 patients with relapsed disease, including 38 paired neuroblastomas at different stages of therapy. Inactivating missense p53 mutations were identified in 6/41 (15%) cases, 5 following chemotherapy and/or at relapse and only 1 at both diagnosis and relapse (Carr-Wilkinson, O'Toole et al., 2010). Studies on neuroblastoma cell lines demonstrated that loss of p53 function due to mutations of p53 gene or gene silencing by p53 shRNA can confer multidrug resistance to neuroblastoma cells (Keshelava, Zuo et al., 2000; Keshelava, Zuo et al., 2001; Xue, Haber et al., 2007). These findings highlight the role of p53 inactivation in tumour progression in some high-risk neuroblastoma patients.

In the majority of neuroblastoma tumours maintaining wild-type p53 gene, an increasing amount of data shows that the p53 pathway may not be functional due to non-mutational mechanisms. For example, a high incidence of abnormalities in the p53/MDM2/p14^{ARF} pathway was found in human neuroblastoma cell lines established at relapse (53%) (Carr, Bell et al., 2006) and patient samples at relapse (49%) (Carr-Wilkinson, O'Toole et al., 2010). MDM2, the essential negative regulator of p53, is transcriptionally regulated by the MYCN oncogene in neuroblastoma (Slack, Chen et al., 2005). Targeted inhibition of MycN leads to reduced Mdm2 expression levels, with concomitant stabilization of p53 protein and stimulation of apoptosis in MYCN amplified neuroblastoma cell lines. These data suggest that Mdm2 is a key player in MycN-mediated suppression of p53. This notion was supported by a later study involving crossing the hMYCN transgenic model of neuroblastoma and the Mdm2 haploinsufficient mouse model (Chen, Barbieri et al., 2009). The *Mdm2*^{+/-}*MYCN*^{+/+} transgenics showed marked delay in tumour development and a lower overall tumour incidence compared to *Mdm2*^{+/+}*MYCN*^{+/+} genotype, strongly implicating Mdm2-mediated blockade of p53 as an essential step in the pathogenesis of

neuroblastoma. Recently, a miRNA (miR-380-5p), has been identified that represses *p53* expression via a conserved sequence in the *p53* 3' untranslated region (Swarbrick, Woods et al., 2010). This miRNA is highly expressed in mouse embryonic stem cells and neuroblastomas, and its expression correlates with poor outcome in *MYCN*-amplified neuroblastoma. *In vivo* delivery of a miR-380-5p antagonist led to a decrease in tumour size in an orthotopic mouse model of neuroblastoma. This new mechanism of *p53* deregulation has uncovered a potential novel therapeutic target for neuroblastoma treatment.

6.2 Small molecule approaches to *p53* modulators

Frequent inactivation of *p53* in cancer and high sensitivity of tumour cells to *p53* suggest that the most straightforward *p53*-based therapeutic approach to cancer treatment involves restoration or imitation of *p53* function in *p53*-deficient tumours, resulting either in a direct (tumour growth inhibition) or indirect (sensitization to treatment) therapeutic benefit. So far the majority of research efforts have been applied to restoration of *p53* function in tumours. However, the development of *p53*-targeting therapeutic approaches also takes advantage of the fact that this important signalling pathway is relatively well studied, making it possible to develop tools affecting individual components or interactors within the pathway. *p53* function is inactivated in tumours either by mutations or deletions within the *p53* gene itself, by viral *p53*-inactivating proteins (i.e., E6 of papilloma virus), or through deregulation of other members of the pathway such as inactivation of positive (Arf) and overexpression of negative (Mdm2) regulators. Thus, modulation of *p53* pathway activity may target any of the above factors. This general strategy is being extensively explored through a variety of approaches (reviewed in (Gudkov & Komarova, 2007; Lane, Cheok et al., 2010; Cheok, Verma et al., 2011)).

6.2.1 Mutant *p53* as a target for inhibition

The transcriptional regulatory and tumour suppressor activity of *p53* is dependent on the ability of the protein to maintain DNA binding conformation. Human *p53* protein containing the most common mutations cannot bind significantly to the DNA-binding sequence of *p53*-responsive genes (Kern, Kinzler et al., 1991). Normal activity of mutant *p53* might be restored, at least in part, by application of antibodies and peptides to a negative regulatory domain at the *p53* COOH-terminus (Hupp, Sparks et al., 1995; Selivanova, Ryabchenko et al., 1999). Alternatively, restoration of *p53* activity might be realized by stabilizing the active conformation of the DNA-binding domain by chemicals. This idea was confirmed by Foster and colleagues (Foster, Coffey et al., 1999). A chemical library (100,000 compounds) was screened in the study and active chemicals, which promoted conformational stability of wild type *p53* as judged by binding with mutant-specific antibodies, were found. These compounds were also effective *in vivo* by slowing tumour growth in mice. A number of small molecules with the potential to reactivate mutant *p53* are currently under preclinical development (including CP-31398, MIRA-1 and STIMA-1) or Phase I/II clinical trial in patients with haematologic and prostate neoplasms (PRIMA-1MET/APR-246) (reviewed in (Cheok, Verma et al., 2011)). Recently, more novel *p53* activating small-molecule compounds have been identified in cell-based screening. Six lead compounds (BMH-7, BMH-9, BMH-15, BMH-21, BMH-22, and BMH-23) were able to activate *p53* and repress the growth of human cancer cells. Two tested compounds suppressed *in vivo* tumour growth in an orthotopic mouse model of human B-cell

lymphoma. All compounds interacted with DNA, and two activated the p53 pathway in a DNA damage signaling-dependent manner (Peltonen, Colis et al., 2010).

6.2.2 p53 regulatory proteins as targets

In addition to p53, other members of the p53 pathway (i.e., Mdm2, E6, Arf) make attractive targets for screening purposes. The development of small-peptide effectors that can inhibit Mdm2 binding to p53 protein provides a potential drug target for reactivating the p53 pathway in cancers overexpressing Mdm2 (Böttger, Böttger et al., 1997). Similar inhibition of Jnk binding to p53 by small peptides derived from Jnk/p53 interface can also reduce Jnk-dependent ubiquitination and degradation of p53 (Fuchs, Adler et al., 1998). p14Arf blocks the degradation of p53 by MDM2 through the inhibition of its ubiquitin ligase-associated function (Lowe, 1999). The use of small peptides derived from p14Arf, which map at the p14Arf /Mdm2 interface, can activate p53, providing an additional target for modulating the Mdm2-degradation pathway (Midgley, Desterro et al., 2000). A number of small molecules that indirectly activate the p53 response have also reached clinic trial, of which the most advanced are the p53/Mdm2 interaction inhibitors (Nutlin, MI-219/AT-219, RITA, JNJ-26854165; PXN727 and PXN822) (Cheok, Verma et al., 2011).

Nutlins are cis-imidazoline analogs that inhibit the interaction between Mdm2 and p53, and were discovered by screening a chemical library by Vassiliev and colleagues (Vassiliev, Vu et al., 2004). Nutlin-1, Nutlin-2 and Nutlin-3 were all identified in the same screening, of which, Nutlin-3 is the one most well studied. Crystallization data have shown that nutlin-3 mimics the three residues of the helical region of the transactivation domain of p53 (Phe19, Trp23 and Leu26) that are conserved across species and critical for binding to Mdm2. Thus, Nutlin-3 displaces p53 by competing for binding to Mdm2.

The effects of Nutlin-3 on p53 reactivation have been extensively studied in a wide range of cancer cell lines and animals, including neuroblastoma (reviewed in (Shangary & Wang 2009)). For example, Nutlin-3 induces cell cycle arrest in both cancer and normal cells, but selective cell death in cancer cells of different origins. *Ex vivo* experiments using AML, B-CLL and multiple myeloma patient specimens have shown that inhibition of Mdm2 by Nutlin-3 effectively triggers apoptosis. Nutlin-3 synergizes with doxorubicin and cytosine arabinoside in killing myeloblasts in AML and with doxorubicin, chlorambucil, and fludarabine in killing leukemic cells in B-CLL patient specimens. Importantly, both the single agent and the combination effect of Nutlin-3 are selective for cancer versus normal cells. In neuroblastoma cell lines with wild type p53, targeted disruption of the p53-Mdm2 interaction by nutlin-3 stabilizes p53 and selectively activates the p53 pathway, resulting in a pronounced antiproliferative and cytotoxic effect regardless of the MYCN amplification status (Van Maerken, Speleman et al., 2006). Furthermore, oral administration of nutlin-3 to mice bearing xenografts of chemoresistant neuroblastoma cells with wild type p53, resulted in inhibition of tumour growth and reduction in metastatic disease (Van Maerken, Ferdinande et al., 2009). Thus, targeting p53/Mdm2 by nutlin-3 or other Mdm2 antagonists may be a viable treatment option for neuroblastoma patients with wild type p53, perhaps particularly for high-risk MYCN-amplified disease, since a recent study demonstrated that overexpression of MycN sensitizes neuroblastoma cell lines with wild type p53 to Mdm2/p53 antagonists (Gamble, Kees et al., 2011). To date, the most promising small molecule Mdm2 modulators are still in preclinical development or early stage clinical trial.

7. Targeting kinases and the kinase pathway in neuroblastoma

Deregulation of kinase activity is one of the major mechanisms by which tumour cells evade normal physiological constraints on proliferation. One of the advantages of targeting kinases is that inhibition of kinase activity in normal cells can often be tolerated, which favours selective killing of cancer cells. Small-molecule kinase inhibitors have been intensively pursued as new anticancer therapeutics for many years, with approximately 30 distinct kinase targets having been identified. The vast majority of these inhibitors are being investigated for the treatment of cancer (reviewed in (Zhang, Yang et al., 2009)).

7.1 ALK targeted inhibition

Anaplastic lymphoma kinase (ALK), also known as ALK tyrosine kinase receptor or CD246, is an enzyme that in humans is encoded by the *ALK* gene. ALK plays an important role in the development of the brain and exerts its effects on specific neurons in the nervous system. Recent studies have shown that heritable mutations of *ALK* are a major cause of familial neuroblastoma, and that germline or acquired activation of this cell surface kinase is a tractable therapeutic target for this lethal paediatric malignancy (Mosse, Laudenslager et al., 2008). Specific and potent ALK inhibitors have been discovered and described in the literature. One of the promising ALK inhibitor, PF-2341066 (Pfizer), is currently in clinical development both for c-Met and ALK driven cancer indications (Ardini, Magnaghi et al., 2010).

7.2 Trk targeted inhibition

Trk (Tropomyosin receptor kinase) receptors are a family of tyrosine kinases that regulate synaptic strength and plasticity in the mammalian nervous system. Among the three Trk receptors (A, B and C), TrkB is preferentially expressed in aggressive neuroblastoma tumours and the BDNF/TrkB signaling pathway has been shown to form an autocrine loop in these tumours (Schramm, Schulte et al., 2005). TrkB mediates chemoresistance in neuroblastoma by activation of PI3/AKT survival pathway (Ho, Eggert et al., 2002). Given the apparent roles of *Trk* genes in the biological and clinical behaviour of neuroblastomas, inhibiting Trk receptors may be an important adjunct to therapy. Lestaurtinib (CEP-701) is a Trk-selective tyrosine kinase inhibitor that blocks Trk activation by its ligand. It has been shown that precursor compounds to Lestaurtinib have efficacy in treating neuroblastomas in preclinical xenograft models. Lestaurtinib is currently in a phase I clinical trial in neuroblastoma patients. It is presumed that this agent, by blocking an important survival pathway, will render tumour cells more susceptible to cytotoxic drugs, as suggested by preclinical studies (Brodeur, Minturn et al., 2009).

7.3 PLK1 targeted inhibition

The Polo-like kinase 1 (PLK1) is highly expressed in many human cancers and is a target of the novel small-molecule inhibitor BI 2536, which has shown promising anti-cancer activity in adult malignancies. A recent study showed that elevated PLK1 expression is significantly associated with high-risk neuroblastoma and unfavourable prognosis. Inhibition of PLK1 using BI 2536 exhibits strong anti-tumour activity on human neuroblastoma cells *in vitro* and *in vivo* (Ackermann, Goeser et al., 2011). In addition, BI 2536 significantly inhibited tumour growth in a therapeutic xenograft model of tumour initiating cells, both as a single agent

and in combination with the therapeutic agent irinotecan (Grinshtein, Datti et al., 2011). BI 2536 has progressed into clinical studies in patients with locally advanced or metastatic cancers in adults.

7.4 Aurora kinase targeted inhibition

Aurora kinases are a family of three highly homologous serine/threonine kinases (Aurora A, B and C) that play a critical role in regulating many of the processes that are pivotal to mitosis. Each isoform has a different function in the control of mitosis. Aurora A, encoded by the *AURKA* gene, appears to be involved in microtubule formation and/or stabilization at the spindle pole during chromosome segregation and has a critical function in regulating turnover of MycN protein. Aurora A interacts with both MycN and the SCFFbxw7 ubiquitin ligase that ubiquitinates MycN and counteracts degradation of this oncoprotein, thereby uncoupling MycN stability from growth factor-dependent signals (Otto, Horn et al., 2009). Overexpression of *AURKA* in neuroblastoma tumours is associated with high risk, late-stage tumours, unfavorable histology, *MYCN* amplification, disease relapse and decreased progression-free survival. knockdown of this gene by siRNA results in growth inhibition and enhanced chemosensitivity in neuroblastoma cell lines. Similarly, treating neuroblastoma cells *in vitro* with MLN8054, a small molecule Aurora A inhibitor, causes dramatic growth inhibition (Shang, Burlingame et al., 2009). Several small molecule Aurora kinase inhibitors have been developed and are currently undergoing preclinical or clinical trials. However, the clinical activity of the aurora kinase inhibitors in patients with solid tumours has been rather disappointing (reviewed in (Gautschi, 2008; Boss, Beijnen et al., 2009)). Currently, a promising Aurora A inhibitor MLN8237 is in phase II clinical trial for treating young patients with recurrent or refractory solid tumours or leukemia (NCT01154816, USA). Future studies with aurora kinase inhibitors should focus on the possibility of combining these agents with radiotherapy, chemotherapy, or other targeted anticancer agents.

7.5 PI3K/Akt/mTOR pathway as therapeutic target

The prognostic value of the phosphatidylinositol 3'-kinase (PI3K)/Akt/mTOR pathway was first investigated through analysis of 116 primary neuroblastoma samples. Akt activation was identified as a novel prognostic indicator of both decreased event-free and overall survival, and this was correlated with a number of well-established prognostic markers, including *MYCN* amplification, 1p36 aberrations, advanced disease stage, age at diagnosis, and unfavourable Histology (Opel, Poremba et al., 2007). *In vitro* studies also demonstrated that activation of Akt by Insulin-like Growth Factor I protected neuroblastoma cells against TRAIL- or cytotoxic drug-induced apoptosis, indicating that activation of this survival cascade can rescue neuroblastoma cells from cell death. In preclinical studies, the bisphosphonate inhibitor of osteoclasts, zoledronic acid, showed anti-neuroblastoma activity in bony metastases by inhibiting osteoclasts and tumour cell proliferation. Its antitumoral activity involves inhibition of the activation of Ras, c-Raf, ERK1/2 and Akt (Peng, Sohara et al., 2007). Inhibitors of PI3-K and mTOR have been shown to be anti-proliferative and pro-apoptotic in neuroblastoma cells *in vitro* and *in vivo* (Chesler, Schlieve et al., 2006; Johnsen, Segerstrom et al., 2007). The importance of the PI3K/Akt pathway in maintaining neuroblastoma cell growth has attracted interest in the design of molecular targeted therapies for this disease (Sartelet, Oligny et al., 2008). Several strategies have been

developed to interfere with distinct components of PI3K/Akt/mTOR pathway signalling (LoPiccolo, Blumenthal et al., 2008; Fulda, 2009). To date, few specific inhibitors of AKT have been discovered. The novel Akt inhibitor, Perifosine, has demonstrated both clinical efficacy and safety in several tumour types (LoPiccolo, Blumenthal et al., 2008). It is currently in Phase 1 clinical trial for paediatric tumours, including neuroblastoma. Thus, drugs targeting PI3K/Akt/mTOR pathway may eventually open novel avenues to improve the poor prognosis of patients with advanced stage neuroblastoma.

8. Future perspectives

Recent advancements in targeted therapy for neuroblastoma using small molecule drugs are not limited to the above-mentioned studies. There are a number of other molecular targets and inhibitors that are being intensively investigated in various cancer types, including neuroblastoma. One such example involves inhibitors of histone deacetylases (HDACs), which are currently in early phase clinical trial for neuroblastoma as well as other cancers (Reviewed in (Witt, Deubzer et al., 2009)). We can anticipate that current molecular genetic research in neuroblastoma will enable the identification of more prospective molecular targets that will provide opportunities for the development of new therapeutic strategies. In this regard, new targets that have recently been identified include S-phase kinase-associated protein 2 (SKP2) (Westermann, Henrich et al., 2007), Endosialin (Rouleau, Smale et al., 2011), cycle checkpoint kinase 1 (CHK1) (Cole, Huggins et al., 2011), and c-Met (Crosswell, Dasgupta et al., 2009). By integrating the analysis of MycN binding sites derived from ChIP-chip experiments with mRNA expression microarrays and clinical data, Murphy and colleagues in a recent comprehensive study on the role of MycN in tumorigenesis, have found a large set of clinically relevant cell cycle genes that are critical to neuroblastoma tumorigenesis. Many of these clinically relevant MycN target genes may warrant further functional study as potential neuroblastoma therapeutic targets (Murphy, Buckley et al., 2011).

In recent years, a number of strategies have been developed to identify synthetic lethal interactions between genes for use in anticancer drug discovery, and a few drugs are currently being investigated in preclinical and clinical studies (reviewed in (Chan & Giaccia, 2011)). Synthetic lethality screening involves searching for genetic interactions between two mutations, whereby the presence of either mutation alone has no effect on cell viability, but the combination of the two mutations leads to cell death. The presence of one of these mutations in cancer cells but not in normal cells can therefore create opportunities to selectively kill cancer cells by mimicking the effect of the second genetic mutation with targeted therapy. One such example of this strategy in neuroblastoma involves cyclin dependent kinase 2 (CDK2), which is found to be a synthetically lethal molecule to MycN (Molenaar, Ebus et al., 2009). Inactivation of CDK2 by either siRNA or small molecule drugs induces apoptosis in *MYCN*-amplified neuroblastoma cell lines, but not in *MYCN* single copy cells, and silencing of *MYCN* by siRNA can abrogate this apoptotic response in *MYCN*-amplified cells. Thus, the synthetically lethal relationship between CDK2 and MycN suggests CDK2 is a potential *MYCN*-selective target in neuroblastoma therapy.

Another research area that warrants exploration for targeted therapy is that aimed at neuroblastoma differentiation. Conventional chemotherapy often results in dramatic reduction in tumour mass after initial treatment, suggesting a strong primary apoptotic response. However, histopathology reveals that, in many cases, neuroblastoma tumours are composed of both pathogenic undifferentiated neuroblasts and neuronal lineage cells at

various stages of differentiation. This suggests that neuroblastoma tumours retain the biochemical pathways mediating both programmed cell death as well as multilineage terminal differentiation and this represents the rationale for involvement of differentiating agents such as retinoic acid derivatives in neuroblastoma therapy. Identification of key molecules (apart from MycN) involved in the differentiation of neuroblastoma will be essential for future development of potent differentiating agents.

The rapid screening of collections of compounds for which the majority have already been approved for clinical use as anticancer agents is another area that holds considerable promise for the development of compounds with potential therapeutic value in treating paediatric cancers. Virtually all the new anticancer drugs that are currently being tested in clinical trials as targeted therapies, have been developed for specific adult cancers. Given the size of the adult cancer population by comparison with the total number of paediatric cancer patients, the vast majority of these drugs will never be administered to childhood cancer sufferers. However, compound libraries containing marketed drugs are available for purchase and bioavailability and toxicity studies have already been performed for any promising candidates that might be identified. Recently, a number of promising compounds for treatment of children with neuroblastoma were identified using this strategy and further studies are warranted (De Preter, De Brouwer et al., 2009). As previously discussed, the use of DFMO for inhibiting Odc1 in neuroblastoma is a good example of an off patent drug demonstrating renewed clinical promise as both an anticancer drug as well as a cancer chemopreventive agent (Hogarty, Norris et al., 2008; Rounbehler, Li et al., 2009).

In looking towards future clinical application, targeted therapies may be useful in combination with conventional chemotherapeutic agents in order to achieve synergistic effects, or alternatively, the combination of two targeted therapeutics may also prove efficacious. For example, recent studies have shown that DFMO acts synergistically with PI3 kinase inhibitors to increase apoptosis in neuroblastoma cells (Koomoa, Yco et al., 2008). Overall, despite the existing challenges in the discovery of new drugs, an increased understanding of cancer genetics is allowing the development of powerful drug or drug combinations that may increase the selectivity and safety of chemotherapy by selective killing of cancer cells and protecting normal cells. It is hoped that with this increased understanding and the development of new therapeutic strategies, this will ultimately lead to improved outcomes for children with neuroblastoma and other cancers.

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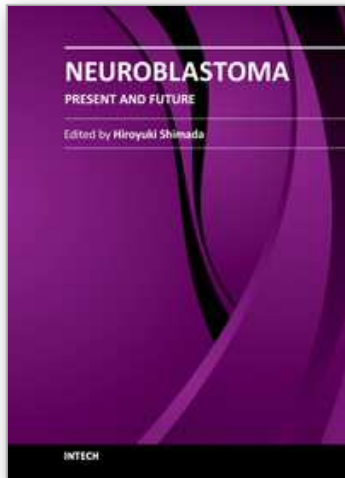
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Neuroblastoma, once called "enigmatic", due to "unpredictable" clinical behaviors, is composed of biologically diverse tumors. Molecular/genomic properties unique to the individual tumors closely link to the clinical outcomes of patients. Establishing risk stratification models after analyzing biologic characteristics of each case has made a great success in patient management. However, the trend of improving survival rates in neuroblastoma over the last 30 years has started to level off, and currently available treatment modalities have almost reached to their maximized intensity. Furthermore, aggressive treatment causes significant long-term morbidities to the survivors. We really need to make the next step to the level of personalized medicine with more precise understanding of neuroblastoma biology. This book includes useful data and insights from the world's experts in this field. I believe this book can make an excellent contribution to all the investigators working hard and fighting for the children stricken by this disease.

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