

Karolinska Institutet, Stockholm, Sweden

**EXPERIMENTAL COMBINATION
THERAPY OF BRAIN CANCER CELL
MODELS**

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To the greatest man I have ever met my grandfather Carlos
Vargas

*Before you judge my life, my past, or my
character,
walk in my shoes, walk the path I have
traveled,
live my sorrow my doubts, my fear, my pain
and my laughter!
Remember: "judge not lest ye be judge"
Everyone has their own story!
When you've lived my life, then you can judge
me*

Lizamarie

ABSTRACT

Epigenetic alterations and aberrant expression of genes controlling epigenetic mechanisms have been identified in several cancers, including medulloblastoma and glioma, the most common primary brain tumors in children and adults, respectively. We have been investigating if combination therapy using histone deacetylase inhibitors (HDACi) and receptor tyrosine kinase inhibitors (RTKi) will enhance glioblastoma and medulloblastoma cell killing. In medulloblastoma studies we combined a DNA methylation inhibitor, an HDAC inhibitor together with tyrosine kinase inhibitors.

In the first study (Paper I) we have shown that combining HDACi with the RTKi gefitinib and vandetanib resulted in enhanced cell killing and reduced clonogenic survival. Mono-therapy using HDACi sodium 4-PB induced minor cell killing effects in neither of the analyzed cell lines. Similar results were observed after mono-therapy using gefitinib or vandetanib. However the combination of 4-PB with gefitinib resulted in significantly increased cell death compared to mono treatment in both cell lines. Furthermore, the double therapy resulted in a significant decrease in colony formation.

The second study (Manuscript) showed that combination of drugs that inhibit two of the most important epigenetic factors (gene methylation and post-translational modifications of protein histone-associated DNA with small molecule inhibitors of receptor tyrosine kinase) enhances cell killing in two medulloblastoma cell lines. The HDACi, 4-phenylbutyrate (4-PB) and the demethylation agent, 5-Aza-2'-deoxycytidine (5-Aza-dC) had minor effects on medulloblastoma cell cytotoxicity when used as single agents. A significant enhancement in cell cytotoxicity was seen when these drugs were combined with imatinib or sorafenib. Triple combinations resulted in accumulation of cells with subG1 DNA content and were associated with a decrease in the expression of histone deacetylase genes and reduced global methylation. This occurred together with an increase in apoptosis.

Taken together these results suggest that combinations of these drugs may be beneficial in the treatment of medulloblastoma.

LIST OF PUBLICATIONS

- I. Marino, A. M., A. Sofiadis, Baryawno, N., Johnsen, J.I., Larsson, C., Vukojevic, V., Ekstrom, T.J. Enhanced effects by 4-phenylbutyrate in combination with RTK inhibitors on proliferation in brain tumor cell models. Biochem Biophys Res Commun (2011) 411(1): 208-212.
- II. Marino, A.M., Frijhoff, J., Baryawno, N., Ostman, A., Johnsen, J.I. Effects of Epigenetic modifiers in combination with small molecule inhibitors of receptor tyrosine kinases on medulloblastoma cells. Manuscript

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LIST OF ABBREVIATIONS

AKT	Protein Kinase B
AML	Acute Myeloid Leukaemia
BCOR	BCL-6 co-repressor
CNS	Central Nervous System
DIPG	Diffuse Intrinsic Pontiac Glioma
DKK	Dickkopf Family of Proteins
DNMT	DNA Methyltransferase
DNMTi	DNA Methyltransferase Inhibitors
EGFR	Epidermal Growth Factor Receptor
EGL	External Granular Layer
ER	Estrogen Receptor
GCP	Cerebellar Granular Progenitors
GIST	Gastrointestinal Stromal Tumors
GNP	Granular Nuclear Progenitors
GPS2	G Protein Suppressor 2
HAT	Histone Acetyl Transferases
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase Inhibitor
LRLP	Lower Rhombic Lip
MB	Medulloblastoma
MBEN	Medulloblastoma with Extensive Nodularity
MDS	Myelodysplastic Syndrome
MLL	Mix Lineage Leukaemia
PDGFR	Platelet Derived Growth Factor Receptor
RTK	Receptor Tyrosine Kinase
RTKi	Receptor Tyrosine Kinase Inhibitors
SAHA	Sureroylanilide Hydroxamic Acid
SAM	S-adenosylmethionine
SHH	Sonic Hedgehog
SMO	Smoothed
TGF β	Transforming Growth Factor Beta
TSA	Trichostatin A
VEGFR	Vascular Endothelial Growth Factor Receptor
Wnt	Wingless
4-PB	Sodium 4 Phenylbutyrate

1 THESIS SUMMARY

1.1 BRAIN TUMORS

1.1.1 Adult Brain Tumors

Brain tumors in both adults and children are among the most deadly diseases in human despite the recent increase in biological understanding and management of the diseases. Hence, there is a great need for further investigation and development of new therapeutic modalities. Current therapeutic techniques need to be improved, both in order to cure more patients and to reduce secondary effects [1].

According to the American cancer society there is a higher incidence of brain tumors in men than in women 7.6 vs 5.3 respectively, and the age peaks between 65 and 79 years [2].

There are many different types of brain tumors in adults (fig 1). Among these, gliomas are most frequent accounting for 70% malignant brain tumors [3]. The most severe subtype of gliomas, glioblastoma multiforme accounts for 50% (20% of all adult brain tumors) of diffuse gliomas [3]. Glioblastoma is more common to occur in white persons than in black persons [2].

Gliomas are divided into four groups according to the World Health Organization grade I and grade II are low grade where grade III and grade IV are high grade gliomas, the most common glioma is glioblastoma multiforme [2, 3]. Treatment for gliomas requires surgery, radiation, and chemotherapy [2, 3].

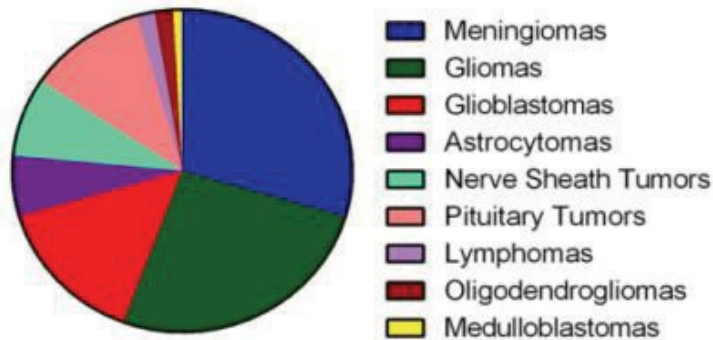


Figure 1 Graph representing % of adult brain tumor types (statistics taken from the ABTA)

1.1.2 Pediatric Brain Tumors

Although brain tumors are rare in children, pediatric brain tumors are the most common solid cancers of childhood representing 25% of all pediatric cancer cases, and the leading cause of death due to disease in western countries [4].

According to the USA Central Brain Tumor Registry (CBTRUS) the annual incidence of pediatric brain tumors between the ages of 0-19 years is of 4.84 per 100.000 population [4]. There are different types of pediatric brain tumors (fig 2): gliomas, astrocytomas, ependimomas, embryonal tumors (medulloblastoma), Choroid plexus tumors, germ cell tumors, and craniopharyngiomas, the therapy for these tumors requires surgery followed by radiation and chemotherapy [4].

Medulloblastoma (MB) is the most common form of pediatric brain cancer accounting for 15% of all CNS cases [5].

Even though current therapeutic protocols have improved the overall survival of patients, there is a need to develop new therapeutic modalities for medulloblastoma, since the current therapies often cause long-term side effects with high risk of severe morbidity even if cured from the tumor.

In summary primary brain tumors of adults and children need to be further investigated to achieve a better understanding of these tumors that can help to develop new therapies, improve survival and quality of life for the patients.

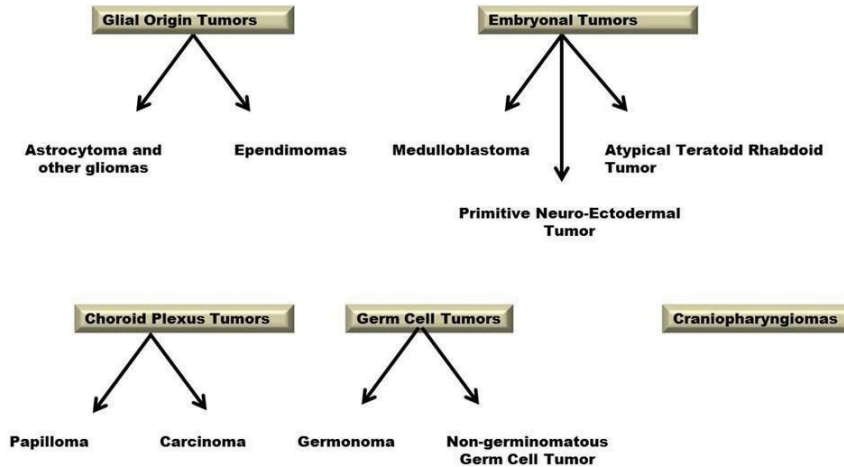


Figure 2 Types of pediatric brain tumors

1.2 MEDULLOBLASTOMA

Medulloblastomas are a compilation of molecular and clinical diverse tumor types that together encompass the most frequent malignant brain tumors in children [6]. The majority of medulloblastomas arises in the cerebellum and are usually diagnosed before the age of 10 years [5, 7]. Medulloblastomas are currently classified according to five histological subtypes: desmoplastic/nodular, classic, large cell, medulloblastoma with extensive nodularity (MBEN) and anaplastic medulloblastoma [7-9]. Classic medulloblastoma is the most common variant followed by desmoplastic [7]. Risk stratification of patients diagnosed with MB is based on patient age, surgical tumor resection, presence of metastasis and histology (presence or absence of diffuse anaplasia). Based on these parameters patients are classified as standard risk patients, who are older than 3 years, with localized disease, without anaplasia, and all the rest are considered as high risk [8].

1.2.1 Medulloblastoma Origin

Different subtypes of medulloblastoma have distinct cellular origins. One subtype originates from cerebellar granule neural precursor (GNP) cells located in the external granular layer (EGL) of the cerebellum as a result of aberrant Shh signalling [10, 11]. A subpopulation of cells from these tumours is positive for the progenitor markers Math1 and CD15 [12]. A different medulloblastoma subtype arises outside the cerebellum from cells of the dorsal brainstem and is dependent on Wnt signalling. These tumours contain aberrantly proliferating Zic (+) precursor cells [13]. Finally, a third medulloblastoma subtype deriving from prominin1-positive (Prom1) cerebellar stem cells has been proposed. These tumours contain elevated Myc expression [14, 15].

Abnormal activation of the developmental signalling cascades Shh and Wnt is observed in approximately half of all medulloblastomas. Also, Notch, ERBB2, PI3K/Akt and TGF β 3 signalling has been shown to contribute to the development of medulloblastoma. [16-18].

1.2.2 Molecular Subgroups of Medulloblastoma

Medulloblastomas have recently been stratified into four distinct molecular subgroups based on transcriptional signatures, mutational spectra, copy number profiles and clinical features. These groups have been named: WNT, sonic hedgehog (SHH), group 3 and group 4 (fig 2) [19]. The different subgroups are briefly described below.

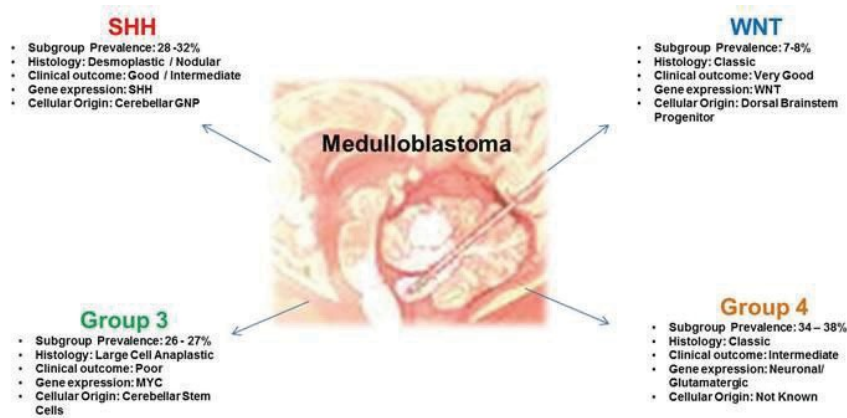


Figure 3 Representation of medulloblastoma subgroups

1.2.2.1 Sonic Hedgehog (SHH)

Sonic hedgehog (SHH) is a morphogenic factor that has been shown to be in control of central nervous system progenitor's proliferation. It is known that SHH acts on gene expression through the activity of a transcription factor family of proteins called GLI. When SHH is absent the transmembrane receptor Patched 1 will inhibit the seven-pass transmembrane protein Smoothed (SMO), preventing its translocation to the primary cilia [20]. Aberrant expression of key proteins in the SHH signal transduction pathway may lead to tumorigenesis and tumors classified in the SHH subgroup have been shown to develop from cerebellar granular progenitors (GCPs) [21, 22].

Approximately 25% of MBs originate from GNP due to the aberrant alteration of SHH pathway [13]. SHH pathway is directly connected to the cell cycle control and the induction of cyclins D1 and D2 through the mediation of MYCN [18]. MYCN acts downstream of the SHH pathway and its expression has been seen in both nodular and anaplastic MBs. Patients with nodular type of MB have a better survivor rate than those with anaplastic [23].

Desmoplastic/nodular tumors belong to the SHH subgroup. These tumors are found mainly in infants and adults and account for more than 60% of patients from either group [24]. The MBEN subtype is seen mainly in very young infants and has a relatively poor prognosis. It has been linked to Gorlin syndrome an autosomal dominant disorder that exhibits germline mutations in the SHH receptor Patched [24].

1.2.2.2 WNT

WNT tumors develop outside the cerebellum possibly from cells of the dorsal brainstem and differ from SSH tumors [13]. Publishes series has shown that the genes marking this subtype are highly expressed at the lower rhombic lip (LRLPs) [25]. Deregulations of the WNT signaling pathway occur in about 10-15% of medulloblastomas. The activation of the WNT pathway occurs when Wnt binds to the seven transmembrane receptor Frizzled [21]. Frizzled activates the Disheveled protein resulting in inhibition of the destruction complex and translocation of β -catenin from the cytoplasm to the nucleus. In the nucleus β -catenin interacts with members of the T cell factor/lymphoid enhancer factor (Tcf/Lef) family of transcription factors and activation of target genes such as cyclin D1 and c-Myc [21]. Tumors in this subgroup have a classic histopathology, and it affects older children [26].

1.2.2.3 Group 3

The majority of tumors seen in this subgroup are mostly large cell/anaplastic. Like WNT tumors, subgroup 3 tumors present a high expression of MYC and the amplification of MYC influences patients survival [19, 27]. The majority of patient with group 3 tumors are males, and it occurs in both infants and older children [19, 26]. Investigation has showed that the highest frequency of metastatic MB falls into this subgroup (30%) and also into subgroup 4 (31%) [27].

1.2.2.4 Group 4

Tumors classified as group 4 are large cell/anaplastic MB. This is the only group of tumors that does not present high expression of any member of the MYC family [24, 27]. Like WNT tumors most of the patients are between the ages of 9 and 10 years at the time of diagnosis [19, 24, 27]. The presence of isochromosome 17q is quite high in this sub-group of MB (66%) [27]. There is not much known of this group and no animal models have been developed to further investigate its pathogenesis.

1.3 EPIGENETICS IN MEDULLOBLASTOMA

Epigenetics are modifications of the DNA or associated proteins, other than DNA sequence variation, that carry information content during cell division [28]. DNA methylation and histone modifications are coordinately regulated processes which regulate the cell-specificity of intracellular signalling, the two processes are intimately involved in the epigenome together with acetylation, methylation, and phosphorylation [28, 29].

Epigenetic alterations are early events in the loss of cellular homeostasis. A deeper general understanding of the epigenetic mechanisms and patterns will provide a foundation for the future clinical therapy of MB and other types of pediatric and adult tumors. Researchers have shown epigenetic events to be involved in medulloblastoma development by promoter methylation [30].

The WNT signalling pathway is activated due to epigenetic events, which lead to the inactivation of SFRP and DKK, which enable β -catenin to translocate to the nucleus switching on WNT signalling [31].

Mutations in many genes involved in chromatin remodeling complexes have been shown, some of these genes are MLL2, GPS2, KDM6A, BCOR, SMARCA4 [32]. Among the genes found to suffer mutations in group 4 tumors are the MLL3 and HDAC2, the two genes are involved in histone modifications [32]. A study performed by Parsons DW et. al. in a study of 22 MB tumors revealed common inactivation mutations of the MLL2 and MLL3 in 16% of the tumors.[33, 34]

Recent research of the Bmi1 signaling pathway, has showed that its deregulated in MB [35].

The Bmi1 gene a member of the chromatin Polycom-group recently proven to be a regulator neural stem cells via the p16^{Ink4a} and p19^{Arf} pathways, seems to also play a role as an epigenetic regulator of fate in normal cells and has been implicated in MB development [35].

1.4 CURRENT THERAPY FOR MEDULLOBLASTOMA

The current therapeutic protocol for medulloblastoma includes multimodal therapy, surgery, radiation therapy and chemotherapy. The treatment is based on risk assessment based on the age of the patient, infants (<3 years) have a poorer outcome and are classified as high risk these patients are treated only with chemotherapy. Patients older than 3 years receive radiation therapy after tumor resection followed by adjuvant chemotherapy[36]. Patients older than 3 years that belong to high risk group are treated with higher radiation dosage and adjuvant chemotherapy.

Tumor resection is of great importance in risk stratification if the tumor is resected completely than the patient will fall into average outcome and low risk, if the tumor can only be partially resected than the patient will fall into a high risk poorer outcome group [37, 38]. Based on these classifications the therapeutic modality will be decided [37-39].

Commonly therapeutic modalities for low and high risk patients include cisplatin; N-(2-chloroethyl)-N-cyclo-hexyl-N-nitrosourea (CCNU); vincristine; etoposide and

cyclophosphamide [36, 37]. Craniospinal radiation in average risk patients is of 23.4 Gy craniospinal and 54 Gy radiation of the posterior fossa, high risk patients receive 36 Gy craniospinal radiation followed by 54 Gy radiation of the posterior fossa [36, 39].

New therapeutic strategies are being investigated, that will include the use of proton radiation and different combination of therapeutic agents, such as target molecules and epigenetic modifiers [36, 39].

To avoid the risks of developing secondary malignancies, and learning disabilities, and organ damage, research is being focused on proton therapy [40].

1.4.1 Therapy Resistance

This is one of the biggest drawbacks in medulloblastoma and cancer therapy in general. Many patients develop resistance after treatment has started and others might never enter remission. Patients can develop resistance not only to drug therapy but also to radiation therapy.

It is believed that resistance to therapy is multifactorial and it involves a number of factors such as autocrine/paracrine signaling which involves the local tumor microenvironment; loss of therapeutic target and other factors [41]. A loss of response to therapies has been attributed to an attenuation of cancer death pathways (apoptosis); which can occur due to suppression of cell cycle arrest/apoptosis cascades [41].

Recently research has shown how resistance can be overcome in the treatment of medulloblastoma and other forms of pediatric brain tumors with the use of epigenetic drugs [18, 42]. For instance it has been shown how promoter hypermethylation can modulate cancer sensitivity to drug and radiation therapy [43]. A specific predictive biomarker of tumor response to therapy is O6-methylguanine DNA methyltransferase (MGMT), it has been shown that MGMT promoter methylation can be used to predict glioblastoma response to alkylating agents [43]. Adults whose tumors have an MGMT deficiency have shown to have a better outcome to alkylator chemotherapy [44]. In medulloblastoma studies have shown that one way to overcome MGMT mediated chemo resistance could be by depletion of MGMT [45].

Epigenetic modulators (HDACi) have recently been shown to overcome resistance to drug therapy, the mechanism by which HDACi exert their action has not been elucidated but some investigators think it might be through the hyperacetylation of histones after treatment with an HDACi [46, 47].

1.5 HDAC INHIBITORS, DNMT INHIBITORS AND RECEPTOR TYROSINE KINASE INHIBITORS AS PRIMARY MB TARGETS

1.5.1 Histone deacetylase inhibitors

The most studied histone modifications are acetylation of lysines. This is an important process in the regulation of gene expression and involves histone deacetylases (HDACs) and histone acetyl transferases (HATs) [28, 48]. HDACs have been divided into 4 different classes. Class I HDACs (1, 2, 3 and 8). Class II is subdivided into two classes: class IIa HDACs (4, 5, 7, and 9), class IIb HDACs (6 and 10). Class III are named sirtuins and these enzymes differ from the class I II and IV in that their function is NAD-dependent. Class IV includes HDAC11 [49, 50].

Inhibitors of HDACs, HDACi are potent inducers of differentiation and apoptosis by shifting the equilibrium of acetylation and deacetylation in favour of acetylation of core histones.

HDAC inhibitors activated either one or both apoptotic cell death pathways (intrinsic and extrinsic pathways) [51, 52].

An example of a non-toxic HDACi is sodium 4-phenylbutyrate (PB) – an aromatic fatty acid which can reach biologically active serum borders in the mM levels, with oral administration. PB has been evaluated in medulloblastoma cell lines where it increased acetylation of histones, with subsequent differentiation effects [53, 54]. PB can induce cell cycle arrest, involving induction of p21^{Waf1} in G1 and G2, and induce differentiation or apoptosis in a wide range of cell types [54]. Among the varieties of HDACi:s, valproic acid and Sodium butyrate, are two other short chain fatty acids that have been reported to be well tolerated by patients [50]. These drugs have a very short life time in plasma and therefore, high dosages are required in order to achieve good therapeutic effects [50, 55, 56]. The hydroxamic acids Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) on the other hand, have high therapeutic effects at nanomolar concentrations [54-56]. SAHA has been reported to down regulate the expression of the ErbB family [55].

HDACi induce downregulation of anti-apoptotic proteins and upregulation of pro-apoptotic proteins [55]. HDACi have also been shown to inhibit medulloblastoma cell growth, induce cell death and enhance the therapeutic effects of ionizing radiation (IR) [56]. One advantage of HDACi is their induction of cancer cell death, while normal cells are relatively in-sensitive to the drugs [54].

However, HDACs are also involved in the deacetylation of non-histone proteins, and transcription factors [57]. There is a lot of research focusing on the deacetylation of non-histone proteins since they are known to play a big role in cancer progression and development due to aberrant alterations involving HDACs [58]. Further research on HDACs will aid in the development of new and more specific HDAC inhibitors for the treatment of cancer.

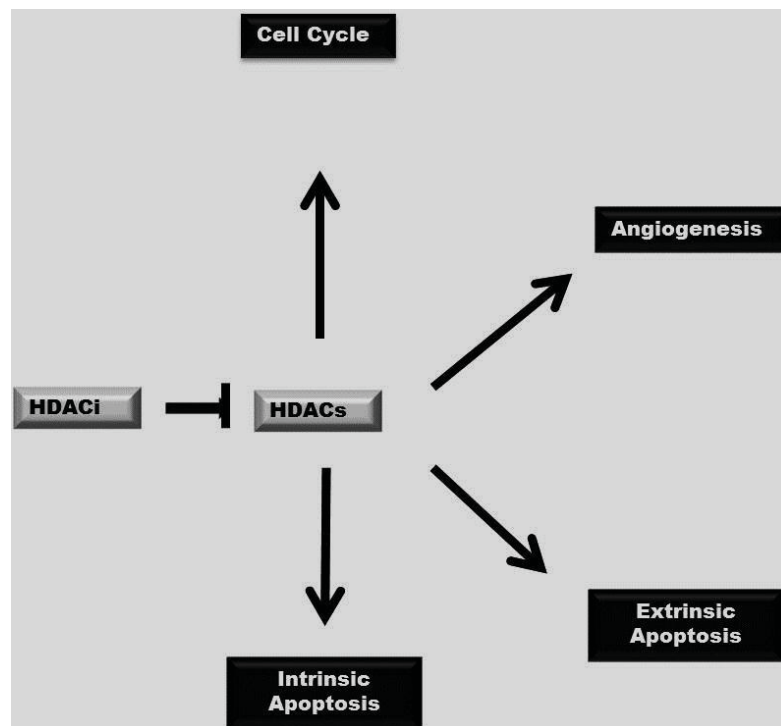


Figure 4 Pathways affected by HDACi

1.6 DNA METHYL TRANSFERASES INHIBITORS

The occurrence of epigenetic silencing during tumor development can be exploited by using epigenetic drugs to potentiate chemo sensitivity and response of the tumor to chemotherapeutic drugs [59]. Tumors show an increased methylation of CpG islands leading to gene silencing, since these regions often are associated with gene promoters [59].

DNA methylation is the enzymatic addition of a methyl group in the fifth position of the cytosine ring in the CpG dinucleotide context [59, 60]. The enzymes catalysing this enzymatic addition are the DNA methyltransferases (DNMTs) using the methyl donor S-adenosylmethionine (SAM). The products are 5-methylcytosine (5meC) and S-adenosylhomocysteine [60, 61]. There are three enzymatically active isoforms of DNMTs; which methylates cytosin; the de novo methyltransferase DNMT1, and the maintenance methyltransferases DNMT3a, DNMT3b, [62, 63].

The two DNMTs inhibitors decitabine (5-Aza-2'deoxyctidine) and azacitidine (5-azacytidine) are currently under clinical trials for solid tumors after their success in the treatment of MDS and AML. Both are cytidine derivatives, and are incorporated into DNA where they bind and lock the DNA methyltransferase [62, 63]. The two compounds are now in phase II clinical trials for solid tumors, in monotherapy as well as in combination with HDACi [62, 63].

Both decitabine and azacitidine are incorporated into DNA that causes an irreversible inactivation of DNMTs, which results in the reactivation and expression of tumor suppressor genes that previously had been aberrantly silenced by DNA methylation [64]. Research studies of haematological malignancies have shown that in bone marrow mononuclear cells previously collected from patients with MDS in a phase II study with decitabine silenced tumor suppressor genes had been reactivated [64].

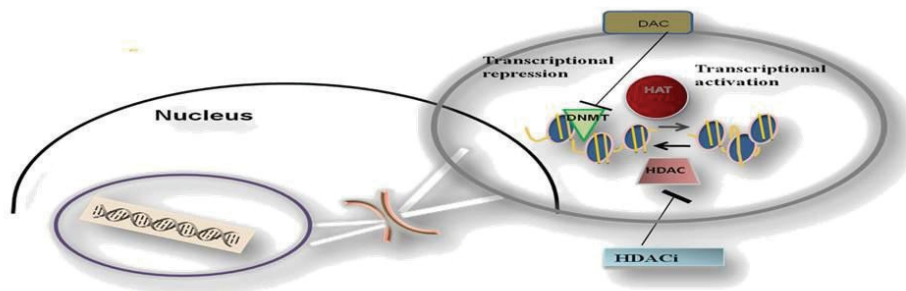


Figure 5 Represents rational of epigenetic combination targeting

1.6.1 Combination of DNMT and HDAC Inhibitors

The cytotoxicity of the DNMT inhibitors can be dramatically reduced when they are administered in combination with HDACi since the effective dosages are lowered [62, 63, 65]. In addition, clinical trials for triple negative metastatic breast cancer (lack of expression of the estrogen and progesterone receptors, and the Her2/neu) have demonstrated that combined administration of DNMTi and HDACi to patients before treatment with tamoxifen (an antagonist of the estrogen receptor) has resulted in the restoration and tamoxifen sensitivity of the estrogen receptor (ER) [63, 65]. The ER is silenced by methylation and histone deacetylation in this type of cancer, and therefore by reverting these epigenetic alterations the ER becomes active and the patients respond to therapy [63, 65, 66]. Combination therapy using DNMT and HDAC inhibitors in

solid tumors is promising, since it restores gene activities that have been epigenetically silenced, making the gene products responsive to targeting drugs. However the selection of epigenetic modifiers to be incorporated in cancer therapy should be based on the epigenetic profile of the tumor.

Due to the promising effects seen by their use during adult solid tumor therapy, the combination of epigenetic therapies is a promising therapeutic strategy for the treatment of metastatic aggressive pediatric cancers.

1.6.2 Receptor tyrosine kinase inhibitors

Current therapy for MB includes the use of cytotoxic agents that interfere with cell division and result in cell death. Molecular target drugs act by targeting specific aberrant features of the cancer cell. The identification of MB subgroups will enable clinicians to include molecular target drugs into the therapeutic protocols for MB therapy. Drugs in such clinical studies should be selected according to the molecular characteristics of the different tumor subgroups. The large number of novel drug candidates also offers the possibility of multiple targeting.

RTKs are a large family of enzyme-linked surface receptors that interact in order to form a complex signalling network [67, 68]. Some members of this class of surface receptors are the epidermal growth factor receptor (EGFR), the platelet derived growth factor receptors (PDGFRs) and the vascular endothelial growth factor receptors (VEGFRs). All these receptors have been implied in pediatric cancers, including medulloblastoma, where they potentially contribute to tumor cell growth and to angiogenesis [67, 68].

1.6.2.1 EGFR

The EGFR receptor is over-expressed in many types of cancer and has also been reported to be expressed in MB [69].

Additional members of the EGFR-family are erythroblastic leukemia viral oncogene homolog 2 (erbB2), erythroblastic leukemia viral oncogene homolog 3 (erbB3), and erythroblastic leukemia viral oncogene homolog 4 (erbB4), which are also expressed in medulloblastoma [23, 70].

Medulloblastoma expresses high levels of ErbB-2, whereas in the normal cerebellum the ErbB-2 level is undetectable [68, 71]. The ERBB2 is a marker for survival and has recently been pointed as a marker for poor prognosis [72, 73].

Gefitinib (Iressa) and Erlotinib (Tarceva) are the first approved inhibitors of the EGFR. Gefitinib is in clinical trial for the treatment of diffuse intrinsic pontiac gliomas (DIPG), an aggressive form of pediatric brain tumor and has been assessed for the treatment of medulloblastoma [68, 74-76].

Gefitinib was also in clinical trials for pediatric gliomas where studies have demonstrated that aberrant overexpression of the EGFR is responsible for cell proliferation [77].

1.6.2.2 PDGFR

The PDGFR family comprises two members; the PDGFRA and PDGFRB [70, 78, 79]. This receptor also signals through a surface receptor, and has been implicated in MB development [78, 80]. The PDGFRA and PDGFRB are upregulated in metastatic forms of MB [36, 81, 82]. Their expression has been associated with poor survival [36].

Imatinib (Gleevec) an inhibitor of the PDGFRA and β receptors; the ABL; BCR-ABL; KIT and CSF-1R, it has been tested in clinical trials for medulloblastoma [74, 83]. Imatinib showed excellent results during clinical trials against gastrointestinal stromal tumors (GIST), that show a high expression of KIT, and mutations of the PDGFRA [84]. Some forms of metastatic MB tumors have an overexpression of KIT besides upregulation of the PDGFRA and PDGFRB [85].

1.6.2.3 VEGFR

receptor-1; vascular endothelial growth factor receptor-2; and vascular endothelial growth factor receptor- 3, which regulate angiogenesis after activation by members of the vascular endothelial growth factor family.

Several VEGFR inhibitors have undergone and are currently undergoing clinical trials for pediatric forms of brain tumors [86]. It has been demonstrated that some MB tumors express VEGFR [68, 87].

Sorafenib is a multi-kinase inhibitor presently approved for use in renal cell cancer and liver cancer [88]. This drug is undergoing clinical trials for medulloblastoma and other types of pediatric brain tumors [89].

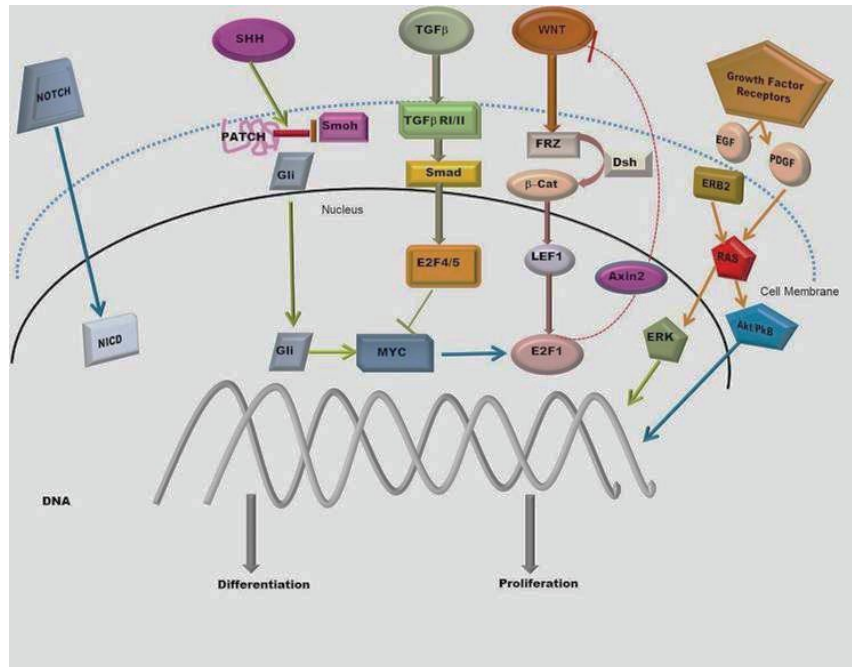


Figure 6 Some of the cell signalling pathways involved in MB development

1.6.2.4 NOTCH

During cerebellar development Notch signalling plays an important role, different members of this family are involved in GCP expansion [9].

Notch deregulation has been shown to result in brain tumor development [21, 90]. Notch signalling promotes medulloblastoma stem like cell survival, and Notch it's over express in medulloblastoma [91, 92].

1.6.2.5 TGF β

Recently, research has linked the canonical TGF β pathway to the pathogenesis of medulloblastoma. The transforming growth factor beta (TGF β) was found by Aref et. al. to be a potential contributor to medulloblastoma progression and metastasis [93]. Previous research had described the involvement of this pathway in the physiological and pathophysiological processes of the normal brain and investigators observed resistance to growth inhibitors in glioma cell was linked to growth receptors [18]. It has also been described by Subkhankulova et. al. that there is difference in expression of a number of genes involved in the TGF β pathway [94].

TGF β in normal tissue is secreted by platelets in cancer is secreted by surrounding environment and is overexpressed, its secretion serves as a signal for the cancer cells proliferation, which ha been indicated by Aref et. al. [93, 95].

2 AIMS OF THE THESIS

The present thesis was aimed to investigate the effects of epigenetic drugs as monotherapy and in combination with tyrosine kinase inhibitors on the growth of brain tumor cells.

The main rationales are:

1. To find new therapeutic modalities for the therapy of medulloblastoma with the use of epigenetic drugs in combination with tyrosine kinase inhibitors.
2. To acquire a better understanding of the epigenetic markers involved in the silencing of key genes of the apoptotic pathways.

3 RESULTS

3.1 EFFECTS OF EPIGENETIC MODIFIERS AND TYROSINE KINASE INHIBITORS ON MEDULLOBLASTOMA CELL GROWTH

3.1.1 Paper 1

The rationale for this was to achieve enhanced cytotoxic effects by combining 4-PB with gefitinib or vandetanib two receptor tyrosine kinase inhibitors, using a medulloblastoma cell line (DAOY) and a glioblastoma multiforme cell line (U343MGa). The cytotoxic effects using 4-PB in combination with gefitinib or vandetanib showed increase cell death, and clonogenic assays showed a loss in the ability for colony formation. These result suggested that a combined treatment with the HDACi 4-PB and RTKi may be beneficial in the treatment of brain tumors.

3.1.2 Manuscript

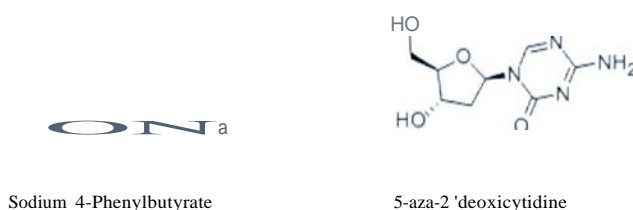
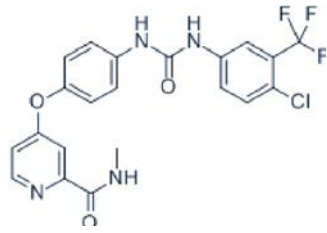


Figure 7 Molecular structures 4-PB and decitabine

Based on the results obtained in paper I, we decided to expand the repertoire of tyrosine kinase inhibitors to include imatinib and sorafenib, two inhibitors that have been widely used in the treatment of different cancers and is currently undergoing clinical trials for pediatric brain tumors. The epigenetic modifiers, 4-BP and 5-Aza-dC had minor effects on medulloblastoma cell growth when used as single agents. However, combining these drugs with the small molecule inhibitors of tyrosine kinases, imatinib and sorafenib, significantly enhanced the medulloblastoma cell cytotoxicity. We also observed an arrest in subG1 after therapy that correlates with the increase in apoptosis that was observed after we carried out a cell cycle study and a caspase 3/7 activity assay.

The epigenetic assays showed a difference in methylation and HDAC activity after triple therapy that can as well be correlated to increase cell death and an arrest in subG1. After we investigated the expression of key genes in non-treated cells and triple treated cells we as well observed a difference in their expression levels that could be used to explain the enhanced cell death and increase in apoptosis.



Sorafenib



Imatinib

Figure 8 Molecular structures of Imatinib and Sorafenib

The use of epigenetic modifiers and tyrosine kinase inhibitors are therefore a good alternative for the treatment of medulloblastoma. The combination as we have shown will activate caspases 3/7 and decrease the expression of anti-apoptotic genes as we have as well demonstrated.

4 DISCUSSION AND FUTURE PERSPECTIVES

4.1 THESIS DISCUSSION

Our results shows that a combination therapy using HDAC inhibitors together with receptor tyrosine kinases should be further evaluated as a treatment option for adult and pediatric brain tumors (Paper 1).

Results after the incorporation of 5-aza-dc resulted in enhanced cell killing after combination of the two epigenetic drugs, and using lower dosages of the drugs after triple combination. These results suggest that DNMTs play a role in MB progression, and are involved in inhibition of apoptosis together with HDACs. Their inhibition has led to enhance cell death by sensitization of MB cells. Further studies of the cell cycle showed increase number of cells in sub-G1 and increase activity of caspases 3/7. Our conclusion is that the epigenetic modifiers might restored the expression of genes involved in the induction of apoptosis and cell cycle activity, therefore enhancing cell death when imatinib or sorafenib are added. The epigenetic modifiers may sensitize medulloblastoma cells to tyrosine kinase inhibitors resulting in increased cell death. We showed in the manuscript that HDAC1 mRNA expression was reduced after triple treatment and HDAC1 has been pointed to be involved in drug resistance [49, 96].

These studies indicates the importance of epigenetics in drug resistance due to silencing of genes important in the regulation of cell proliferation and apoptosis. After the use of 4-PB in the triple treatment we showed the down regulation of Bcl-2 an anti-apoptotic member of the BCL2 family. Others have previously reported that HDACis down regulated the expression of BCL2 family members [97].

4.2 FUTURE PERSPECTIVES

- All our studies were conducted in vitro therefore it is of great importance to conduct an in vivo investigation of our findings. In vivo models will indicate if our findings are relevant and will also allow us to get a better knowledge of mechanisms involved in drug resistance. This will be mandatory in order to transfer these potential new drug combinations into clinical trials.
- We are planning to investigate novel receptor tyrosine kinases that together with epigenetic modifiers might result in enhance cell death. At the same time we are planning to screen novel HDAC and DNMT inhibitors not only for medulloblastoma therapy but also for other forms of aggressive pediatric brain tumors.
- To further study the effects of these combination of drugs, more molecular analysis on the mechanisms of action of these drugs used in combination will need to be performed. These will include analysis of important pro-/anti-apoptotic genes and the expression analysis in genes involved in cell cycle progression.
- Since most patient with medulloblastoma and other malignant brain tumors are subjected to radiation therapy. The drug combinations used in this study should be investigated together with different modes of radiation therapy.

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