HISTONE DEACETYLASE INHIBITOR MS-275 INHIBITS NEUROBLASTOMA CELL GROWTH BY INDUCING CELL CYCLE ARREST, APOPTOSIS, DIFFERENTIATION AND BY TARGETING ITS TUMOR STEM CELL POPULATION

by

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A thesis submitted in conformity with the requirements
for the degree of Masters of Science

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Histone Deacetylase Inhibitor MS-275 Inhibits Neuroblastoma Cell Growth By Inducing Cell Cycle Arrest, Apoptosis, Differentiation and By Targeting Its Tumor Stem Cell Population

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Abstract

Objective: MS-275, a phase trialed histone deacetylase inhibitor will be characterized for its ability reduce neuroblastoma (NB) viability and to target the tumor stem cell (TSC) population in neuroblastoma.

Methods: Ability of MS-275 to reduce NB growth is characterized using a tumorigenic NB N-type cell line that has high differentiation potential. TSC enriched side population from NB and a reference teratocarcinoma cell line was analyzed as a model of TSC. The potential of MS-275 to modulate functional characteristics and markers of TSC was also investigated.

Results: MS-275 induces a G1 cell cycle arrest, the intrinsic apoptosis pathway in NB and can potentially differentiate NB into a more terminal phenotype. NB TSC-like population is reduced following MS-275 treatment by the targeting of their self-renewal and drug pumping ability.

Conclusions: By targeting both the NB and its TSC population, MS-275 has therapeutic potential for neuroblastoma. This warrants further in-vivo investigations.

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Introduction and Rationale

1.1 Neuroblastoma

1.1.1 Epidemiology and clinical presentation of neuroblastoma

Neuroblastoma (NB) is a tumor of the primitive neural crest cells that form the sympathetic nervous system. NB can arise wherever sympathetic tissue naturally occurs. NB has also been noted in locations such as the thymus, lung, kidney and stomach. Annually, approximately 600 cases of NB are diagnosed in North America [1]. This makes NB the 3rd most common pediatric malignancy behind central nervous system tumors and leukemia. While NB only represents 8% of all pediatric tumors, because of its aggressive nature, it accounts for 15% of pediatric cancer deaths. Because NB clinical behavior varies broadly, it is classified based on its clinical phenotype into stages. Diagnosis is based on staging, clinical presentation, histology, genetics and other molecular and biological information. Despite treatment regimens having significantly improved over the last few decades, the prognosis for children with high-risk clinical phenotypes has barely improved, with long-term survival still less than 40%. Two-thirds of patients have metastatic bone disease at presentation [1]. In older children with disseminated NB (stage 4) that NB becomes difficult to combat, resulting in poor survival rates [1].

1.1.2 Neuroblastoma, ganglioneuroblastoma and ganglioneuroma

Because it is derived from the neural crest, NB, collectively with ganglioneuroblastoma (GNB) and ganglioneuromas (GN), is classified as a neuroblastic tumor. While sharing similar origins, the three differ in their degree of cellular and extracellular maturation. NB is found histologically to be immature, containing large amounts of undifferentiated neuroblasts and little differentiated ganglion tissue and Schwannian stromal content. GN in contrast is completely differentiated in stromal and cellular content. GNB is intermediate in phenotype in that it has more differentiated stroma. When a neuroblastic tumor is composed of less than 50% differentiated (Schwannian) stroma, it is classified as NB. Tumors with more undifferentiated immature elements are usually more aggressive

and portend a poor prognosis. NB which has more immature tissue is considered malignant, while fully differentiated GN is considered benign. Clinically it has been observed that NB and GNB can mature and regress spontaneously into a GN phenotype. While the mechanism by which this occurs is not fully understood, it is theorized to be a result of the activation of a combination of differentiation and apoptosis pathways which are normally silenced or inactive in the undifferentiated and malignant tumors [2]. GN has been noted to arise spontaneously, it has also been noted to occur following chemotherapy of NB and GNB [2].

1.1.3 Neuroblastoma cytology

In-vitro, NB has been classified into three different morphologically and biochemically distinct cell types: N(neuroblastic), S (substrate-adherent) and I (intermediate) types [3]. N-type cells have been observed to be immature neuroblasts that have small cell bodies, high nuclear to cytoplasmic ratio, and short neurites. They are weakly adherent and attach well to each other to form cell aggregates. S-type NB adhere tightly to substrate, have large flattened cell morphology, have no neurites and have low nuclear to cytoplasmic ratio. I type cells have phenotypes of both N and S types and grow equally well as aggregates or on substrate [4]. N-type cells represent an immature neuroblastic cell with expression of, but not limited to, tyrosine hydroxylase, chromogranin A, secretogranin II, and all three neurofilament (NF) proteins. They also express enzymes and receptors commonly associated with neuroblasts. S-type cells represent Schwannian, glial and melanoblastic precursor cells that lack neuronal proteins and express proteins such as tyrosinase, vimentin, epidermal growth factor receptor and fibronectin. They have also been shown to express glial and Schwannian markers, and also sometimes alpha-smooth muscle actin. I-type cells express dual markers expressed by N- and S-type cells and are postulated to be more primitive and the parental cells of the N- and S-types [3-5]. Evidence is accumulating that the three NB cell types observed in culture are reflective of cell types that comprise primary NB tumors, with some groups showing that N-type cells account for the neuroblastic component and the S-types account for the stromal component of tumors[6]. I-type like cells have been theorized to be tumor stem cell like and give rise to the neuroblastic and stromal components of NB tumor [4, 6, 7].

It has also been hypothesized that NB tumors with predominantly more S-type cells are more likely to regress to a benign GN state [4].

1.1.4 Genetic and molecular abnormalities associated with neuroblastoma

Genomic amplification of MYCN is the most common genetic abnormality associated with advanced disease and resistance to treatment in NB and occurs in nearly 20% of primary tumors [1]. Other genetic abnormalities include deletions of the short arm of chromosome 1 which predicts an increased risk of relapse in patients with localized tumors. Allelic loss of 11q is also associated with high-risk phenotypes even though this loss most commonly occurs in non-MYCN amplified tumors. Loss of segments of chromosomes 1 and 11 is sometimes associated with translocation to chromosome 17. Gain of chromosome 17 is associated with more aggressive phenotypes and is theorized to be due to amplification of genes such as survivin [1] [8]. Finally DNA content is also a prognostic indicator for NB, with hyperdiploid NB having better prognosis than normal or near diploid NB. Hyperdiploid NB has been associated with NB regression and stromal differentiation [2].

Signaling proteins, such as INK4A, RAS and p53 altered in adult cancers are very rarely disregulated in NB. Nonetheless, mutations in the neurotrophin pathway has been associated with NB disease. Neurotrophin receptors and their ligands regulate survival, growth and differentiation of neural cells. High TrkA expression is associated with favorable outcome while conversely high TrkB expression is associated with poor prognosis. Experimentally, it has been found that TrkA induces differentiation and apoptosis when neural growth factor (NGF) binds [8]. CD44 expression is inversely correlated with MYCN has also been associated with better prognosis and less metastatic disease in NB. This is perhaps due to its ability as a cell adhesion protein that it may be able to prevent dissemination [8] [9]. Recently, mutations in the anaplastic lymphoma kinase (ALK) gene has been found in familial NB and also found to be acquired somatically in nearly 25% of NB cases [10].

1.1.5 Therapy of neuroblastoma

The staging of the disease is a critical determinant in choosing the appropriate therapy to be attempted in NB patients. Therapeutic regimens include, in combination or alone, surgery, chemotherapy, radiotherapy and in some low stage cases observation alone. Most commonly, treatment includes a combination of surgery and chemotherapy. However, in high-risk tumors, even with aggressive therapy and subsequent bone marrow transplantation, the 3 year event-free survival rate of these patients is less than 35%. Because of the low rate of survival even after aggressive treatment in high risk NB, there is a pressing need to develop more effective or directed therapeutics [2]. Over the past decade, numerous therapeutics have been proposed, with some going on to clinical trials as treatments alone or in combination with conventional therapeutics. This includes topoisomerase inhibitors, angiogenesis inhibitors, tyrosine kinase inhibitors, immunotherapies, targeted radiotisotopes and retinoids [1]. While these different therapies have all shown varying degrees of efficiency, there is still an urgent need for more effective therapeutics that can increase survival rate for high-risk NB.

1.1.6 Differentiation of neuroblastoma

Being derived from a neural crest origin that produces the sympathetic nervous system, and given its primitive phenotype, NB is still relatively sensitive to differentiating agents. Many in-vitro studies have demonstrated that NB cell lines can undergo a significant degree of lineage specific differentiation [11, 12]. NB cells have been shown to be able to differentiate along the lines of chromaffin cells, neurons, ganglions, and melanocytes. Numerous studies have demonstrated the use of retinoids in differentiating NB toward a more mature neuronal phenotype. The effect of retinoic acid on NB depends on the cell line and type treated [13]. S100 beta, a marker of Schwannian lineage has also been induced in NB treated with 5-bromo-2'deoxyuridine [14]. Agents that can potently differentiate NB into a benign phenotype will be of importance in its treatment. For example, because of their ability to induce more mature neuronal phenotypes, retinoids have been gone through clinical trials and are being investigated in their ability to treat minimal residual disease in NB [15] [16]. However, though they are powerful differentiating agents in-vitro, and have shown to improve survival, the in-vivo the ability

of retinoids to differentiate tumors is attenutated [16]. Thus, there is a need to discover more potent agents of differentiation, or agents that can, in combination, potentiate the effects of retinoids.

Finally, though many agents can only weakly induce differentiation and cannot induce terminal differentiation, they have been shown to be able to possibly induce transdifferentiation of NB from one cell type to another in-vitro [12, 14]. Therefore, in the future, agents that can transdifferentiate N- and I-type NB into the less aggressive S – type will be of importance clinically as well, as they may represent agents that can induce the regression of a NB into a GN.

1.2 Histone deacetylase inhibitors as anticancer agents

1.2.1 Histone deacetylase and histone deacetylase inhibitors

A new class of anti-cancer agents, termed histone deacetylase (HDAC) inhibitors (HDACi) have recently been discovered and are being investigated extensively in various settings. HDACi exert their effects predominantly by altering histone acetylation that is normally controlled by a balance of histone deacetylases HDACs and histone acetyl-transferases (HATs). The acetylation and deactylation of histones occurs rapidly and is easily reversible [17]. DNA packaging is principally based upon the interaction of negatively charged DNA and positively charged histones. HATs and HDACs are enzymes that can modify the N-terminal histone tails of histone octamers. The acetylation of lysine residues on the histone tails neutralizes the charge of the lysine amino group, weakening histone to DNA interaction. This subsequently relaxes chromatin and opens up DNA sites where transcription factors previously were unable to access. HDACi are drugs that by inhibiting HDACs alter the balance of acetylation and deactylation in a cell allowing for transcription of previously blocked genes [18].

HDACs possess two additional major roles; firstly, many hormone receptors, chaperone proteins, transcription factors and cytoskeletal proteins contain lysine residues that can be

acetylated, which play key roles in their control[19-22]. Secondly, HDACs also play as key role in numerous transcription complexes, perhaps effecting control on their trafficking [23] [24]. HDACi have been shown to be able to affect almost every facet of the acetylome [21]. Given their wide spread effects, it has been noted that HDACi can be anti-neoplastic agents, potentiators of differentiation, anti-inflammatory agents, and drugs against auto-immune disorders and neurological disorders [25-29] [21].

In humans, 4 main classes of HDACs have been identified with a total of 18 individual proteins. Classes I, II and perhaps IV are zinc dependent enzymes, while class III HDACs are NAD+ dependent. Despite similar structural homology, HDAC cannot be substituted in function [22]. Class I HDACs are predominantly found in the nucleus and ubiquitously expressed, while class II are expressed only in certain tissue types and are cytoplasmic in expression, with some being able to traffic to the nucleus [20]. The solving of the crystal structure of class I HDACs interacting with HDACi have shown that HDACi prevents the enzymatic activities of HDAC by directly affecting the catalytic site [30].

Numerous HDACi, both natural or synthetic have been discovered. They fit, in most part, into 4 groups, based on their chemical structure: hydroxamates, cyclic peptides, aliphatic acids and benzamides [20]. HDACi do not globally affect all HDAC, but rather have affinity to different HDAC and also at different doses [31]. Work by Chambers et al. have shown that up to 10% of genes could be altered significantly in various cancer types by HDACi [32]. Because of the induction of multiple pathways, there are many secondary and down-stream effects that amplify the effects of HDACi [20]. Increases in chromatin accessibility, and down-stream effects not only lead to upregulation of genes, but also downregulation of others [33]. The ability of HDACi to elicit significant genetic changes accounts for their well documented anti-tumor ability. However, because HDACi are not selective in their targeting, it leads to the possibility of undesired effects, such as the induction of drug resistance [34].

1.2.2 The potential of HDACi as anti-NB agents

Nearly all reported HDACi have been able to able to induce apoptosis and cell cycle arrest in cell lines constituting the N-, S-, and I- neuroblastoma cell types. Nevertheless, in NB it has been noted that S-type Cells are much less sensitive to the apoptotic inducing effects of HDACi treatment [35]. Despite this, HDACi can still induce cell cycle arrest through the attenuation of the pRb pathway and apoptosis through both the intrinsic and extrinsic pathway [21]. In NB, there are variations in the upregulation of different proteins of the pRb pathway between HDACi and also depending on dose. For example, for newly discovered HDACi, Helminthosporium carbonum toxin, it was reported in SH-EP and SKN-BE(C) NB cell lines to upregulate p21(waf1), p27(kip1), p15(ink4a) and p16(ink4b), but expression of the CDK inhibitor varied depending on treatment length, as long term treatments did not upregulate p21 [36]. This highlights that in NB, similar to other cancers, the effects of HDACi can vary considerably depending on the conditions and context. Other HDACi such as M-carboxycinnamic acid bishydroxamide, and BL1521, were able to induce the extrinsic pathway by way of upregulation of FAS and its ligand in SK-N-BE and SK-N-SH cell lines and their subclones[37] [38]. Downregulation of CDK4, and hyperphosphorylated RB and upregulation of p21 appears to occur with all HDACi tested in NB [36]. G1 cell cycle arrest was mostly noted, with G2/M cell cycle arrest reported with longer and higher dose treatments of the more potent HDACi such as TSA, HC-Toxin and MS-275 (lower doses with valproic and butyric acid) [36, 39]. Muhlethaler-Mottet et al., showed that NaB, TSA and SAHA were able to induce apoptosis in both N (LAN-1, IMR32 and SH-SY5Y) & S (SH-EP, SK-N-AS) type NB, while work by Deubzer et al., showed induction of apoptosis in SKN-BE(C)I type cells as well[36]. HDACi was able to induce pro-apoptotic proteins Bid and Bim and inactivate anti-apoptotic proteins XIAP, Bcl-X, RIP and survivin in NB. Their modulation was noted to be due to caspase dependent proteolytic cleavage or increase/decrease degradation via the proteasome pathway. As well, decrease in expression of BCL-2 in N-, S- and I-type cells have been noted [36]. Furthermore, in SH-SY5Y and SK-N-BE cells it was reported that VPA and butyric acid induces p21 cell cycle arrest at low doses, while high doses induces downregulation of BCL-2 with a concomitant increase in BAX and NOXA [40]. Increase in BAX in NB has been

associated with the release of BAX from KU70 control due to acetylation of KU70 [41]. While most NB has wildtype (WT) p53, it is sequestered in the cytoplasm and is non-functional. Therefore, it is also of interest that HDACi can increase acetylation of p53, and induce its nuclear translocation [40] [42]. Downregulation of Skp, Mad2 and survivin survival proteins has been found in NB when treated with HDACi [43]. HDACi in NB cell lines with amplified MYCN, can reduce expression of MYCN [36, 44-46]. Furthermore, proteins that correlate with reduced NB aggressiveness such as TRKA, which induces apoptosis via the modulation of p53 targets, BCL-2 and caspase-3, have also been noted to be upregulated following HDACi treatment in LAN-1-15 and SKN-BE(2) NB cell lines[47] [48]. The extrinsic pathway of apoptosis has also been documented to be induced following HDACi treatment in NB. Caspase 8, which is normally epigenetically silenced in NB, is upregulated following HDACi treatment [40]. Death receptors such as FAS/FAS-L, TNFR1/TNF, TRAIL/DR-4/5 and also their downstream effectors such as c-FLIP have also been shown to be able to regulated by HDACi treatments [37, 39, 49]. Beyond the extrinsic and intrinsic models of apoptosis, HDACi such as SAHA, pyridylmethyl-N-{4-[(2-aminophenyl)-carbamoyl]-benzyl}-carbamate (MS-275) and trichostatin A (TSA) have been shown to be able to increase the accumulation of reactive reactive oxygen species (ROS) in NB cells through the decrease in expression of thioredoxin and increase in thioredoxin binding protein [50, 51] [52]. This ultimately leads to cell death. Despite these documented effects, it has also been found that both broad spectrum and class specific HDACi inhibit BAX-mediated apoptosis to confer neuroprotectivity to normal neurons when co-treated with chemotherapeutics [53]. HDACi SAHA, BL1521, CBHA and MS-275 have shown ability to reduce NB xenograft growth in pre-clinical mice models [46] [54] [39] [38].

Combinatorial usage of HDACi with common therapeutics in NB is also being investigated. In NB, it has been noted high mRNA expression of HDAC1 is associated with multi-drug resistance in cell lines. Pretreatment with HDACi in-vitro sensitized multi-drug resistant NB cell-lines to chemotherapeutics such as carboplatin, etoposide, and vinblastin [55]. However, pre-treatment of NB cell lines with HDACi BL1521 reduced their sensitivity to gemcitabine induced cell cycle arrest and apoptosis.

1.2.3 The potential of HDACi as a differentiating agent

Cancer cells have nearly unlimited proliferative capacity and have also lost the ability to undergo terminal differentiation[56]. When HDACi were first discovered, they were classified as differentiating agents due to their ability to differentiate erythroleukemic cells[57]. Furthermore, clinical studies of HDACi with hematopoietic cancers have documented that in certain cases, remission was accompanied with differentiated precursors[57]. As such, HDACi represent a new type of therapeutic that has the potential not only to function as anti-tumor agents, but also drugs that can reverse the malignant phenotype.

HDACi have been found to be able to differentiate breast, endometrial stromal sarcoma, liver, and small cell lung cancer cells[58-60]. Indirect evidence from in-vivo models also show HDACi able to differentiate prostate cancer cells [61]. While the differentiating capacity of HDACi have not been extensively studied in solid tumors (other than neuroblastoma), it has been noted that certain HDACs are upregulated in different cancer types. These HDACs have also been used as prognostic indicators in pathology. In colon and prostate carcinomas, it has been found that class I HDACs (in particular HDAC3) expression is elevated[62, 63]. This elevation corresponds to the observation that in normal intestinal development, as differentiation occurs class I HDAC expression decreases, further suggesting that an upregulation of HDACs in tumorigenesis leads to a loss of differentiating potential[64].

<u>The potential of HDACi to Differentiate NB</u>

The aggressiveness of neuroblastoma correlates with the degree of differentiation of the cancer[4]. Indeed, it is noted that more Schwannian and neuronally differentiated cancers are often less aggressive and offer better prognosis than undifferentiated ones [65]. Neuroblastoma development has been linked to defects in differentiation of neural crest cells. Furthermore, tumors comprising more S- type cells and possessing more stroma show slower progression and are less tumorigenic than tumors with less stroma and more N –type and undifferentiated I– type NB cells [4]. Numerous studies have shown that HDACi can inhibit NB growth and at the same time differentiate NB. [38, 46, 66-69] Also, HDAC8 has been found to play a prominent role in NB differentiation in that

inhibition of HDAC8 leads to NB differentiation [70]. Furthermore, tissue transglutaminase (TG2) is upregulated often by HDACi in NB. TG2 is often repressed in NB and its upregulation improves retinoid therapy in NB [45]. Also, in NB HDACi is able to induce RARbeta transcription, improving retinoic acid induced differentiation [69].

1.2.4 MS-275

MS-275, the HDACi chosen for this study is a potent synthetic benzamide HDACi with a long half life in serum[71] [72]. It preferentially inhibits class I HDACs. In particular, it inhibits HDAC1 & 2 at 0.3 uM, HDAC3 at 8 uM and does not inhibit HDAC6 and 8 well[31]. Nevertheless, because of its potent in-vivo activity and stability, it is now in phase I and II clinical trials in refractory lymphomas, acute leukemia and metastatic melanoma, breast, colon, prostate cancer and non-small cell lung carcinomas[73-77]. While regimens are still being tested and responses are minimal, results are promising and encourage further study into combinatorial usage with other therapeutics. MS-275 in vitro has been shown to be able to induce cell cycle arrest and/or apoptosis in bladder, clear cell sarcoma, retinoblastoma, non-small cell lung carcinoma, breast, gliomas, endometrial, cholangiocarcinomas, leukemia, Ewing's sarcoma, hepatomas, prostate, and acute myeloid leukemias [52, 78-87]. The mechanism of growth arrest and apoptosis has been mostly attributed to an induction of p21, down regulation of cyclins and a combination of the intrinsic and extrinsic apoptosis pathway. MS-275 has also been shown to be able to induce ROS stress in leukemia[52]. MS-275 has also been noted to be able to inhibit the expression of VEGF, HIF-1alpha and other angiogenic factors and suppress angiogenesis [88]. Its usage in vitro and in in-vivo mouse xenograft models have been coupled with drugs such as proteasome inhibitors, 5-aza-2-deoxycytidine, retinoic acid, CDK inhibitors, chemotherapeutics (such as cisplatin and methotrexate) and interleukins such as IL-2[52, 89-93].

Anti-NB potential of MS-275

In in-vitro and in mouse xenograft models, MS-275 has shown potent anti-NB potential. Jaboin et al., reported that MS-275 was able to induce p21 mRNA expression and decrease MYCN expression in the SK-N-AS NB cell line in vitro [54]. Their in-vitro

findings were reciprocated in in-vivo preclinical mouse models. MS-275 introduced to SCID-beige mice by oral gavage was able to significantly reduce tumor xenograft size[54]. Fuchert et al., similarly showed a time and dose toxicity of MS-275 against NB IMR-5, SHEP-SF and SH-SY5Y cell lines. NB cells also have well documented angiogenic capabilities, and MS-275 has shown ability to downregulate NB VEGF production and VEGFR expression and reduce angiogenesis [39]. However, no study to date has investigated the effects MS-275 or any other HDACi has on NB tumor stem cells, the purported intiating cells of a cancer that can repopulate an entire tumor.

1.3. Tumor stem cells

1.3.1 The definition of tumor stem cells

Over the last decade accumulating evidence has demonstrated that within individual tumors, there is a population of cells that is highly tumorigenic, and when injected into immuno-compromised mice, can form tumors at low cell numbers [94]. This observation has led to the reemergence of the tumor stem cell (TSC) theory. The TSC theory dictates that within a tumor there is a specific subset of cells, the TSC, that drive the growth. Similar to how stem cells are characterized by their ability to divide asymmetrically and self-renew, and to repopulate organs from single cells; TSCs are characterized by their capacity to self-renew and to give rise to the differentiated progeny found in the tumor. Self-renewal denotes the ability of a cell to divide and produce progeny identical to itself, making it able to propagate and maintain the TSC compartment. While over the last century, researchers have noted similarities between cancer and developmental biology, it was not until the advent of molecular techniques such as flow cytometry that further insights into the existence of TSCs could be made [95]. While the genetics, behavior, and genesis of TSC has drawn a lot of controversy, accumulating evidence makes the existence of a highly self-renewal, tumorigenic population that likely drives tumor development irrefutable.

The TSC theory predicts the existence of a normally quiescent, highly tumorigenic compartment that populates a tumor, through asymmetric division of a transit amplifying population that further differentiates into a heterogeneous population [96]. The first

conclusive isolation of TSCs was by Bonnet et al., who demonstrated that in acute myeloid leukemia there is an immature and small subpopulation of cells that expressed surface antigens CD34+ and lacked CD38 were capable at low cell numbers of initiating a tumor in mice with compromised immune systems. Similar populations have been described in other hematological malignancies [97]. In 2003 similar tumor initiating populations in solid malignancies was discovered by Al-Hajj et al., in breast cancers [98]. To date, TSCs have been described in glioblastoma, medulloblastoma, neuroblastoma, colon, breast, lung, pancreas, liver, prostate cancers, and head and neck squamous carcinomas [98-104]. The surface markers that distinguish the TSC compartment in different tumors have been shown to vary considerably.

1.3.2 Identifying tumor stem cells

The isolation of TSCs has primarily been based on the expression, or lack of, surface antigens. No individual marker has been discovered to date that can be attributed to TSCs of all malignancies, and often it takes more than one marker to define the TSC population [96]. Tissue specific stem cell marker expression patterns have often been noted in the TSC of their malignant counterparts (i.e. CD133 in brain malignancies) [99, 105]. However, this does not always hold true (i.e. CD133 in colon carcinoma) [101]. The functions of markers associated with TSCs are not often readily known. For example, CD44, a TSC marker of breast cancers is known to participate in anchorage and cell-cell interactions. Others like CD133, which is also known as prominin, still have not been elucidated [106].

Discovery of markers for TSCs will allow for improved diagnosis and prognostication, as it has been shown that tumors with higher populations of cells that express TSC markers often have poorer prognosis, or have greater metastatic potential [107-115]. Often, the isolation of TSCs is based on the observation that similar to stem cells, TSCs have anchorage independent growth and can form spheroids in non-adherent and serum starved culture conditions. Spheroids are isolated and their tumourigenic potential in immune deprived mice is tested. Gene arrays have also been employed to identify specific gene upregulations [116-120]. Numerous signaling pathways involved in self-

renewal (such as Notch, Wnt/betacatenin, sonice hedgehog) and self-renewal proteins (Oct-4, Nanog, Sox-2, KLF4, BMI-1) that are also implicated in normal stem cell development, have been attributed to TSCs [94, 95]. Another property of TSC is their ability to differentiate into the different progeny of the tumor from which they are derived from. As such following the identification of specific TSC markers, it is also critical to demonstrate that TSCs can be differentiated, and concurrently, the associated TSC marker must also be lost following differentiation [99, 116, 118, 120].

1.3.3 Side population (SP) is enriched in TSCs and is a model to study TSC

Beyond marker discovery and anchorage independent growth to identify TSCs, functional assays have also been employed. It was discovered that stem cell like cells have high expression of ATP binding cassette protein transporters that are drug pumps that can pump out drugs [121]. Based on this principle, it was discovered by Goodell et al., that Hoechst 33342 DNA staining dye is also actively pumped out by cells that express stem cell markers. This population of cells was localized to a small non-stained population when cells stained with Hoescht was analyzed by FACs at two emission wavelengths. This dye excluded population was termed side population (SP) [122]. Further work by multiple groups have shown that the SP is enriched in primitive, undifferentiated and highly stem cell like cells that express multi-drug resistant proteins excluded Hoechst dye [121]. SP has been extensively reviewed and the compiled data has shown that SP has been identified in normal tissues such as lung, heart, liver, brain and also in cancer lines and patient samples [123-125]. SP isolated from cancer cell lines and tumors have enhanced tumorigenic and self-renewal capacity, are highly immature, poorly differentiated, highly tumorigenic, more quiescent, smaller, and have high self-renewal potential as compared to their non-SP counterparts [123, 125, 126]. These observations lend credence to the hypothesis that SP represents a population that is enriched in TSCs. The SP phenotype is most commonly associated with the ATP dependent transporter ABCG2 [121]. However, it has been noted that in some studies that the expression of ABCG2 does not correlate with stem cell like cells [127]. This suggests that the basis of SP is much more complex, and depending on tissue type and

context, multiple pumps are necessary to exclude Hoechst 33342. Indeed, it has been noted that other pumps such as MDR-1 and pumps of the MRP family is associated with SP, and also TSCs [128, 129]. Another evidence that SP correlate to TSC is that they are able to repopulate the non-SP through differentiation [125]. Therefore, the consensus at present is that while SP cells are not necessarily TSCs, the SP is enriched in TSC. While the concept of TSC existing in cultured cell lines seems paradoxical since TSCs are a rare quiescent populations that can repopulate a heterogeneous tumor population, it has been noted that cells within a cell line are not always homogeneous. SP cells isolated from cancer cell lines have shown to express self-renewal proteins and signaling pathways found in stem cells, similar to that of TSCs [127, 130-132]. Coupled with their high tumorigenic and self-renewal capacity, SP cancer like stem cells from cell lines allow for a potential model to study TSCs when primary samples are not readily available.

1.3.4 Neuroblastoma tumor stem cells

I-Type neuroblastoma as candidate NB tumor stem cells

Ross et al., have theorized that because the neural crest is a primitive stem cell compartment that establishes multiple cell lineages in multiple organs, NB, their malignant counterparts are stem cell cancers in themselves[5, 14, 133]. This is supported by evidence that the differentiation of NB recapitulates neural crest development[134]. Furthermore, NB has been shown to be able to differentiate into the different cell types that arise from the neural crest. Walton et al., have demonstrated that I type NB have increased colony formation capacity (not self-renewal capacity), tumorigenic capacity (as determined by how often tumor xenograft is formed with 5X10^6 tumor cells) as compared to N and S type NB and more often expressed neural crest stem cell markers CD133 and CD117 [5]. However, many of their observations and the methodology used suggest that the more primitive I type NB cells are likely to be transit amplifying cells and not the true TSCs. A proposed model of NB initiation from a TSC is shown in Figure 1.

Isolation of NB TSCs

While SP cells that are highly tumorigenic, have high self-renewal potential, and express self-renewal proteins associated with stem cells have been isolated from NB cell lines, it was not until 2007 that Hansford et al., was able to demonstrate TSC population in primary NB samples [116, 130, 135, 136]. By growing cells in a non-adherent surface and in serum starved, bFGF, EGF supplemented media (stem cell culture conditions), Hansford et al., isolated from samples of different risk groups, cells that were able to form tumors from as little as 10 cells. Furthermore, tumors formed were able to form metastases to lung, kidney, liver, spleen and marrow. Further solidifying that these spheres grown are TSCs was that they were able to self-renew and also differentiate and form neuronal networks. These findings provided further evidence that a highly undifferentiated TSC population establishes NB tumors. Highly tumorigenic SP and tumour initiating cells in NB cell lines have been reported to express primitive neural marker nestin, ATP dependent transporter ABCG2 and ABCA3, and self-renewal proteins OCT-4, NANOG and SOX-2 [116] [130] [124] [117]. Expression of neural crest stem cell markers CD133, and CD117 have not been unaminously reported by all NB TSC studies [117] [116] [135]. While the expression of self-renewal proteins has been found in primary samples, CD133, ABCG2 and nestin have not [137] [138]. Hansford et al., also reported no detectable SP in their primary samples. However, NB SP cells from established cell lines have also been used by Tsuchida et al., to investigate the effects of platinum based chemotherapeutics against TSC like cells and Das et al., to investigate the role of hypoxia and oxidative stress in TSC like cells. These studies found that both cisplatin and hypoxia are capable of expanding the NB SP TSC-like population [130, 136]. In normal stem cells and TSC, hypoxic niches regulates differentiation and maintain pluripotency [139, 140]. Similarly so, Hirschmann-Jax et al, in their analysis of SP in multiple SP cell lines found that SP varied dramatically depending on NB cell line investigated (<1 % to > 50%), and that mitoxantrone, a type II topoisomerase inhibitor, was not effective in targeting the SP population, and in fact increased the SP compartment[135]. Taken together, these findings with NB SP strengthens the idea that NB SP is enriched in TSC or TSC-like cells.

Treatment of NB TSCs

While TICs and SP cells have been isolated from both primary tumors and cell lines of NB, the new focus is to find directed therapies that can target these NB TSCs. As predicted by the TSC theory, NB TSCs appear to be resistant to conventional chemotherapeutics (cisplatin, mitoxantrone) [135, 136]. It was reported using a robotic screening of 500 anti-cancer drugs that rapamycin, a mTOR inhibitor has potent effects against NB TICs while having mild to no effects against SKPs (normal skin isolated stem cells derived from neural crest) [141]. Zhang et al, have also demonstrated that NB tumor initiating cells can be targeted by sunitinib, a multi-targeted receptor tyrosine kinase inhibitor that (in this case) targets VEGF receptors. Furthermore, combinatorial use with rapamycin showed prominent anti-NB TIC effects in vitro and in vivo [142]. Finally, Mahller et al, using methods similar to Hansford et al, have isolated tumor initiating cells from NB cell lines that are sensitive to oncolytic viruses directed against nestin. The nestin directed oncolytic virus was able to reduce tumor formation in-vivo [117].

1.3.5 Clinical implications of tumor stem cells

The theory of TSC has significant implications for clinical cancer therapeutics. To date, the most common treatment for cancer is chemotherapeutics and radiation therapy, which targets highly proliferative cells. However, as TSCs are quiescent, they are likely not targeted by such therapeutics [96] Furthermore, similar to normal stem cells, TSCs have been shown to express drug pumps that impart them with further resistance to conventional therapeutics [143]. Toxicity studies against TSCs of different maligancies have indicated as such [143-146]. TSCs have been theorized and linked to minimal residual disease, in which even after successful therapy, some patients see a recurrence of malignancy [96]. Indeed given TSCs ability to evade therapy, the detection of TSCs may provide a way to combat minimal residual disease. Furthermore, there is accumulating evidence that conventional therapeutics can only target the transit amplifying and differentiated populations of a tumor and are not able to eradicate the TSC compartment and can infact expand it. This explains experimentally why there is decrease in sensitivity to therapy in cancers that have relapsed [136, 144]. Given the properties of TSCs, it is also logical to hypothesize that tumor samples with larger TSC compartments

would lead to greater relapse rates and poorer prognosis [147]. Direct and indirect evidence pertaining to this has been obtained for colon, esophageal squamous cell and breast carcinomas, sarcomas, leukemias, and gliomas. Regardless, given TSCs ability to evade conventional therapeutics, there is a pressing need to develop therapeutic drugs and regimen that can target the TSC compartment. Furthermore, given that a single TSC is theorized to be able to repopulate a tumor, it has been hypothesized that TSCs play a critical role in metastasis. Indeed, it has been demonstrated in vitro that TSC have higher invasive capacities and have greater capacity to home to metastatic sites, and in-vivo to induce more distant metastases [130, 148-150]. In breast cancers, it was found that a tumour with greater percentages of TSCs had a higher propensity to develop bone marrow metastasis while cancer cells that have disseminated to the bone marrow has been found in both breast and neuroblastoma to be more stem cell like [116, 151]. With the identification of TSCs in numerous cancer types completed, there is an increasing need to discover therapeutics against TSCs. Indeed therapies against TSCs have been described for neuroblastoma, leukemias, gliomas, pancreatic and colon carcinomas [142, 152-156]. Therapies can be directed at inducing TSCs into apoptosis, sensitizing them to conventional therapies, or inducing their differentiation. Finally, because TSCs have been noted to develop due to both genetic and epigenetic alterations, HDACi, which can alter epigenetic status, is an intriguing therapeutic option against TSCs that has yet to be explored.

1.4 Rationale

Emerging therapies have yet to make a significant impact on the survival rate of children diagnosed with high-risk NB [1]. Given the observations that tumors with poorer prognosis are populated with more TSCs, and TSCs are linked to progression, metastasis and minimal residual disease, there is a pressing need to discover drugs that can target this population[107-115, 147]. It is becoming evident that TSCs are quiescent, express drug pumps, have potent anti-oxidant pathways, and upregulate many anti-cell death mechanisms[121, 123, 125, 157, 158]. In conjunction, this makes TSCs resistant to conventional therapeutics. In NB, a drug that can target the TSCs to enter either the cell

cycle, apoptosis or differentiation will translate to a decrease in dissemination and recurrence and increased patient survival. While there are already possible drugs that can target NB TSC being tested, additional drugs that may be able to work in conjunction and on different pathways need to be developed.

HDACi have been extensively studied over the last decade, individually or in conjunction with multiple conventional therapeutics. The overall in-vitro and in-vivo evidence suggests that HDACi have the potential to induce cell cycle arrest, apoptosis, senescence, and differentiation, and inhibit angiogenesis and metastasis. Given their relative non-toxicity to normal tissue, they are very intriguing drug candidates for many cancers[80, 159]. While anti-cancer potential of HDACi are well known, their anti-TSC capability is unstudied.

The mechanism of action of HDACi is generally attributed to their ability to modify the epigenome and induce transcription[20]. Given that epigenetic alterations are well documented in both cancers and TSCs, HDACi have been theorized to be able to target both compartments. HDACi ability to induce differentiation, sensitize cancer to conventional therapeutics, modify survival pathways, and preferentially target cancers needs to be tested against TSCs.

In NB, HDACi have been shown to be able to differentiate and sensitize NB to differentiating agents. Furthermore, they have shown anti-NB activity and ability to sensitize NB to conventional therapeutics[35]. In NB, if HDACi can induce Schwannian differentiation and overall maturation of the tumor toward more GNB and GN phenotypes, it could also improve survival[5]. If an HDACi can target both the TSC and non-TSC compartment by targeting survival or differentiation, it would prove to be a powerful therapeutic in NB, as reducing the immature neuroblast population within a tumor could have a significant impact on prognosis.

While there are many HDACi commercially available, in this study we chose to study the anti-NB potential of a synthetic benzamide called MS-275. MS-275 has been very well studied, and there exist a wealth of information on this particular HDACi. Furthermore, it is already in phase I and II clinical trials in both solid and hematopoietic

malignancies[73-77]. Because of its synthetic nature, it has been documented to have a long half-life in serum, increasing its worth as a drug that can be used in-vivo[71]. The mechanism by which MS-275 targets a variety of neoplasms has been well studied. In NB, MS-275 has been studied in-vitro in S- and I- type cell lines and also in pre-clinical mouse xenograft models. The anti-NB activity of MS-275 will be confirmed and further characterized on a previously unused N-type NB cell line model.

Because NB is a relatively rare disease, it is more difficult to obtain primary tumor tissue. Therefore, SP TSC-like cells offer a versatile in-vitro model to study drug effects on NB TSCs. Previously, it has been documented that HDACi can reduce clonogenicity of NB in-vitro, suggesting that HDACi might have potential anti-TSC ability[36, 160]. In this study, the ability of MS-275 to target the SP population will be investigated to further substantiate this. HDACi have previously been noted in their ability to alter the expression of ATP-dependent drug pumps, such as the ones that define SP[161]. It will be therefore necessary to investigate whether MS-275 can target the SP and reduce their self-renewal capacity, or just modulate the expression of the drug pumps. Expression of the drug pump ABCG2, and self renewal proteins OCT-4, Nanog and SOX2 have also previously been correlated to TSCs isolated from within cancer populations [110, 131, 146, 162-165].

To investigate the anti-TSC ability of MS-275, three cell lines will be used. Immature neuroblastic like NB cell-lines SH-SY5Y and SKN-BE(2) and the teratocarcinoma NT2/D1 (N-type subclone of Tera-2). While both SH-SY5Y and SKN-BE(2) are tumourigenic, they differ in that SKN-BE(2) is MYCN amplified. It has been noted that MYCN amplified tumors generally reflect a more aggressive phenotype[1]. Thus it is critical to determine whether MS-275 can target the TSC of both MYCN amplified and non-amplified NB. Teratocarcinoma NT2/D1 is used as reference cell line. Teratocarcinomas, cancers of germinal stem cells, share similarities with neuroblastoma, in that they both reflect cancers of primitive stem cells[95]. Furthermore, NT2/D1 similar to neuroblastoma is multipotent and has been well documented to be selected towards a neuronal subtype [166, 167]. Indeed, NT2/D1 cells have been shown to neuronally differentiate, similar in fashion to NB, in the presence of retinoids[167].

Critically, NT2/D1 cells express high levels of proteins linked with self-renewal, and have been documented to have high self-renewal and tumorigenic potential, as greater than 10% of teratocarcinoma cells from cell lines have been demonstrated to be able to form tumors that differentiate into teratomas consisting of many cell types[95]. Thus, teratocarcinomas are tumorigenic primitive ES-like stem cells that represent a good model to study TSCs.

In summary, because MS-275 and other HDACi have shown anti-cancer potential, and there is some indirect evidence that they can target TSCs, this study will characterize whether MS-275 can target both the heterogeneous transit amplifying population of NB and its TSC compartment. A drug that can target both populations could yield a powerful anti-NB therapeutic.

1.5 Hypothesis

The histone deacetylase inhibitor MS-275 inhibits NB tumor growth by inducing cell death and differentiation and by targeting its TSC-like population.

1.6 Objective/Aims

The objective of this study was to investigate whether MS-275 targets both the TSC and transit amplifying compartments, and to characterize its mechanism of activity.

Aim 1: To characterize the apoptotic, cell cycle arresting and differentiating potential of MS-275 in NB in vitro.

Aim 2: Using SP as a model of NB TSC to determine whether MS-275 has anti-TSC potential in NB and to characterize the mechanism by which MS-275 may target NB TSCs.

Figure 1: A Model of Tumor Stem Cells in NB

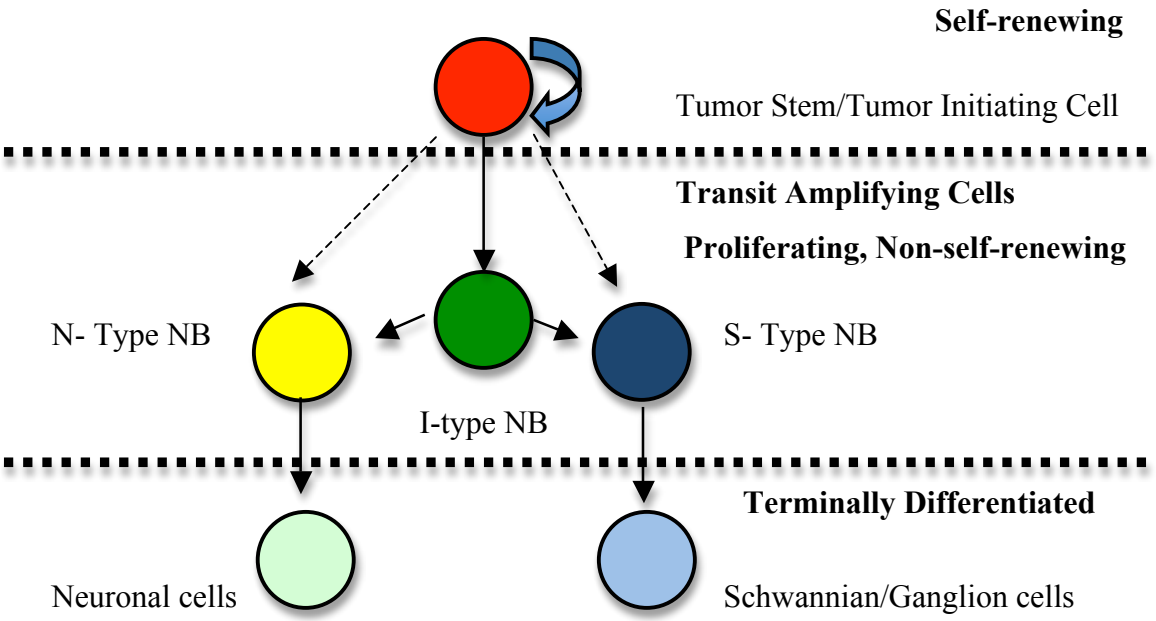


Figure 1: Tumor stem cell model in NB. The purported TSC is highly self-renewing and has the potential to form a tumor by itself. It could be a highly primitive turmorigenic neural crest stem cell, or a differentiated progeny that has aquired self-renewal potential. As this is not well established, arrows are only uni-directional. Its asymmetric division produces transit amplifying progeny which may include I-type cells. I type cells are the purported precursors of both N- and S- type cells, which can differentiate into mature progeny. It is not known whether N and S type necessarily derive from I-type cells and may arise from the TSC directly.

Chapter 2:
HDACi MS-275 induces NB cell cycle arrest, apoptosis and differentiation

2.1 Characterizing the anti-NB potential of HDACi MS-275

2.1.1 Objective

The anti-cancer potential of MS-275 has been extensively reviewed. MS-275 has previously shown potential to inhibit cell cycle progression by inducing histone acetylation to upregulate inhibitors and modulating proteins that ultimately induce hyperphosphorylation of pRB[52, 78-87]. In NB, Fuchert et al., Jaboin et al., and Deubzer et al., have studied the anti-NB ability of MS-275 using in-vitro cell lines. Using MYCN amplified and non-amplified NB cell lines, it was demonstrated that MS-275 can inhibit DNA replication and cell growth by inducing cell cycle arrest and apoptosis. In this study, we aim to first confirm and expand on the previously reported anti-NB potential of MS-275 by characterizing its effects in a N-type NB line not previously used. The N-type cell line SH-SY5Y represents a subset of NB that is MYCN non-amplified, p53 normal and highly metastatic and aggressive. Here we investigated the effects of MS-275 on NB tumor cell growth, apoptosis and differentiation using flow cytometry, in-cell western and western blot analyses.

In this chapter, the histone acetylating ability of MS-275 will be confirmed followed by protein analysis of cell cycle inhibition to characterize the G1 cell cycle arrest previously documented by Jaboin et al. Cell cycle proteins most commonly modulated by HDACi in the literature will be analyzed (i.e. p21). Furthermore, the ability of MS-275 to induce the intrinsic pathway of apoptosis will be investigated.

Finally, the ability of MS-275 to differentiate NB has not been previously studied. SH-SY5Y is the most commonly used model to study the differentiating potential of different drugs in inducing neuronal differentiation of NB[68, 69, 168, 169]. In this study, the potential of MS-275 to induce neuronal and Schwannian differentiation will be explored. This is of importance because it is theorized that the differentiation of NB can reduce minimal residual disease and also improve prognosis[15]. Immature neuroblasts are highly tumourigenic. An agent that can push NB cells toward terminal differentiation or

towards an Schwannian lineage would theoretically have the potential to increase the maturation of NB towards a GNB or GN phenotype[133].

The objective of this section is to determine whether the predominant non TSC like NB population (i.e. the transit amplifying cell fraction) can be targeted by MS-275 into cell cycle arrest, apoptosis and/or differentiation.

2.1.2 Methods

Dose Response:

Cell Culture and Drug Treatments:

N-type NB cell lines SH-SY5Y and SKN-BE(2), and teratocarcinoma NT2/D1 were employed in this study. Cells were cultured in 10% fetal bovine serum, 1% penicillin/streptomycin (Multicell #450-201-EL) in DMEM/F12 (Multicell #319-075-CC) , AMEM (Multicell #310-022-CL) and DMEM (Multicell #319-005-CL) media for SH-SY5Y, SKN-BE(2) and NT2/D1 respectively at 37°C in a humidified atmosphere of 5% CO_2. Cells were grown on adherent T-75 cm^2 flasks and passaged 1to 4 when confluent. For dose response assays cells were grown in adherent 96, 48 or 24 well plates and treated with MS-275 when confluency reached 80%. MS-275 (Sigma #M5568) was solubilized in DMSO and stored in -20°C. Cells were treated for 48 hours at different doses ranging from 10 nM to 20 uM.

Alamar Blue Metabolic Assay:

The Alamar blue assay measures metabolic activity of cell populations. Total metabolic activity has been correlated to cell population and as such this assay is used as a rapid screen of cytotoxicity. Cells were grown in adherent 48 flat bottom well plates and treated with MS-275 at ranges of 10 nM to 20 uM in triplicates for 48 hrs. Following treatment, media is aspirated from wells and replaced with a 10% solution of Alamar blue reagent (ADB Serotec #BUF012B) diluted in culture media. Metabolic reaction was allowed to occur for 4 hours. Plates are then read on a Molecular Devices SpectraMax Gemini fluorescent plate reader at an excitation of 560 nm and emission of 590 nm.

Relative fluorescent units (RFU) were plotted in GraphPad Prism 5 software to extrapolate a dose response curve.

Trypan Blue Exclusion Assay:

Trypan blue dye is taken up by cells with compromised membrane integrity, and as such allows the exclusion of non-viable cells during counting assays. Cells were grown in adherent 24 flat bottom well plates and treated with MS-275 at ranges of 100 nM to 10 uM in triplicates for 48 hrs. Following treatment, cells were washed and trypsinized (0.05% Trypsin Solution; Multicell #325-042-EL) from individual wells, resuspended and treated with a 1:1 solution of trypan blue (0.4% solution; Gibco #15250-061). Cells were then counted under a hemocytometer at 100X magnification on a phase contrast microscope. Total cell counts were plotted in GraphPad Prism 5 software to extrapolate a dose response curve.

DRAQ5/Sapphire700 DNA binding Assay:

Sapphire700 is a non-specific cell stain that accumulates in both the nucleus and cytoplasm while DRAQ5 is a DNA binding dye that allows for stoichiometric analysis of the DNA content. Used in concert, they allow for rapid and quick analysis of cell viability. Cells were grown in adherent 96 flat bottom well plates and treated with MS-275 at ranges of 10 nM to 20 uM in triplicates for 48 hrs. Following treatment wells were washed twice with phosphate buffered saline (PBS) and fixed with a 4% solution of paraformaldehyde (Canemco #0173) for 20 minutes. Cells were then treated with a solution of 1mM DRAQ5 (BioStatus #DR50050) and 1/1000 dilution of Sapphire700 Reagent (Licor #928-40022) and read on the Odyssey Infrared Imaging System at an excitation channel setting of 700 nm. Total fluorescence was quantified also on the Odyssey system and plotted in GraphPad Prism 5 software to extrapolate a dose response curve.

<u>In-cell Western Assay:</u>

In-cell western assays were used to quanity expression of proteins involved in cell cycle arrest, apoptosis and differentiation. Cells were grown in adherent 96 flat bottom well

plates and treated with MS-275 at 1.5 uM. Following treatment, cells were washed twice with PBS and fixed with a 4% solution of paraformaldehyde for 20 minutes. Cells were then treated with a 0.1% Triton-X 100 (Sigma #T9284) detergent solution for 5 minutes to permeabilize cells and washed twice with PBS. Subsequently cells were blocked in 1X Odyssey blocking buffer (Licor #927-40000) for 2 hours at room temperature (RT). Primary antibodies against p16 (Santa Cruz #sc-1661; 1/50), p21 (Santa Cruz #sc-6246; 1/20), p27 (Santa Cruz #sc-528; 1/20), BCL-2 (Cell Signaling #2872; 1/100), cyclin D1 (Neomarkers #RM-9104; 1/10), CDK4 (Neomarkers #MS-299; 1/100), acetylated H3 (Upstate #06-599; 1/200) and H4 (Upstate #06-598; 1/200), pan-neurofilament (pan-NF; Sternberger #SMI-311; 1/200), S100beta (DakoCytomation #Z0311; 1/200), and GFAP (DakoCytomation #N1506; 1/100) were diluted in Odyssey blocking buffer at indicated ratios and added to cells overnight at 4^{o}C. Pan-actin (Cedarlane #CLT9001; 1/100) antibody was also added in conjunction with the other antibodies to serve as a control of cell content. Cell were then washed with a 0.1% Tween 20 (Fisher #BF-337-500) solution for 5 minutes and repeated 5 times. Fluorescently labeled LICOR secondary antibody (Goat-anti-Rabbit IRDye 680, Licor #926-32221; Goat-anti-Mouse IRDye 800, Licor #926-32210) solutions were then added at a dilution of 1/500 and treated for 1 hour at RT. In wells where actin was not used as a cell content control, DRAQ5/Sapphire700 was added at 1mM and 1/1000 dilution respectively. Cells were then again washed with 0.1% Tween solution five times for 5 minutes. Cells were then imaged on the Odyssey Infrared Imaging System at excitation channel settings of 700 nm and 800 nm. Total fluorescence was quantified and adjusted to cell content control of either actin or DRAQ5/Sapphire700. Relative fluorescent numbers were ploted in GraphPad Prism 5 software.

Western Blot Assay:

Western blot assays were used to confirm in-cell western analyses. Cells were treated for 48 hrs with 0.75 or 1.5 uM MS-275 in T75 cm^2 adherent flasks. Subsequently, cells were washed 3X with cold PBS and lysed with RIPA extraction buffer (MBiotech #21308) supplemented with a CompleteMini protease inhibitor tablet (Roche #11836153001) at 100 uL per 5X10^6 cells. Samples were then further lyzed by freeze thawing 3 times.

Protein quantification was done with bicinchoninic acid protein quantification assay (Pierce #23227). Protein samples were then denatured by boiling in a 1X loading buffer supplemented with beta-mercaptoethanol and SDS. 50 ug of protein was loaded onto a 10% acrylamide (30% solution; Biorad #1610156) gel and ran at 100 mV. Protein was then transferred overnight at 20 mV at 4^{o}C to a nitrocellulose membrane (Biorad #162-0115). The membrane was then washed with tris buffered saline (TBS) and blocked with a 5% skim milk (Carnation) TBS solution supplemented with 0.1% Tween for 1 hour at RT. Primary antibodies of p21 (1/100), p27 (1/100), Ki67 (1/1000), Cyclin D1 (1/100), CDK4 (1/500), BCL-2 (1/1000), BAX (1/1000), survivin (Cell Signaling #2802; 1/1000), pan-NF (1/1000), S100beta (1/1000), nestin (Chemicon #MAB5326) were diltuted in 5% skim milk TBST at the indicated ratios overnight at 4^{o}C. Membranes were then washed with a 0.1% TBST 5 times for 5 minutes. Secondary horseradish peroxidase antibodies (Jackson ImmunoResearch) diluted 1/6000 in 5% skim milk TBST solution were subsequently added for 1hr at RT. Following a 5X TBST wash, signal was detected by a chemiluminescence detection system (SuperSignal; Pierce #34080) on film (Amersham #68902839). To compare quantity of expression for each protein band, band density was measured by densitometry (FluroChem FC2) and relative IDV values were determined.

Propidium Iodide Cell Cycle Analysis:

Propidium Iodide stoichiometrically binds DNA and allows for analysis of cell cycle phase by relative DNA content. Cell cycle analysis protocol was adapted from the Hospital for Sick Children flow cytometry protocol. Briefly, 2 X 10^6 cells treated with MS-275 were lifted by citrate saline and fixed in an 80% solution of ice cold ethanol for 48 hours. Cells were then pelleted and resuspended in a 2mg/mL RNase A (Sigma 6514) for 5 minutes. A 0.1 mg/mL propidium iodide solution (Sigma #P-4170) was subsequently added. After 30 minutes of incubation at RT, cells were then filtered through a cell-strainer into a 5 mL polystyrene tube and analyzed on a BD FACSCAN flow cytometer. DNA content analysis was analyzed on Flowjo software and fitted by the Watson-pragmatic model to determine cell cycle phase. Only viable, non sub-G1 and non-doublets were gated for cell cycle analysis. For sub-G1 apoptosis analysis, all cells (except for doublets) were included in gating.

AnnexinV-PE/7-Actinomysin D Apoptosis Assay:

AnnexinV-PE/7-AAD assay was used to determine apoptosis. During early stages of apoptosis, phosphotidyl serine of cellular membranes flips extracellularly. AnnexinV specifically binds phophotidyl serine. 7 actinomysin D is a membrane impermeant DNA binding dye that is used to determine cell viability. Together, they allow for the identification of cells in early and late apoptosis. BD AnnexinV-PE/7-Actinomysin D (7-AAD) kit (BD Pharmingen #559763) was used. Briefly, cells were lifted from adherence by a citrate saline solution and washed twice in PBS. 1 X 10^5 cells were resuspended in annexinV binding buffer and 5 uL of annexinV-PE and 7-AAD for 15 minutes at RT and analyzed on BD FATSCAN flow cytometer. Gating was determined by an etoposide (Sigma #E1383) positive control.

Flow Cytometric Analysis of Cleaved-Caspase 3:

Cleavage activation of of caspase 3 is one of the end stage events in the intrinsic pathway of apoptosis. This assay measures the expression specifically of the cleaved variant of caspase 3. Cells treated with MS-275 were removed with citrate saline. Subsequently, 1 X 10^6 cells were washed and fixed in 4% paraformaldehyde for 20 minutes. Cells were washed and blocked with 5% bovine serum albumim (Multicell #800-095-EG) in PBS for 1 hour at RT. Finally cells were stained with a cleaved caspase 3 antibody conjugated to Alexa Fluor 488 (Cell Signaling #9669; 1/10 dilution) for 45 minutes, and washed 5 times with PBS. Cells were then analyzed on BD FASCAN flow cytometer. Gating was determined with unstained cells.

Statistics

All statistics were analyzed on Graphpad Prism 5 software. Unpaired (student's) T-test was used to analyze statistical significance. Significance was set at $p \leq 0.05$. Asterics, *, ** and *** are used to denote significance of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively.

2.1.3 Results

Establishing the dose response effect of MS-275 in NB cell lines

The 48 hour dose dependent response of the cell lines employed in this study to HDACi MS-275 was established and confirmed with 3 separate methods: Alamar blue metabolic assay, trypan blue exclusion assay and DRAQ5/Sapphire700 DNA quantification. Cells were treated for 48 hrs and the LD50 (lethal dose giving 50% reduction in cell numbers) was determined to be within the 1-2 uM dose range for MS-275 in NB SH-SY5Y and SKN-BE(2) and teratocarcinoma NT2/D1 (Figure 2A, B, C). Data is summarized in the table 1D. The LD50 will hence be set at 1.5 uM. LD20 was also determined to be approximately 0.75 uM in NB cell lines. The LD50 is physiologically relevant as it corresponds to the low range used in clinical trials against solid malignancies[170].

MS-275 induces Histone Acetylation

MS-275 induction of histone acetylation was analyzed by measuring expression of acetylated histone H3 and H4. Histone tails of histone H3 and H4 are most commonly modified and relevant to epigenetic modulation, and their acetylation serves as a good representation of the acetylation potential of an HDACi[18]. Expression of acetylated histone H3 and H4 was measured by in-cell western assay and confirmed by western blotting. Following 1.5 uM MS-275 treatment in NB SH-SY5Y, acetylated H3 and H4 relative fluorescence significantly increased nearly two fold and 1.5 fold compared to control with $p = 0.0020$ and 0.0033 respectively (Figure 3A). Western blot analysis of MS-275 treatments at 0.75 and 1.5 uM confirmed the increase in acetylated H3 and H4 expression (Figure 3B). Western blot analysis were only performed once as a check of in-cell western data, as experiments could not be repeated because of lack of drug reagent. Acetylated H3 and H4 were also measured in reference to cell line NT2/D1 with in-cell western analysis. Following 1.5 uM MS-275 treatment acetylated H3 and H4 expression significantly increased nearly 3.5 and two fold respectively ($p < 0.0001$ and $p = 0.0028$ respectively) (Figure 3C).

MS-275 induces cell cycle arrest in NB by modulating proteins associated with pRB cell cycle regulation

The ability of the LD50 dose of 1.5 uM MS-275 to induce cell cycle arrest in N-Type NB SH-SY5Y was assayed with propidium iodide cell cycle FACS analysis. DNA quantification by PI was mapped and fitted according to the Watson Pragmatic model

(Figure 4A). Following MS-275 treatment, cells with DNA content indicating that they are in G1 increased significantly from approximately 40 to 70% ($p = 0.0003$), while cells in S phase decreased from 45 to 20 % ($p=0.0001$). Percentage of cells within G2/M did not change significantly (Figure 4B). Results are summarized in Table 4C. This suggests that MS-275 is inducing a G1 cell cycle block, preventing entry into S-phase.

G1 cell cycle arrest is most commonly associated with alterations to the cyclinD/CDK cell cycle progression complexes [171]. In the case of HDACi, p21, p27 and p16 are the most commonly associated cell cycle inhibitors that are upregulated[172]. Thus, cyclinD1, CDK4, p21, p27, p16 and the Ki67 proliferative indicator were analyzed by in-cell western and confirmed by western blot. Western blots were only performed once as a check of in-cell western results because of the limitation of MS-275 reagent. Following treatment with 1.5 uM MS-275, Cyclin D1 and CDK4 were found to be significantly downregulated by approximately 30% of control ($p = 0.0006$ and $p = 0.0020$ respectively) while p16 cell cycle inhibitor was found to be upregulated 2.5 fold as compared to untreated control ($p = 0.0002$). p21 and p27, cell cycle inhibitors most commonly associated with HDACi were not altered significantly in their expression (Figure 4D). These results were confirmed by western blot analysis (Figure 4E). Furthermore, western blot analysis of the expression of the Ki67 proliferative marker showed a down regulation of Ki67(Figure 4E). Ki67 is expressed at all points during the cell cycle, except in G0.

MS-275 induces the intrinsic apoptosis pathway

The potential of MS-275 to induce apoptosis was investigated by first determining the percentage of sub-G1 cells in a PI cell cycle analysis that was data-fitted by the Watson-Pragmatic model on the flow cytometry analysis program Flowjo. Cells treated for 48 hrs at 0.75 and 1.5 uM MS-275 were found to have a significant increase in the population of cells that are sub-G1. Following treatment with the lower dose an increase in cells in sub-G1 was detected from a baseline value (untreated cells) of 5.5 to 9.3% ($p = 0.0187$). Cells treated with the higher 1.5 uM dose had an increase from 5.5 to 28.1% ($p = 0.0066$). The difference between 0.75 uM and 1.5 uM MS-275 treatment was significantly different ($p = 0.0132$) (Figure 5A).

While the sub-G1 population analysis from propidium iodide stained populations allow for rapid analysis of cell death, it may not be specific for apoptosis. Furthermore the number is usually skewed, as the sub-G1 population would also account for cells that were mechanically damaged. Therefore, Annexin/7-AAD and cleaved-caspase 3 expressions were also analyzed by FACs to assess apoptosis. Annexin binds phosphatidyl serine in the plasma membrane, which is flipped to the extracellular side in the early stage of apoptosis, while 7-AAD stains for cells that have lost membrane integrity. Single staining of annexin would identify cells in the early stage of apoptosis, while single staining of 7-AAD would identify cells that have lost membrane integrity, and double staining of both annexin and 7-AAD would identify cells in end stage apoptosis. This FACs analysis demonstrated that MS-275 indeed is able to induce cells into early (10.7%) and late stage apoptosis (7.66%). This was compared to a 1 uM dose of etoposide (the LD50 dose) that induces 2.51% and 8.58% early and late stage apoptosis respectively when assayed in parallel (Figure 5B). This data is summarized in Table 5C.

Caspase 3 is cleaved near the end of the intrinsic pathway of apoptosis and thus serves as an indicator of apoptosis. After 48 hour treatment with 0.75 and 1.5 uM MS-275, 1% and 3% respectively of total cells were found to express cleaved caspase 3 ($p = 0.0003$ and $p < 0.0001$ respectively as compared to control and $p = 0.0001$ when comparing doses)(figure 5E). Representative FACs profiles are shown in Figure 5D. Furthermore, analysis of BCL2 and BAX expression by western blot revealed that MS-275 can reduce the BCL2/BAX ratio (15% of control ratio), which is indicative of a potent apoptotic induction in cells (Figure 5G). This was due to the increase in expression of apoptotic protein BAX since western blot analysis showed that the expression of anti-apoptotic protein BCL2 was unchanged following MS-275 treatment (Figure 5F). This result differed from in-cell western analysis that demonstrated MS-275 reduced BCL2 expression by greater than 60% ($p = 0.0035$)(Figure 4D). The discrepancy in the methods of analysis might be due to the fact that in-cell western analysis depends on antignen presentation and access. This will be further discussed in the following section. Western blot analysis also demonstrated that survivin, a potent inhibitor of apoptosis,

previously shown to be upregulated in NB, is reduced in expression following MS-275 treatment (Figure 5F).

MS-275 may induce neuronal and Schwannian differentiation in NB

MS-275, like other HDACi, is known to be able to induce differentiation of tumor cells[87]. Using in-cell western and western blot analysis, we assessed the potential of a 48 hr treatment with 1.5 uM MS-275 to differentiate N-type NB SH-SY5Y. Similarly, we assessed the potential of MS-275 to differentiate NT2/D1 cells, since NT2/D1has been shown to be sensitive to differentiating agents.

Phase microscopy of N-type NB cell lines dosed with MS-275 showed an increase in cell cytoplasm, and a loss of neurites (Figure 6A, arrows). Neurites commonly associated with N-type SH-SY5Y disappeared following treatment, replaced with filapodia-like processes. This was similarly seen in SKN-BE(2). Cell size nearly increased by 1.5 fold. Increased adherence to substrate was also noted. Phase microscopy of NT2/D1 similarly showed an increase in cell size, but also a development of neuritic like processes (Figure 6A arrows). These morphological observations demonstrate the need to explore the neuronal and Schwannian differentiation potential of MS-275.

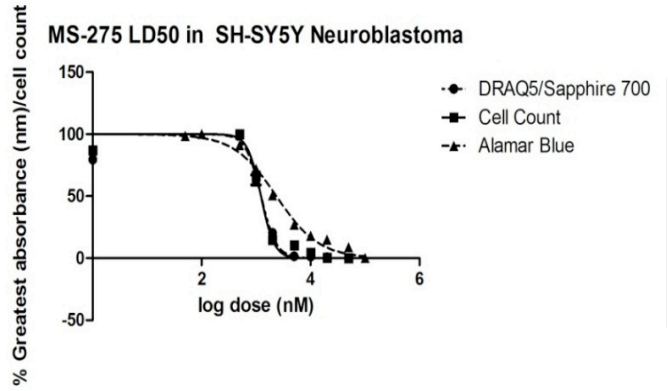
In-cell western assays demonstrated that a 48 hr treatment with 1.5 uM MS-275 was able to increase the overall expression of all three neurofilament (NF68, NF150, NF200) isoforms by 1.5 fold ($p = 0.0002$), whereas Schwannian and glial markers S100beta and GFAP were not significantly altered (Figure 6B). To further characterize the neuronal differentiation, western blot analysis was performed. NF68 constitutively expressed by SH-SY5Y, was increased with MS-275 treatment. Interestingly, while the more mature isoform NF150 was expressed in SH-SY5Y and expression increasedwith 0.75 uM MS-275, at the higher 1.5 uM dose, it was not increased. In contrast, NF200, a marker of neuronal maturity, did not increase at the lower 0.75 uM dose but was highly expressed at the higher dose of 1.5 uM MS-275. This suggested that MS-275 works dose dependently in regulating NF expression. Contrary to in-cell western analysis, S100beta expression was also detected to increase. This may indicate Schwannian differentiation. Surprisingly, nestin, a primitive neural marker, which has previously been reported to be

expressed at low levels in NB and in neural crest stem cells [134, 173] was shown to be upregulated by 0.75 and 1.5 uM MS-275 (Figure 6D). In NT2/D1, a teratocarcinoma line that is selected for its potential to differentiate neuronally, 1.5 uM MS-275 reduced overall expression of NF by 40% ($p = 0.0122$)(Figure 6C). Upon western blot analysis, the decrease appears to be due to a downregulation of NF68 and NF150. However, NF200 was upregulated, perhaps indicating maturation of cells, as expression of NF200 indicates functional maturity of neurites[174]. Nestin and S100beta expressions in NT2/D1 was also decreased following MS-275 treatment (Figure 6D). The implications of these findings will be discussed below in the discussions.

Figures

Figure 2: MS-275 Dose Response of NB and Teratocarcinoma cell lines

A



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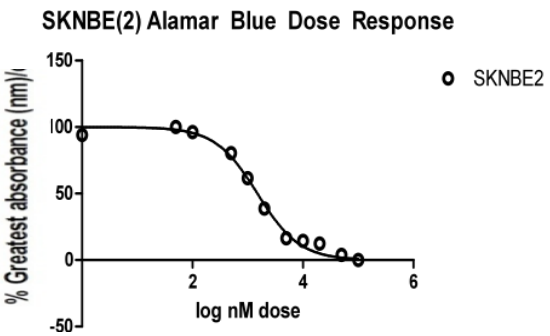
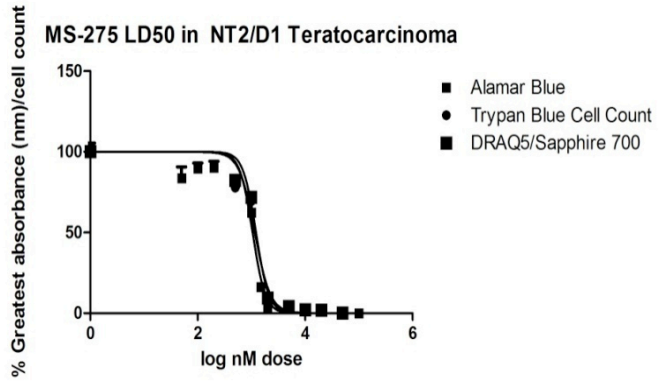


C



D

	LD50		
Cell line	**Alamar Blue**	**DRAQ5/ Sapphire-700**	**Trypan Blue Cell Count**
SH-SY5Y	2.362 uM	1.236 uM	1.227 uM
SKN-BE(2)	1.497 uM	N/A	N/A
NT2/D1	1.05 uM	1.215 uM	1.156 uM

Figure 2: 48 hour MS-275 Dose response against NB and teratocarcinoma reduced cell vialility as analyzed by Alamar blue metabolic assay, trypan blue exclusion assay and Draq5/sapphire700 DNA binding assay. **A)** Survival plots for SH-SY5Y, **B)** SKN-BE(2) and **C)** NT2/D1 were plotted as log nM MS-275 against greatest absorbance/fluorescence/cell count. NB line SKN-BE(2) was analyzed singly with Alamar blue assay because it was found it had a similar dose response as NB cell line SH-SY5Y. The LD50 was calculated to be 1.5 uM and LD20 was 0.75 uM. **D)** Summary of LD50 dose for all three cell lines using the different methods. Experiments were repeated in triplicates.

Figure 3: MS-275 Induces Histone H3 & H4 acetylation

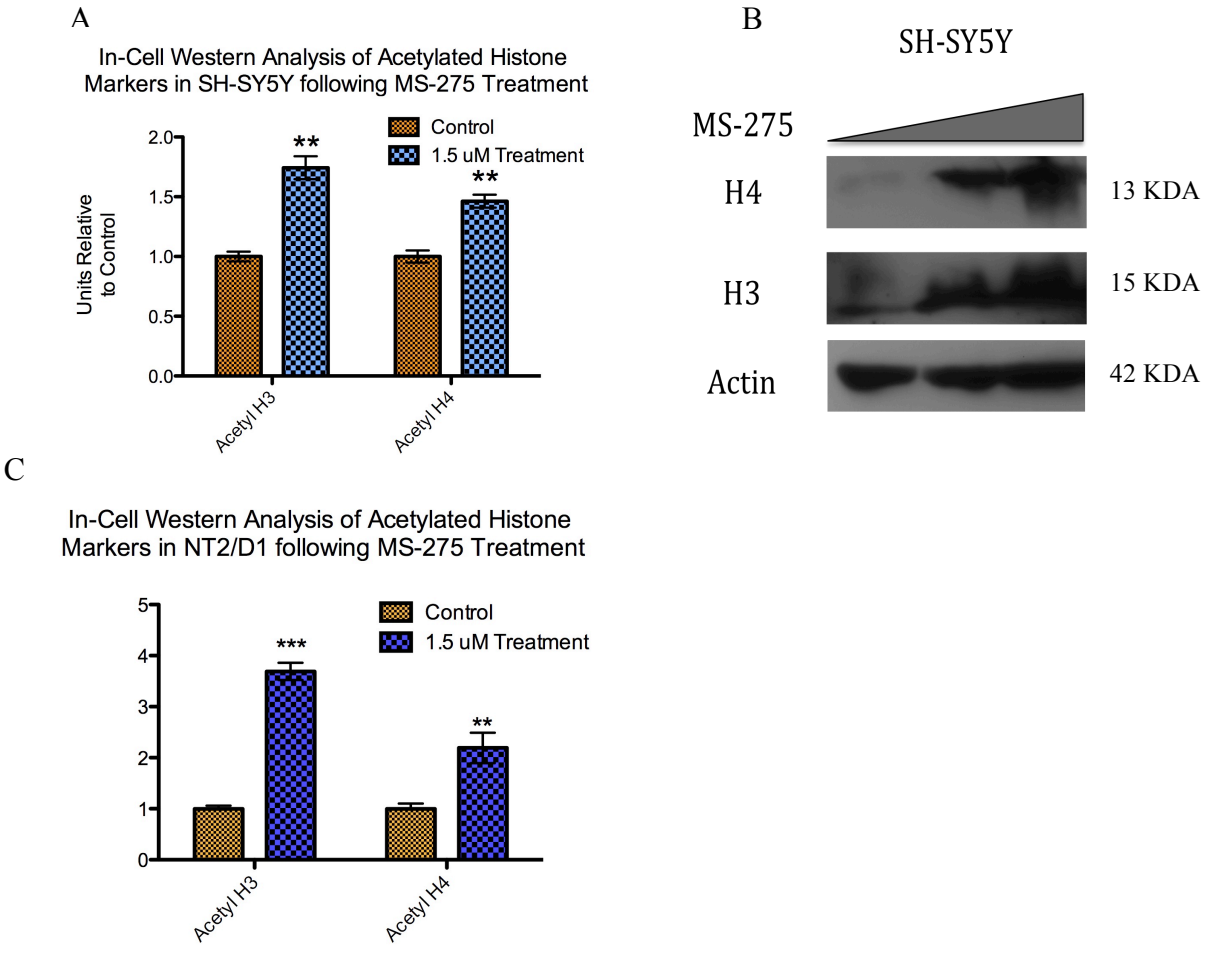
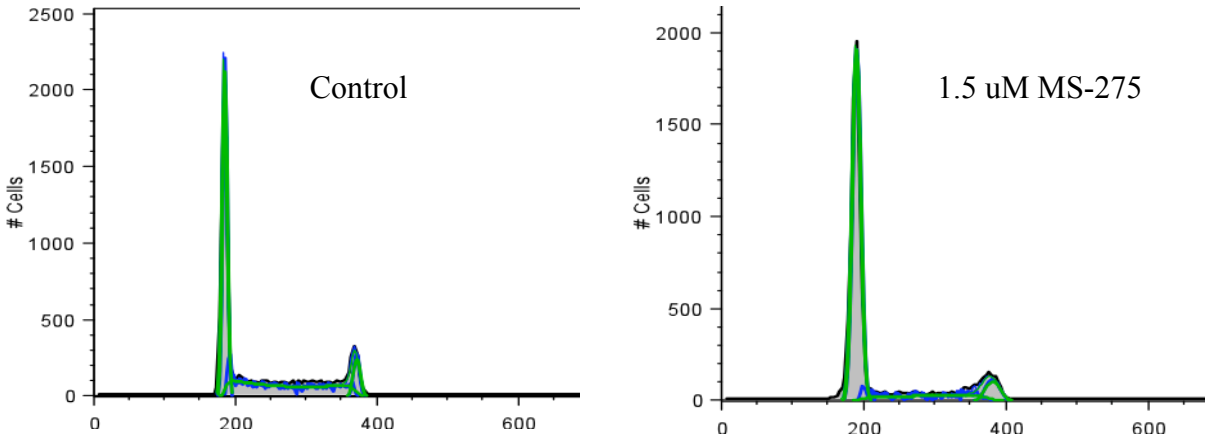


Figure 3: Histone H3 and H4 acetylation expression was increased following MS-275 treatment. **A)** Relative fluorescence expression compared to control of acetylated H3 and H4 expression increased significantly following 1.5 uM MS-275 treatment by a multiple of 1.74±0.096 1.46±0.054 respectively ($p = 0.0020$ and 0.0033 respectively). **B)** Increase in expression of acetylated H3 and H4 following treatment of MS-275 at 0.75 uM and 1.5 uM is confirmed by western blot with the same antibodies used in figure 2A. **C)** Relative fluorescence expression compared to control of acetylated H3 and H4 expression increased significantly following 1.5 uM MS-275 by a multiple of 3.691±0.097 and 2.193±0.172 respectively ($p < 0.0001$ and $p = 0.0028$ respectively). In-cell western analaysis were repeated in triplicates and unpaired 2-tailed t-tests were performed. Astericks, *, ** and *** are used to denote significance of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively. Western blots were performed only once as a check of validity of in-cell western data.

Figure 4: MS-275 induces G1 cell cycle arrest in NB SH-SY5Y by inhibiting cyclinD1/CDK4 complex and inducing p16 cell cycle inhibitor.

A



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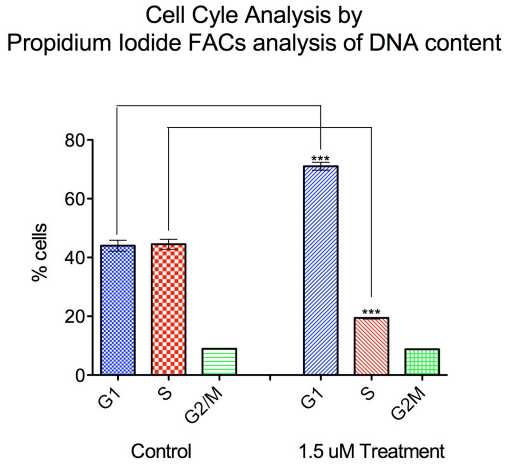
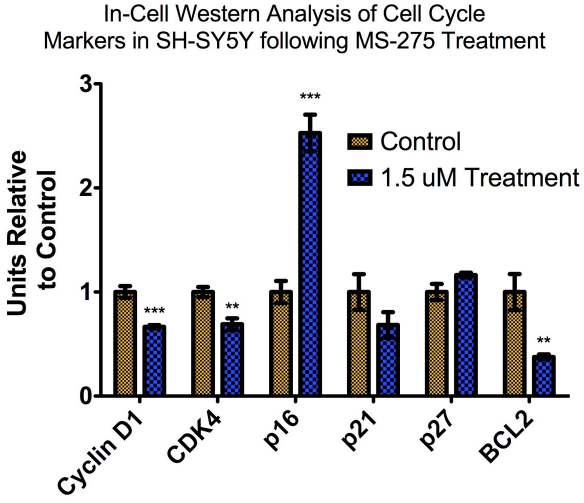


C

	%G0/G1	%S	&G2/M
Control	44.03±1.855	44.5±1.680	8.94±0.041
1.5uM MS-275	71.03±1.328	19.43±3.353	8.79±0.073

D



E

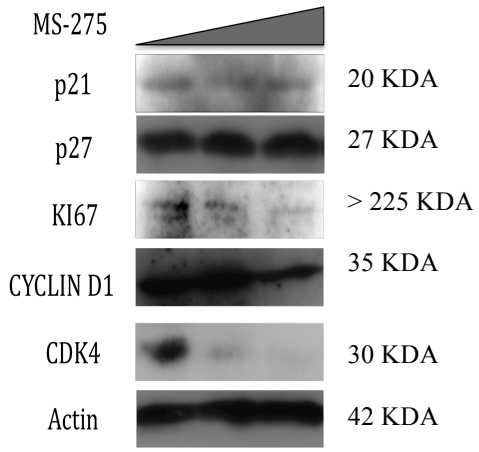
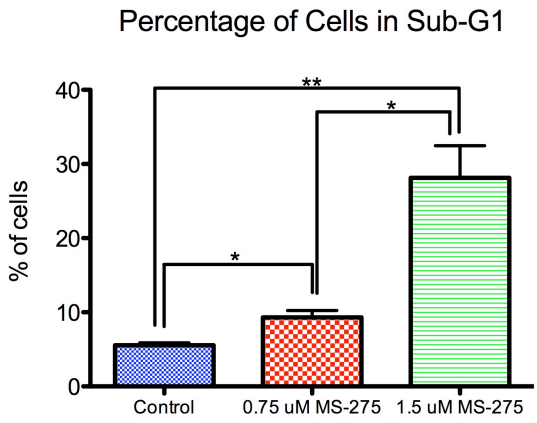


Figure 4: MS-275 induced G1 cell cycle arrest. SH-SY5Y cell cycle analysis was analyzed by propidum iodide DNA staining using flow cytometry. Measured DNA content of cells was fitted according to the Watson-pragmatic model by Flowjo. **A)** Representative DNA content profile of control and cells treated with 1.5 uM MS-275. **B)** 48 hour treatment of 1.5 uM MS-275 induces a G1 cell cycle block which corresponds with a increase in cells in G1 from 44.03±1.855% to 71.03±1.328% ($p = 0.0003$) and decrease in cells entering S phase from 44.5±1.680% to 19.43±3.353% ($p = 0.0001$). G2/M content was not significantly altered. **C)** Table summarizing changes in cell cycle profile. **D)** In-cell western comparing relative fluorescence compared to control value demonstrating that the G1 block predicted by cell cycle profile corresponds to a significant decrease in proteins of the cyclinD/CDK4 complex (0.666±0.010% and 0.690±0.033% of control respectively)($p = 0.0006$ and 0.0020 respectively) and increase in cell cycle inhibitor p16 by a multiple of 2.528±0.101 ($p = 0.0002$). p21 and p27, cell cycle inhibitors most commonly associated with HDACi regulation of cancer were not significantly induced. Anti-apoptotic protein BCL2, which is normally upregulated in NB SH-SY5Y was significantly reduced to 0.376±0.014% of control ($p = 0.0035$). **E)** Western blot confirmation of in-cell western data using lysates from cells treated 48 hours with 0.75 and 1.5 uM MS-275. p21 and p27 showed constant levels following treatment whereas cyclin D1 and CDK4 were reduced in expression. Expression of Ki67 proliferative marker decreased as well. In contrast to in-cell western data, BCL-2 expression was unaffected with treatment. p16 expression could not be confirmed by western blot because antibody used works only with immunofluorescence. In-cell western analaysis were repeated in triplicates and unpaired 2-tailed t-tests were performed. Astericks, *, ** and *** are used to denote significance of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively. Western blots were performed only once as a check of validity of in-cell western data.

Figure 5: SH-SY5Y N-type NB cell line is induced into the intrinsic pathway of apoptosis by MS-275 treatment.

A



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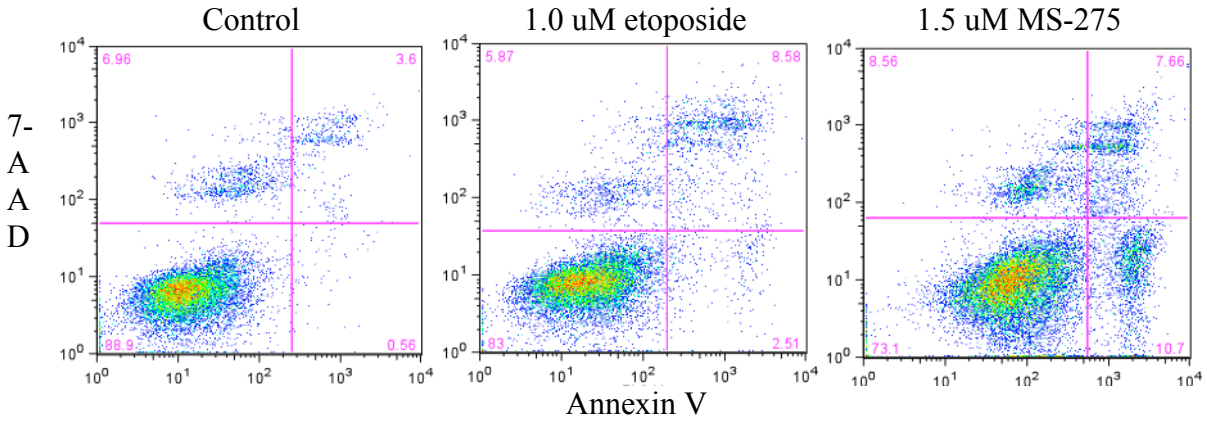
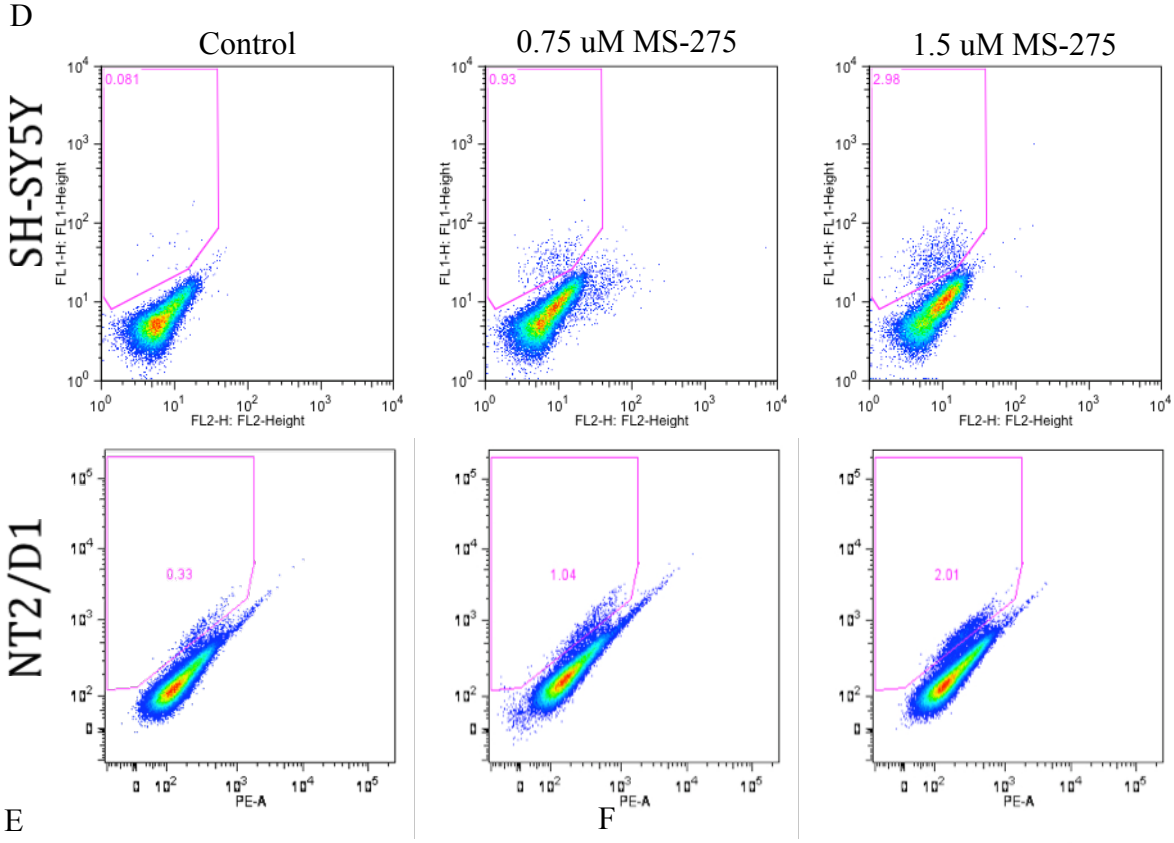


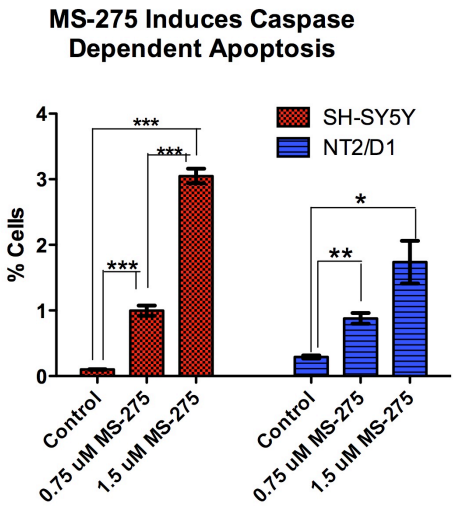
C

	Unstained (Lower left)	Annexin (Lower Right)	7-AAD (Upper Left)	Annexin -7AAD (Upper Right)
Control	88.9	0.56	6.96	3.6
1mM Etoposide	83	2.51	5.87	8.58
1uM MS-275	73.1	10.7	8.56	7.66

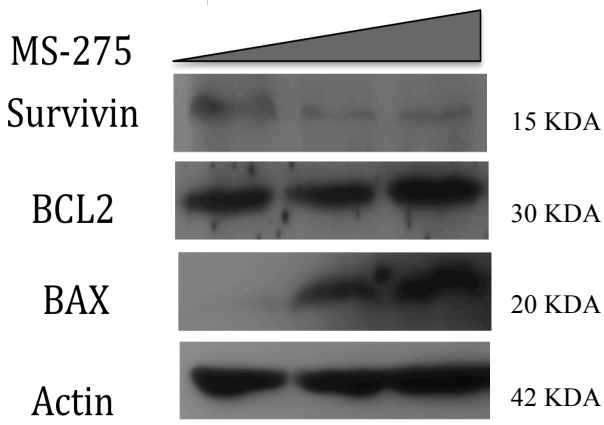
D


Control
0.75 uM MS-275
1.5 uM MS-275
SH-SY5Y
NT2/D1
0.081
0.93
2.98
0.33
1.04
2.01
FL1-H: FL1-Height
FL2-H: FL2-Height
PE-A

E


MS-275 Induces Caspase Dependent Apoptosis
SH-SY5Y
NT2/D1
% Cells
Control
0.75 uM MS-275
1.5 uM MS-275

F


MS-275
Survivin
15 KDA
BCL2
30 KDA
BAX
20 KDA
Actin
42 KDA

G

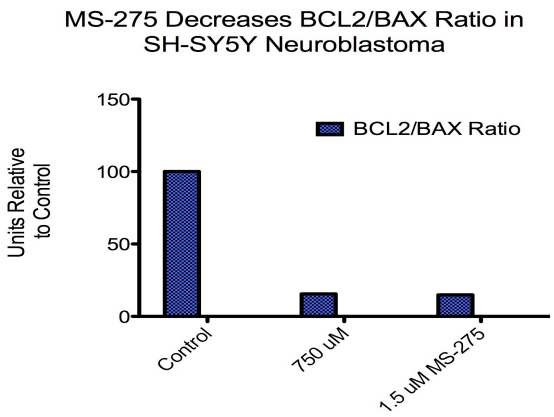
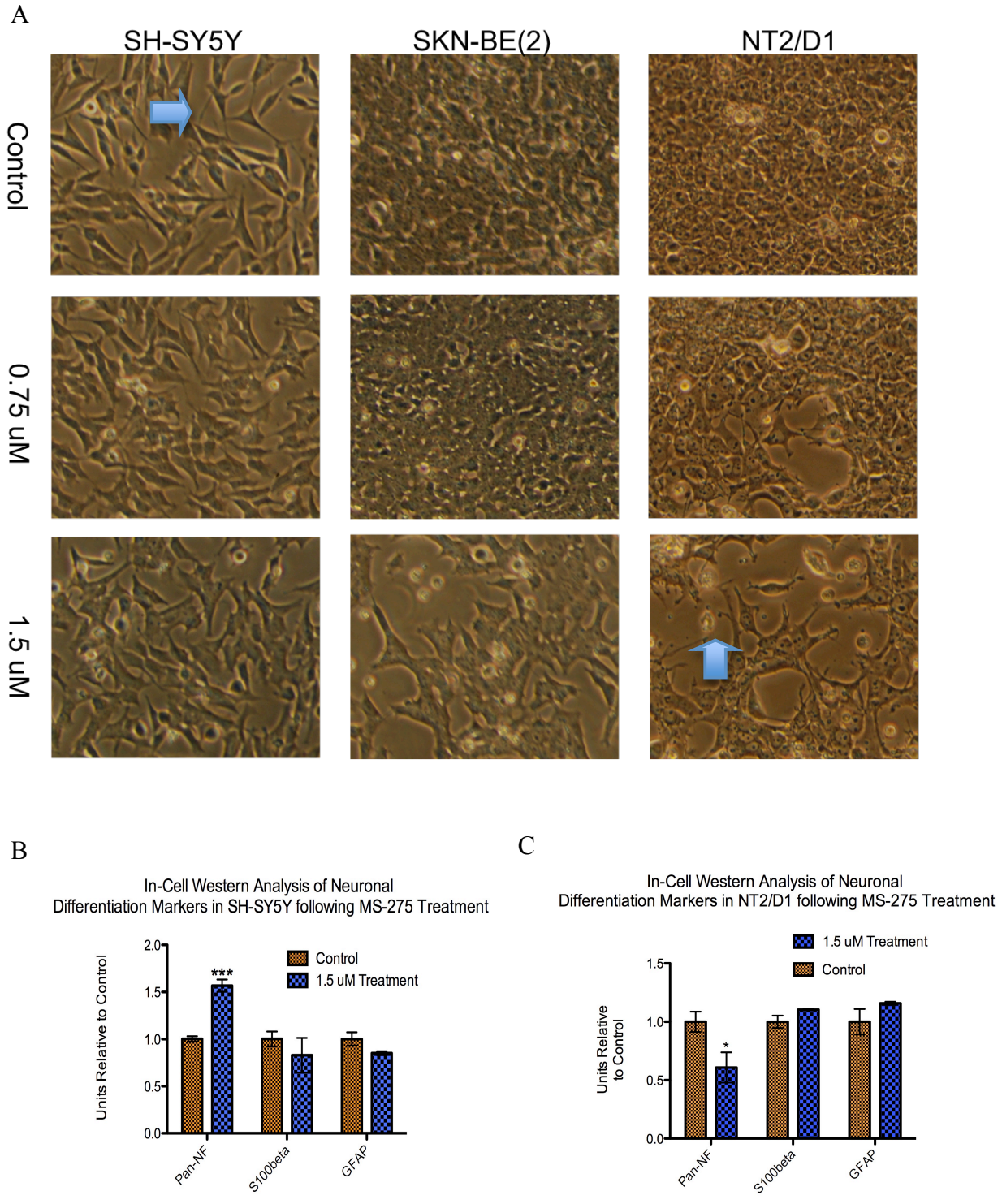

MS-275 Decreases BCL2/BAX Ratio in SH-SY5Y Neuroblastoma
BCL2/BAX Ratio
Units Relative to Control
Control
750 uM
1.5 uM MS-275

Figure 5: MS-275 induces apoptosis through the intrinsic pathway. **A)** sub-G1 population increases significantly following 0.75 uM and 1.5 uM MS-275 treatment from a control percentage of 5.540±0.3365 to 9.303±0.9238 ($p = 0.0187$) and 28.13±4.339 ($p = 0.0066$) respectively. The change in sub-G1 population of the low dose to the high dose is significant of each other as well ($p = 0.0132$). **B)** AnnexinV/7-AAD analysis demonstrates that MS-275 is a potent inducer of apoptosis as percentage of cells in early apoptosis (stained with annexin) increase from a control value of 0.56% to 10.7%, as compared to a comparable dose of etoposide (2.51%). Cells in endstage apoptosis increased from control of 3.6% to 7.66% with treatment of MS-275. This is as compared to 8.58% with comparable dose of etoposide. **C)** table summarizing data from figure B. **D)** Representative FACs profile of cleaved caspase 3 staining for SH-SY5Y and NT2/D1. E) FACs analysis of cleaved caspase 3 expression shows that MS-275 significantly increased the percentage of cells expressing the functional end stage variant of caspase 3 from control of 0.101±0.005% to 0.997±0.077% ($p = 0.0003$) and 3.050±0.112% ($p < 0.0001$) with 0.75 uM and 1.5 uM doses respectively. The difference between the low and high dose was significant of each other ($p = 0.0001$). This was observed in NT2/D1 was well with 0.293±0.023 percentage of control cells expressing cleaved caspase 3 in control. This percentage significantly increased to 0.880±0.081 ($p = 0.0022$) and 1.737±0.325 ($p = 0.0114$) with 0.75 and 1.5 uM MS-275 treatment. **E)** Western blot analysis of proteins involved in the intrinsic pathway of apotosis. BCL2 expression did not change, while BAX was upregulated and survivin expression was decreased. **F)** BCL2/BAX ratio was calculated from densitometry values obtained from western blot analysis in figure 5E. BCL2/BAX ratio, an indicator of apoptosis, decreased following 0.75 and 1.5 uM MS-275 treatment to 15.59 and 14.86% of control. In-cell western analaysis were repeated in triplicates and unpaired 2-tailed t-tests were performed. Astericks, *, ** and *** are used to denote significance of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively. Western blots were performed only once as a check ofvalidity of in-cell western data.

Figure 6: MS-275 can induce neuronal/Schwannian differentiation in NB and teratocarcinoma



D

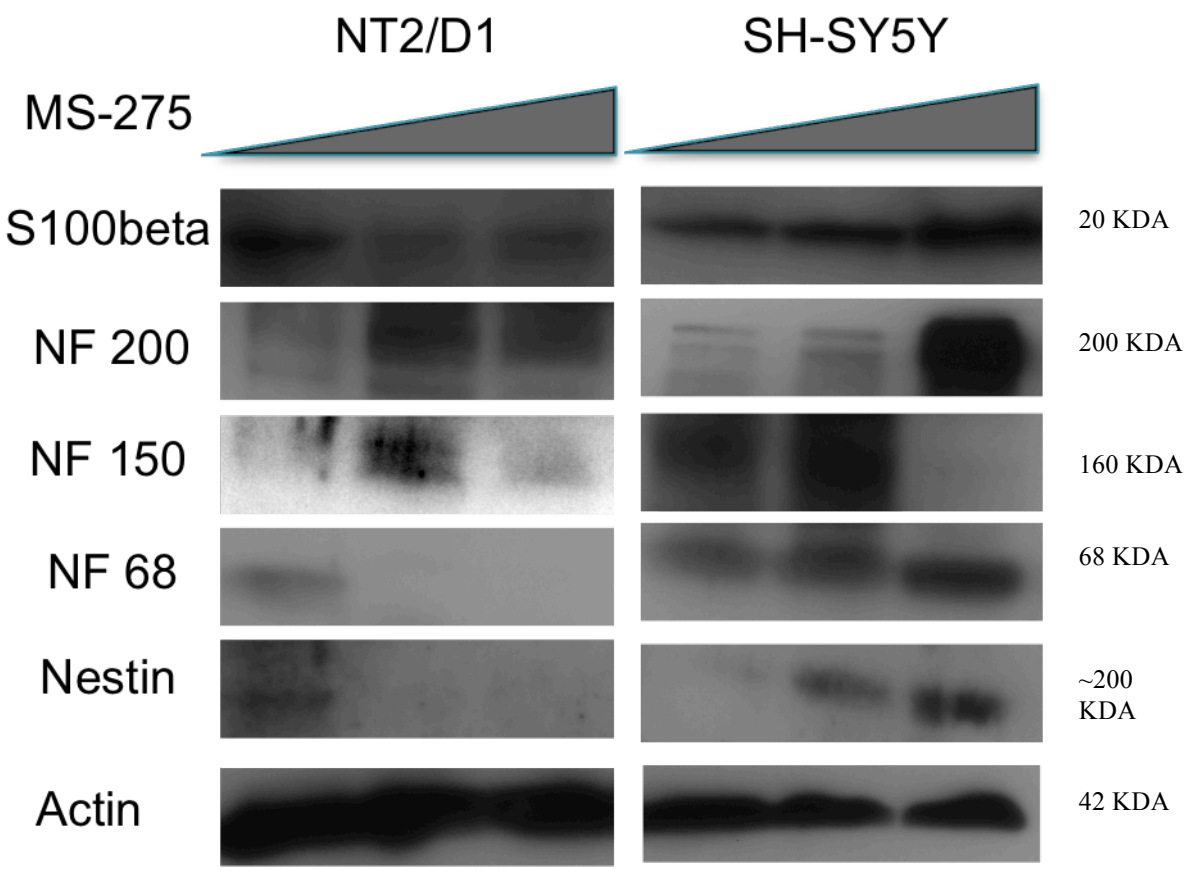

NT2/D1
SH-SY5Y
MS-275
S100beta
NF 200
NF 150
NF 68
Nestin
Actin
20 KDA
200 KDA
160 KDA
68 KDA
~200 KDA
42 KDA

Figure 6: MS-275 displays potential as a differentiator of NB SH-SY5Y and teratocarcinoma NT2/D1. **A)** phase contrast images at 200X magnification, after 48 hours, of control, 0.75 and 1.5 uM MS-275 treated SH-SY5Y, SKN-BE(2) and NT/2D1. Arrows indicate neurites that either disappear in the case of SH-SY5Y or appear in NT2/D1. Cells achieve a flattened and larger cell body following treatment as well. **B)** In-cell western analysis of the expression of proteins of neuronal lineage following 1.5 uM MS-275 treatment. Pan-NF analyzes neuronal, S100beta analyzes Schwannian and GFAP analyzes glial differentiation. Pan-NF increased significantly by 1.567±0.038 folds compared to control ($p = 0.0002$). **C)** In-cell western of the same proteins in figure 6B in NT2/D1. Overall pan-NF expression decreased to 0.608±0.075% that of control ($p = 0.0122$). **D)** Western blot analysis of S100beta, the three isoform of NF (NF68, NF150 and NF200) and neural progenitor marker nestin. Higher molecular weight form NF are associated with more mature neuronal phenotypes. In SH-SY5Y, MS-275 treatment induces expression of NF68 dose dependently. NF150 expression increased with low doses of MS-275, but not at high doses. NF200 is upregulated at high doses only. For NT2/D1, overall NF expression decrease observed in figure 6C is explained by a decrease in NF68 and NF150 at high treatment. However, NF200 is upregulated at high doses. Schwannian marker S100beta expression is increased in SH-SY5Y while it decreased in NT2/D1, corresponding to phenotypic changes in figure 6A. Nestin expression expectedly is decreased in NT2/D1, but surprisingly increased in SH-SY5Y following MS-275 treatment. In-cell western analaysis were repeated in triplicates and unpaired 2-tailed t-tests were performed. Astericks, *, ** and *** are used to denote significance of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively. Western blots were performed only once .

2.1.3.7 Summary of results

The results in this chapter demonstrates that the HDACi MS-275 is able to reduce growth dose dependently in the N-Type NB SH-SY5Y. This reduction in growth was found to be due to three factors: a potent G1 cell cycle block as demonstrated by a propidium iodide cell cycle analysis; apoptosis, demonstrated by an increase in sub-G1 population and an annexinV/7-AAD assay; and induction of differentiation, suggested by lineage specific and morphological changes observed by phase contrast microscopy.

The induction of a G1 cell cycle block was indicated by the a loss in expression of cyclinD1 and CDK4 and an induction of the CDKi p16. Induction of the intrinsic pathway of apoptosis, was shown by the cleavage of caspase 3 and coincident with decrease in expression of survivin, increase in expression of BAX and a decrease in BCL-2. BCL-2/BAX ratio was found to decrease dramatically, further suggesting a potent induction of the intrinsic pathway of apoptosis. Morphological observations of differentiation were confirmed, as there was an increase in expression of mature isoforms of neurofilament. Interestingly, an increase in expression of Schwannian marker S100beta was also noted. Surprisingly, nestin, a marker of a primitive neural lineage was also induced.

The differentiation potential of MS-275 was also tested on NT2/D1 teratocarcinoma, which is a common model of neuronal differentiation. Mature isoform of neurofilament was observed to increase while immature isoforms decreased. In contrat to SH-SY5Y, nestin, commonly associated with an undifferentiated NT2/D1 state decreased in expression.

2.1.4 Discussion

While the potential of MS-275 to reduce growth of NB cell lines has been documented before by Jaboin et al., and Deubzer et al., [36, 54] this thesis confirms and builds upon their results using a tumorigenic N-type non-MYCN amplified cell line not previously investigated in detail. Jaboin et al., used a MYCN amplified S-type SMS-KCNR and MYCN non-amplified SK-N-AS to characterize MS-275 induction of cell cycle arrest and apoptosis, while Deubzer et al., studied S and I type SH-EP and SKN-BE(C) lines and at a much lower drug concentration (400 nM).

HDACi activity of MS-275: Histone H3 and H4 acetylation

The increase in expression of acetylated histone H3 and H4 in Figure 3 confirms that MS-275 indeed acts upon the histones. The acetylation of the histone tails of H3 and H4 is well documented to induce chromatin release and induce an overall increase in transcription[18]. However, the results documented in this chapter may not necessarily be due only to a change in acetylation of histone, as HDACi have been shown to target the entire acetylome as well[19]. Regardless, the observation of histone acetylation serves as a basis for subsequent analysis and is critical in that the development of cancer is now recognized to involve both genetic and epigenetic events. Furthermore, the CDK inhibitors investigated (i.e. p21, p16) for their involvement in NB cell cycle blocks have been documented to be upregulated as a result of relaxation of transcription at their promoters following histone acetylation due to HDACi treatment[175, 176].

MS-275 reduces cell viability

Jaboin et al., and Deubzer et al., and Fuchert et al., have previously demonstrated the potential of MS-275 in reducing NB cell viability [43, 54, 177]. However, it was important to confirm their findings for several reasons. Jaboin et al., determined cell viability based on tritiated thymidine incorporation (^{3}H-TdR) that measured DNA synthesis. However, there is growing evidence that that ^{3}H-TdR itself induces cell cycle arrest and apoptosis[178]. Deubzer et al., used a substantially lower uM concentration of MS-275 (400 nM) that does not reflect concentrations being used in clinical trials of solid

tumors (when calculated at mg/kg to be 1-5 uM) [74]. However, HDACi are known to have differential responses depending on the dosage and regimen[31, 33, 177]. Furthermore, the dose of MS-275 they used would only have inhibited HDAC 1[31]. We used a dose range of 10 nM to 5 uM and settled on doses (0.75 and 1.5 uM) of MS-275 for subsequent studies that reflect what is being used already in phase trials. The 1.5 uM dose employed was determined to be approximately the LD50 dose for all three cell lines employed in this study. Separate assays were employed to verify and to remove bias associated with individual techniques. For example, Alamar blue reacts to mitochondrial metabolic activity which could be modified by HDACi treatment; trypan blue exclusion is simple to perform but can incur a larger experimental error; the DRAQ5/Sapphire700 DNA quantification method can be skewed if HDACi induces/reduces polyploidy. This is of importance because SH-SY5Y is near diploid, and NB that are less aggressive are known to hyperdiploid[179] [1]. The Alamar blue assay was found to yield a substantially higher LD50 value. This could be because Alamar blue measures metabolic activity and that HDACi, given their broad effects, have also been shown to affect mitochondrial metabolism[180].

MS-275 induces G1 Cell Cycle Arrest that correlates with p16 CDKi expression increase

Both Jaboin et. al, and Deubzer et al., noted that MS-275 induced a potent G1 cell cycle arrest. Our own propidium iodide cell cycle analyis confirmed this observation. However, NB is an incredibly heterogenous neoplasm. Since the N-type phenotype is prominent and a tumourigenic N-type cell line had not been previously studied, we sought to characterize the effect of MS-275 on the N-type SH-SY5Y model. The p21 cell cycle inhibitor is most commonly observed to be induced by HDACi treatment[175]. In contrast to Jaboin et al., and Deubzer et al., we found that p21 was not upregulated and p27, another commonly induced CDKi is also unchanged in expression. However, the p16 CDKi was dramatically induced. While p16 has been noted previously to be induced by HDACi in certain cancer types such as colon carcinoma (HCT116), it is a relatively rarer event[176]. In fact, MS-275 has previously been documented to induce p16 expression [43, 176, 181]. Furthermore, while p16 is very commonly deregulated in neoplasms, and genetic mutations and epigenetic modifications are involved with p16 as

a possible cause of neoplastic transformation[182], it is rarely mutated or disregulated in NB [8]. This may explain why p16 induction is less commonly seen. The cell line employed by Jaboin et al., SK-N-AS is known to have a p16 loss of heterozygosity[183]. As such, it is plausible that unlike other HDACi, MS-275 preferentially induces expression of p16, except in the instances where p16 is mutated or functionally compromised. Deubzer et al. did however document p16 induction in their NB cell lines, but only with longer treatment times at a low dose. Finally, as the models used by both Jaboin et al., and Deubzer et al., were S and I type cells, p16 induction by MS-275 might indicate a N-type specific event. More cell lines are needed to verify this hypothesis. G1 cell cycle arrest is most commonly associated with the modulation of the cyclinD/CDK4 complex by cell cycle inhibitors [17]. As such we sought to investigate whether MS-275 treatment leads to a significant reduction in expression of this complex. Both CDK4 and cyclin D1 expression was significantly reduced at similar levels relative to controls. Whether this is due to a direct effect by MS-275, or a more downstream effect is not known.

MS-275 induces the intrinsic apoptotic pathway

In addition to cell cycle arrest, HDACi are commonly known to induce apoptosis [21]. MS-275 was documented to induce apoptosis through an induction of oxidative stress, the intrinsic pathway and the extrinsic pathway of apoptosis [52, 83, 85, 90, 184]. In NB, Muhlethaler-Mottet demonstrated that HDACi such as NaB, SAHA and TSA employs multiple complex mechanisms to induce apoptosis; most commonly the intrinsic pathway is induced [39]. As such we sought to charaterize the ability of MS-275 to induce the intrinsic apoptotic pathway [39]. Furthermore, the HDACi induction of the extrinsic pathway of apoptosis was demonstrated in MYCN amplified NB where caspase 8 is deleted and preferentially silenced [42]. BCL-2 has previously been demonstrated to be downregulated in NB following MS-275 treatment [36, 54]. We showed here that MS-275 induces the intrinsic apoptotic pathway evident by a concomitant decrease in expression of BCL-2 and increase in BAX. While our in-cell western data and western blot data did not correlate (perhaps due to a difference in antigen presentation), it is still evident that the BCL-2/BAX ratio was dramatically reduced. A decrease in the BCL-

2/BAX ratio is a well-documented indicator of the induction of the intrinsic pathway of apoptosis, predictor of drug efficacy and is in fact used as a prognostic indicator of cancer aggressiveness [185-187]. While BCL-2 reduction is a sign of reduction in cell growth and induction of apoptosis, we also sought to characterize whether caspase 3 is cleaved following MS-275 treatment. Cleavage of caspase 3 is one of the endstage events in apoptosis. We found cleavage of caspase 3 correlating with the tunel assay results of Jaboin et al [54]. However, cleavage of caspase 3 was found only in approximately 3% of cells, which is as apposed to data from AnnexinV/7-AAD which showed nearly 15% of cells entering apoptosis. Also, our 48 hr treatment with MS-275 induced a percentage of cells into early apoptosis, as shown by the AnnexinV/7-AAD assay. Taken together, while MS-275 can induce apoptosis, the low percentage of cells entering apoptosis may indicate that the drug should be used in longer treatment doses.

Induction of apoptosis by MS-275 needs to be further studied in NB. There are many other documented effects and proteins that HDACi are associated with that can induce apoptosis. For example, HDACi have previously been found to be able to directly induce acetylation of p53 and mediate its effect as a tumor suppressor [188]. SH-SY5Y expresses wild-type p53. HDACi can promote p53 acetylation with functional translocation to nucleus which can inhibit the anti-apoptotic protein survivin [40, Mirza, 2002 #50, 189]. While p53 was not assayed in this study, survivin expression was noted to be decreased by MS-275. In this study, we showed for the first time that survivin expression was downregulated by MS-275 in a NB cell line. Ku70 acetylation similarly has been demonstrated to induce the release of BAX, inhibit BCL-2 and induce apoptosis [41]. Caspase 8, which is normally silenced in NB is induced by HDACi to potentiate the extrinsic pathway of apoptosis [42]. HDACi functions at multiple levels, and further work needs to be completed to characterize the overall effects MS-275 on NB. Other inhibitors of apoptosis such as XIAP and BCLX have been noted to be regulated by HDACi in NB [39].

Differentiation potential of MS-275

Finally, the ability of MS-275 to differentiate NB was characterized. While HDACi such as CBHA, valproate, and BL1521 have previously shown to induce neuronal differentiation in NB cell lines or used in conjunction to potentiate the response to differentiating agents, MS-275 has not been tested [66] [38] [38] [46]. Jaboin et al., noted that MS-275 did not induce differentiation in the NB cell lines they employed [54]. However, they assessed this based on morphological observations and only on the basis of neurite extension. Differentiation in NB cell lines does not necessarily have to be neuronal to be of significance. Ross et. al, have demonstrated that the three cell phenotypes in NB correlate with tumorigenic ability; a higher prevalence of S-type cells is associated with a better prognosis [5] [133]. Similarly, the greater percentage of I-type and N-type cells populating a tumor, the poorer the prognosis. Accordingly, a differentiating agent that can potentiate the transdifferentiation of a more tumorigenic neuroblastic N-type NB to a more Schwannian S-type would be of clinical importance [12].

To further characterize and confirm the ability of MS-275 to induce NB lineage type related differentiation, NT2/D1 (a subclone of Tera-2) a common neuronal differentiation model was evaluated. NT2/D1 is a teratocarcinoma cell line that is developmentally closer to ES cells and extremely sensitive to differentiating agents such as retinoids[167]. While overall expression of neurofilaments was decreased (as assessed by in-cell western), western blot demonstrated an increase in the 200 KDa form of NF that is associated with neuronal maturation. This is correlated with an observed increase in neuronal processes in culture. In conjunction with the SH-SY5Y studies, these results suggest that MS-275 can be a potent differentiating agent.

NB succeptibility to differentiation by MS-275 was analyzed in N-type SH-SY5Y. SH-SY5Y is a commonly used model to investigate the potential of HDACi, retinoids and other therapeutics to induce differentiation in NB [68, 69, 168, 169]. This is because of its selected neuronal features and the ability of SH-SY5Y to differentiate into a mature neuronal phenotype that can form action potentials. However, in most studies the differentiation potential of HDACi in SH-SY5Y is only examined in terms of induction of NF68, an immature NF marker or NF150, as criteria [37, 40, 66, 190]. To characterize

differentiation of more mature and functional neuronal forms, we assayed the expression of NF200. Unlike NF68 and NF150, NF200 has previously been shown to not be expressed by SH-SY5Y [3]. Schwannian markers S100beta and GFAP were also assayed to investigate transdifferentiation and differentiation into the Schwannain phenotype. In-cell western analysis demonstrated an overall increase in neurofilament expression, while S100beta was not induced. Western blot data confirmed that neurofilament expression was increased, and that the 200 KDa isoform is dramatically upregulated at the LD50 dose. Interestingly, while the LD50 dose only increased the 200 KDa isoform, the LD20 (0.75 uM) dose only induced the mid 150 KDa isoform. While 150 KDa NF is also used as a marker of neuronal maturation, the 200 KDa isoform is a marker of neurite functionality. The induction of different isoforms of NF by MS-275 at different doses highlights the critical importance dose has in the usage of HDACi. Regardless of the upregulation of the mature NF isoforms, neurite extension was not noted in culture. In fact neuritic extensions normally associated with SH-SY5Y were lost and cells appeared more S-type like. This may indicate that while MS-275 is inducing the production of the high KDa forms of NF, it is not potentiating their organization. An agent such as retinoic acid may be needed to induce NF assembly and neurite extension. These results suggest that MS-275 may be a potent potentiator of neuronal programming as opposed to a potent differentiator. Co-treatment of MS-275 with retinoic acid would demonstrate whether MS-275 could potentiate the neuronal programming of differentiation. Hahn et al., have shown HDACi have potent induction of neuronal differentiation in NB lines when used in conjunction with retinoic acid, when compared to other agents [67]. It has been shown that MS-275 used in conjunction with retinoic acid induced PC4 and LNCaP prostate cancer cell differentiation into a more benign phenotype and reduced xenograft growth by upregulating RARbeta expression[191, 192]. This was also similarly shown in the RCC1 renal carcinoma cell lines [193]. This is further substantiated in that HDACi have been documented to increase expression of the retinoid receptors, and help dissociate them from HDAC that are part of the transcriptional complexes residing on the promoters and thereby prevent their association with DNA to induce transcription [69].

As mentioned above, MS-275, in this study, was observed to induce SH-SY5Y into a phenotype with larger cell bodies, reduced neuritic processes, and increase adherency. This correlates with induction of a S-type phenotype. Examining the expression of S100beta, a Schwannian marker, it was noted that MS-275 was able to increase its expression, when assayed by western blot. This may suggest that MS-275 has ability to transdifferentiate an N-type NB cell into a S-type [12]. Transdifferentiation of the NB SKN-N-SH and other lines has been previously documented [12]. Our laboratory has previously found that MS-275 at low nM doses can potentiate differentiation of NB into a more Schwannian like cells when induced (unpublished data). This further substantiates that MS-275 can transdifferentiate NB cells.

Nestin is a marker commonly associated with neural progenitors and the more primitive embryonic phenotype of neural crest stem cells [95]. Here we actually found that MS-275 induced nestin expression. This may signify several possibilities. Ross et al., have previously demonstrate that I-type NB cells express markers of both Schwannian and neuronal lineages, and increased expression of primitive neural crest markers such as nestin, c-kit and CD133. Given this, it maybe possible that MS-275 is transdifferentiating N-type SH-SY5Y into a more I-type like. Furthermore, nestin has previously been used as a marker of the undifferentiated state that is associated with TSCs of C6 glioma cell lines and TIC cells from N-type NB cell line LA-N-5 [117, 194]. If this is indeed the case, it could possibly have adverse effects in NB patients, as I-type cells and TIC are associated with more primitive tumors with poor prognosis [5, 116, 133]. Furthermore, I-type cells are noted to have higher tumorigenic capacity in in-vivo xenograft assays [133]. However, in-vivo xenograft studies by Jaboin et al., have shown that MS-275 has the potential to nearly completely reduce tumour growth and size [54]. Therefore, it may also be possible that MS-275 epigenetically reversing N-type SH-SY5Y into a more neural crest state that is not tumorigenic. Neural crest cells can proliferate and differentiate into neuronal, Scwhannian and myogenic lineages [134]. Since, nestin is a marker that has also documented to be expressed in myogenic cells and NB can be induced along the myogenic line, upregulation of nestin and a correlating flattened phenotype, may suggest muscle differentiation [195].

Discrepancies between in-cell western and western blots

Finally, it is necessary to address the inconsistincies with in-cell western and western assays noted our study. Although the difference in culture format may be factor, where for in-cell western assays, cells were grown and treated in a 96 well plate, while for western blotting, cells were grown and treated in a T-75 cm^2 culture flask these difference may not be critical. It is more likely that the in-cell western assay is based on the quantification of immunofluorescence detection of native non-linearized protein, while the western blot assay is based on linearized proteins and a non-native conformation. The antigen presentation and access can differ significantly. As such these assays are complimentary and provide a more comprehensive analysis of expression. Finally, because of the licensing of MS-275 by a private company, it was not possible to secure more drug. This lead to a lack of replication of western blots.

Summary

The results presented in this chapter confirm that MS-275 has a potent ability to modulate NB-growth. Together with the fact that MS-275 has a long half-life and an effective dose that ranges from nM to low uM concentrations, it is a very attractive therapeutic to be used in-vivo [72]. However, further work has to be conducted to characterize the full effects that MS-275 has in NB. The model used predominantly in this chapter was a MYCN non-amplified N-type neuroblastoma that is commonly used as a model of NB differentiation. Experiments should be repeated in other cell type NB cell lines that are MYCN amplified as well, as MYCN is a clinically significant prognostic indicator of tumour aggressiveness and stage. While these results confirm and further characterize the ability MS-275 to target NB growth and survival, it is becoming evident that to develop effective therapeutics, the TSC component must be targeted as well. Therapies that can only target the prevalent transit amplifying population and not the TSC would likely result in tumor recurrence. The ability of MS-275 to target the TSC population will be presented in the following chapter.

Chapter 3:
MS-275 targets the TSC-like population of NB cell lines

3.1 Characterizing anti-NB TSC ability of MS-275

3.1.1 Objective

In the previous chapter, we demonstrated the ability of MS-275 to induce histone acetylation and target a NB cell line into cell cycle arrest, apoptosis and differentiation. However, the effects of MS-275 were analyzed on the entire cell population which is heterogeneous and comprises both progenitors and transit amplifying cells. We therefore wanted to determine if MS-275 could target the TSC like subpopulation which is responsible for the repopulation of the cancer. The potential anti-NB TSC effect of MS-275 is not known. Of present there has only been one study that has investigated the ability of HDACi to target progenitors of cancers [196]. Bug et al, demonstrated that valproic acid may expand one subset of acute myeloid leukemic progenitors while reducing another [196]. TSC/TICs have recently been discovered in NB primary tumors and in cell lines [116, 117, 124, 130, 135]. The isolation of SP TSC-like cells has proven to be an effective means to study the molecular biology of NB TSCs. Furthermore, NB SP cells have previously been shown to have increased tumorigenicity and self-renewal capacity, similar to that of TSCs [117, 124, 130, 135]. Though the origin of the NB TSC is not yet well defined, and whether TSC alone can generate tumors is a point of contention and continuing studies, it is becoming evident that TSCs exhibit the genetic and epigenetic alterations of the parent tumor [197]. This is the reason why MS-275, with its ability to modulate epigenetic status through histone acetylation should be investigated. If it is able to modulate the NB TSC-like population, together with its anti-NB activity shown in the previous chapter, MS-275 could prove to be a powerful anti-NB therapeutic and would have clinical implications toward reducing tumorigenicity, residual minimal disease and metastatic disease.

We first took the approach of assessing the ability of MS-275 to reduce clonogenicity and self-renewal of NB SH-SY5Y and SKN-BE(2), and teratocarcinoma NT2/D1. Clonogenicity, the ability of cells to form colonies(spheroids) in a viscous suspension media has been associated with an origin from TSCs [43, 131, 145, 155, 162, 196, 198,

199]. Cells with a propensity to self-renew in a clonogenic assay are theorized to be TSC-like as they can initiate colony growth from a single cell. Self-renewal, is assayed by the ability of the colonies to reform following dissociation. NT2/D1 can serve as a critical reference line because of its high self-renewal potential and was therefore included in the analysis.

In the event MS-275 can reduce the SP population of mycN amplified and non-amplified NB cell lines, the mechanism by which this occurs will also be characterized. It has been shown previously in leukemia cell lines that HDACi have the potential to alter the expression of ATP dependent drug pumps that are associated with the SP phenotype [34]. It has also been postulated that HDACi can affect the expression of proteins in the self-renewal pathway [200]. Therefore, we will investigate whether the main drug pump that defines NB SP is affected, and whether the self-renewal machinery linked with TSCs, is affected. In NB, ABCG2 is the predominant ATP-dependent drug transporter associated with SP [135]. The stem cell markers OCT-4, Nanog and SOX-2 have been linked to the self-renewal of stem cells [200]. Furthermore, OCT-4, SOX2 and ABCG2 have also been shown to be upregulated in TSCs [130, 131, 162-164].

In summary, the objective of the studies described in this chapter was to examine whether the HDACi MS-275 can target and reduce the NB TSC population, and to characterize the mechanism by which this occurs.

3.1.2 Methods

Methylcellulose Clonogenic Assay

The clonogenic assay, using methylcellulose as the suspension medium measures the potential of cells to form colonies. The colony formation potential is a reflection of the ability of cells to self-renew. However, it has been noted that transit amplifying cells slightly removed from TSCs can form colonies as well. Regardless, high self-renewal has been associated with with TSCs, as such this assay identifies the population that is most TSC-like. NB SH-SY5Y, SKN-BE(2) and NT2/D1 were cultured, trypsinized and resuspended in Methocult methycellulose media (Stem Cells # M3134) to quantify their

clonogenic potential. Briefly, 1.2 X 10^4 SH-SY5Y, 7.5 X 10^3 SKN-BE(2) and 2 X 10^3 NT2/D1 cells/mL was placed into a 40% methycellulose solution supplemented with 10% FBS, 1% antibiotics and 49% of corresponding media (as identified in section 2.1.2 Methods). MS-275 doses ranging from 10 nM to 3 uM was added to methycellulose solution as well. Cells were vortexed gently and distributed into non-adherent 10 mm dishes with a blunt end 16 guage needle. Samples were then place in a 37^{o}C incubator in 5% CO_2 for 2 weeks. Colonies formed were documented and counted on a phase contrast microscope using a grading dish. Number of colonies formed was divided by number of cells seeded to determine colony forming potential. Samples were assayed in triplicate. Cell numbers was then plotted in GraphPad Prism 5 to determine dose response and LD50.

Hoechst 33342 dye Exclusion Assay

Cells expressing drug pumps that exclude Hoechst 33342 in a heterogeneous population have been previously demonstrated to have high self-renewal and are most primitive. In tumors, the SP population is enriched in highly tumorigenic TSC-like cells. Cells grown in adherent culture flasks were lifted using a citrate saline solution, washed and resuspended in 10% FBS supplemented media at 1 X 10^6/mL. Verapamil (verapamil hydrochloride; Sigma #V4629), an ABCG2 pump inhibitor, was added to a final concentration of 50 uM to control samples for 15 minutes and incubated in a 37^{o}C water bath. Hoechst 33342 (bisBenzimide H 33342 trihydrochloride; Sigma #B2261) was then added to a final concentration of 5 ug/mL and incubated for 90 minutes in a 37^{o}C water bath. Samples were then washed twice with cold PBS and resuspended in 2% FBS supplemented PBS with 1ug/mL propidium iodide. Samples were subsequently placed on ice and run on a BD LSRII flow cytometer equipped with an UV laser. Samples were analyzed with Hoechst Red (630 nm) and Hoechst Blue emission wavelengths (450 nm). Gating for the SP was determined from the verapmail negative control. Cells were also treated with Trichostatin A, a hydroxamic acid HDACi, (TSA; Sigma T8552) to determine the effect other known HDACi may have on the SP population of NB.

Flow Cytometric Assessment of ABCG2

ABCG2, is the ATP-dependent drug pump that is most commonly associated with the SP phenotype. Highly primitive stem cells and TSCs have also been shown to upregulate this protein. Cells grown in adherent culture flasks were lifted using a citrate saline solution, or with trypsin in negative control samples. 1 X 10^5/mL of cells were resuspended in a cold 5% BSA/PBS solution at 4^{o}C for 15 minutes to block. Cells were then treated with an ABCG2 antibody conjugated to phycoerythrin (R&D Systems #FAB995P) for 45 minutes. Cells were then washed with cold PBS 3 times and resuspended in a PBS solution containing 7-AAD (BD Pharmingen #51-68981E) and analyzed on a BD FACSCAN flow cytometer. Only cells negative for 7-AAD was included in the gating to exclude non viable cells. Gating was determined from the negative trypsin controls.

Immunofluorescence

Immunofluorescence was used to confirm the nuclear expression of the stem cell markers OCT-4, SOX2 and Nanog. Cells were grown on glass coverslips placed in a 12 well adherent substrate plate until they were 75% confluent, and then treated with MS-275. Following a 48 hour treatment, coverslips were fixed with a 4% paraformaldehyde solution, placed in a weight boat, washed and permeabilized with a 0.3% Triton-X/PBS solution. Following a two time wash, cells were blocked with a 5% BSA/PBST solution overnight at 4^{o}C. Primary antibodies of OCT-4 (Cell Signaling # 2890S; 1/300), SOX-2 (R&D Systems #MAB2018; 1/250) and Nanog (Cell Signaling #3580S; 1/200) were diluted in a 5% BSA/PBST solution and added to cells overnight at 4^{o}C. Cells were subsequently washed 5 times in PBST, and then a 1/500 dilution of secondary Alexa Fluor-488 antibody (chicken-anti-rabbit or donkey-anti-mouse; Molecular Probes #A21441; #A21202) was added for 1 hour at RT. Following washing, cells were treated with a 0.5 ug/mL solution of DNA counterstain DAPI. Coverslips were inverted and mounted onto a glass slide with Universal Mount acrylic mounting agent (Research Genetics #750105) and visualized via fluorescence microscopy. ABCG2 immunofluorescence was similarly performed, but without permeabilization and secondary antibody incubation as it is already conjugated to phycoerythrin. Coverslips treated only with secondary were used as a negative control.

Flow Cytometic Assessment of OCT-4, SOX2 and Nanog

FACs analysis was used to quantify the cell fraction expression OCT-4, Nanog and SOX2. As the expression of these proteins have been associated with TSCs, the fraction expressing highly these proteins represents the cells that are TSC-like. Cells were lifted from adherent cultures by trypsin, washed and fixed with a 4% paraformaldehyde solution. 3 X 10^6 cells were then permeabilized with a 0.1% solution of PBS/Triton-X, washed twice with PBS and blocked with a 5% BSA/PBS solution for 1 hour at RT. Primary antibody of OCT-4 (1/200), SOX2 (1/200) and Nanog (1/200), made up in 5% BSA/PBS was then added overnight at 4^{o}C. Cells were subsequently washed 3 times with PBS and stained with a chicken-anti-rabbit Alexa Fluor-488 (1/3500) or goat-anti-mouse R-Phycoerythrin (Caltech #M30004-1; 1/500) secondary antibody made up in 5% BSA/PBS, for 1 hr RT. Cells were then washed and analyzed on BD FACSCAN flow cytometer. Gating was determined from negative control samples that were stained only with the secondary antibody. This controls for autofluorescence and non-specific antibody binding of the secondary.

Western Blotting of Stemness Proteins

Western blots were used to quantify the expression of stem cell markers before and after treatment, to determine whether MS-275 could target the self-renewal pathway. Western blotting was performed similar to section 2.1.2 Methods section, with the exception that 20 ug of lysate was loaded for NT2/D1 and 100ug of lysate was loaded for SH-SY5Y. Primary antibodies of OCT-4 and Nanog are used at dilutions of 1/1000.

Statistical Analysis

All statistical analysis was performed on GraphPad Prism 5 software. Unpaired (student's) t-test was used to analyze samples, or the paired T-Test was used when samples were paired. Statistical significance cut-off is as described in section 2.1.2 Methods.

3.1.3 Results

MS-275 reduces clonogenicty of NB and Teratocarcinoma cell lines.

Clonogenicity is an important in-vitro functional indicator of the self-renewal potential of cells [199]. In a viscous methylcellulose media on an non-adherent surface, cells are deprived of substrate adherence, and only single cells that have high self-renewal capacity have been shown to be able to grow as colonies. Highly primitive stem cells and TSCs have previously been shown to have high clonogenic potential. A reduction of clonogenicity suggests a targeting of the TSC population. In in-vitro cell lines, the percentage of cells that have clonogenic capacity is usually quite low [102, 131, 136, 152]. SH-SY5Y, SKN-BE(2) and NT2/D1 cells were placed into clonogenic media and treated with 0 to 3 uM MS-275. Colonies were counted after 2 weeks and dose response curves were established and a clonogenic LD50 was calculated.

NT2/D1, SH-SY5Y, and SKN-BE(2) have a clonogenic potential of approximately 13.2%, 0.8% and 1.9% respectively. The high colony forming potential of NT2/D1 is expected due to its high self-renewal potential (Figure 7A) [95]. MS-275 was able to significantly reduce the number of colonies in all three groups at the dose of 200 nM and above ($p < 0.005$) (Figure 7A). Under phase microscopy we observed that in the methylcellulose medium NT2/D1 formed large and highly spherical colonies, SKN-BE(2) formed smaller colonies, while SH-SY5Y formed small poorly formed and dispersed colonies. Treatment with MS-275 reduced colony size as well (figure 7B). In the monolayer LD20 dose (0.75 uM)(figure 2A), no colony formation was observed in all three cell lines despite the colony formation potential of all three cell lines being different (figure 7A, 7B). Interestingly, when normalizing all numbers to a percentage of control, MS-275 was able to reduce colony formation potential nearly identically in both teratocarcinoma and NB cell lines (Figure 7B). The LD50 dose of MS-275 for all three cell lines was calculated to be approximately 200nM in the methylcellulose medium(Figure 7B). Self-renewal assay was not performed, because no colonies were formed at 0.75 and 1.5 uM MS-275.

MS-275 reduces the SP component

While NT2/D1 and SKN-BE(2) have previously been shown to contain a SP population, this has not been shown for N-type SH-SY5Y [130, 136]. Using the dye exclusion of Hoechst 33342 and FACs, we showed that SH-SY5Y has a Hoechst low population, indicating that it has an SP population (Figure 8A). The SP fraction in NT2/D1, SH-SY5Y and SKN-BE(2) was determined to be 0.75, 3.32 and 0.32% respectively (Figure 8B). Following the determination of an SP population, MS-275 at either the LD20 (0.75 uM) or LD50 (1.5 uM) dose, previously determined to inhibit all colony formation in colony formation assays (Figure 7), were for effects on the SP population. 0.75 and 1.5 uM MS-275 were able to reduce the SP population in all three cell lines significantly. In NT2/D1, a 48 hr treatment with 0.75 and 1.5 uM of MS-275 reduced the SP percentage to approximately 0.17% and 1.27% of control ($p < 0.0001$ for both). In SH-SY5Y the same treatment reduced SP percentage to approximately 18.55 and 22.89% of control ($p = 0.0098$ and $p = 0.0177$ for low and high dose respectively). In SKN-BE(2), 0.75 and 1.5 uM MS-275 reduced SP percentage to approximately 29.71% and 1.08% of control respectively ($p = 0.0237$ and $p < 0.0001$). Gating of the SP was determined for all three cell lines using a negative control sample treated with verapamil, an inhibitor of ABCG2 (Figure 8A). Comparatively, trichostatin A (TSA), another potent HDACi that is routinely used in in-vitro HDACi studies, increased the percentage of SP population in SH-SY5Y by greater than 266.6% at an LD50 dose of 100 nM ($p = 0.0212$) (Figure 8E). This was observed as a large increase in the SP tail of the FACs profile (Figure 8D). This increase was significantly different from the effect an equivalent dose of 1.5 uM MS-275 had on the SP population ($p = 0.0089$).

MS-275 reduces the SP component by decreasing ABCG2 expression

Since the SP fraction is defined by the ability of cells pump out Hoechst 33342, and ABCG2 is a key drup pump in this process, we analyzed the percentage of cells expressing ABCG2 after the 48 hr MS-275 treatment.

A phycoerythrin conguated ABCG2 antibody specific to an external epitope of ABCG2 was employed to analyze ABCG2 expression. Cells were exposed to ABCG2 antibody

and 7-AAD. To exclude the non-viable population of cells, a 7-AAD negative population was included in the analysis. Gating was determined based on negative control of trypsinised cells which removes the ABCG2 antigen. The ABCG2 antibody is specific to an extracellular epitope that is sensitive to trypsin cleavage (Figure 9A). Interestingly, NT2/D1, SH-SY5Y and SKN-BE(2) all demonstrated an ABCG2 positive population of 0.19% (Figure 9B). Following normalization to controls, a 0.75 uM dose of MS-275 was found to reduce the percentage of NT2/D1, SH-SY5Y and SKN-BE(2) cells expressing ABCG2 to approximately 39.35, 47.48 and 37.38% of control ($p = 0.0111$, $p = 0.0131$ and $p = 0.0086$ respectively). A 1.5 uM dose of MS-275 reduced the ABCG2 expressing percentage of NT2/D1, SH-SY5Y and SKN-BE(2) cells to approximately 32.69, 36.72 and 27.16% of control respectively ($p = 0.0270$, $p = 0.0022$ and $p = 0.0084$ respectively). The trypsin negative control reduced the percentage of control cells expressing ABCG2 to 4.86, 17.44 and 12.12 % respectively in NT2/D1, SH-SY5Y and SKN-BE(2) ($p = 0.0006$, $p = 0.0040$, and $p = 0.0061$) (Figure 9C). To confirm that the ABCG2 antibody employed is specific to an extracellular epitope, immunofluorescence labeling was performed and showed an extracellular membranous staining (Figure 9D).

MS-275 reduces the number of cells expressing stem cell markers

Stem cell markers have been associated with highly self-renewal stem cells and TSCs and shown to play a key role in maintaining its undifferentiated phenotype with a high self-renewal potential. Stem cell marker expression has also been correlated with the NB TSC population and increased tumorigenicity. The reduction in percentage of SP and clonogenicity following MS-275 treatment suggested that self-renewal was being affected. Therefore, OCT-4, Nanog and SOX2, three proteins linked with self-renewal in TSCs and stem cells were analyzed in SH-SY5Y and NT2/D1. The ability of MS-275 to reduce the expression of OCT-4, Nanog and SOX-2 was first analyzed by immunofluorescence in teratocarcinoma cell line of NT2/D1 since it is developmentally close to an ES cell line and greater than 90% of cells in NT2/D1 express the stem cell markers markers[95]. A 48 hour 1.5 uM MS-275 treatment against NT2/D1 cells showed a dramatic reduction in expression of nuclear OCT-4, Nanog and SOX2 as assayed by immunofluorescence (Figure 10A). This reduction was confirmed by FACs staining of

fixed NT2/D1 cells with the same antibodies (Figure 10B, 10C and 10D). Following subtraction for the percentage of autofluorescence determined from a control stained only with secondary antibody, the percentage of control NT2/D1 cells that expressed OCT-4, SOX2 and Nanog was all 90.10, 91.96 and 91.70% respectively(Figure 10E). OCT-4 and SOX2 appeared to drop significantly and dose dependently following 0.75 and 1.5 uM MS-275 treatment to 78.07 and 78.93% ($p = 0.0024$ and $p = 0.0031$) and 56.37 and 64.23% ($p = 0.0096$ and $p < 0.0001$) respectively, while the percentage of cells expressing Nanog dropped to 32.77 and 32.97% ($p < 0.0001$)(Figure 10E). The difference in percentage of cells expressing OCT-4 and SOX2 between the low and high dose was also significant at $p = 0.0365$ and $p = 0.0024$ respectively. This decrease was further confirmed by western blotting for OCT-4 and Nanog in NT2/D1 (Figure 10H). Using the highly stem cell like NT2/D1 as a reference, similar studies were performed on NB SH-SY5Y. 0.39 and 0.33% of SH-SY5Y cells expressed OCT-4 and SOX2 respectively(Figure 10F). Following 0.75 and 1.5 uM MS-275 treatment, relative to control untreated cells, the percentage of cells expressing OCT-4 and dropped significantly to 53.80 and 45.78 respectively and the percentage of cells expressing SOX2 dropped to 51.96 and 45.78% respectively ($p = 0.0480$ and $p = 0.0003$ for OCT4 and $p = 0.0391$ and $p = 0.0066$ for SOX2) (figure 10G). This reduction in expression was confirmed by western blot analysis for OCT-4 in SH-SY5Y cells (figure 10I).

<u>The percentage of cells that express clonogenic capacity, SP, ABCG2 and stemness proteins differ.</u>

While clonogenic capacity, SP, ABCG2 and expression of OCT-4 and SOX2 have all been linked to TSC, the percentage of cells expressing these can differ from each other. This raises the possibility that not all of these may correctly or completely label TSC in a population. In NB SH-SY5Y, 0.35% of cells express stemness proteins OCT-4 and SOX2, 0.7% of cells exclude Hoecsht 33342, 0.1% express ABCG2 and 0.75% of cells have clonogenic capacity (Figure 11A). These numbers do not differ significantly. In contrast, in NT2/D1, 90% of cells express OCT4 and SOX2, 3% are SP, 1% express ABCG2 and 13% possess clonogenic capacity (Figure 11B) This observation will be further discussed in the discussion of this chapter.

Figures

Figure 7: MS-275 is able to reduce the colony forming potential of NB and teratocarcinoma cell lines.

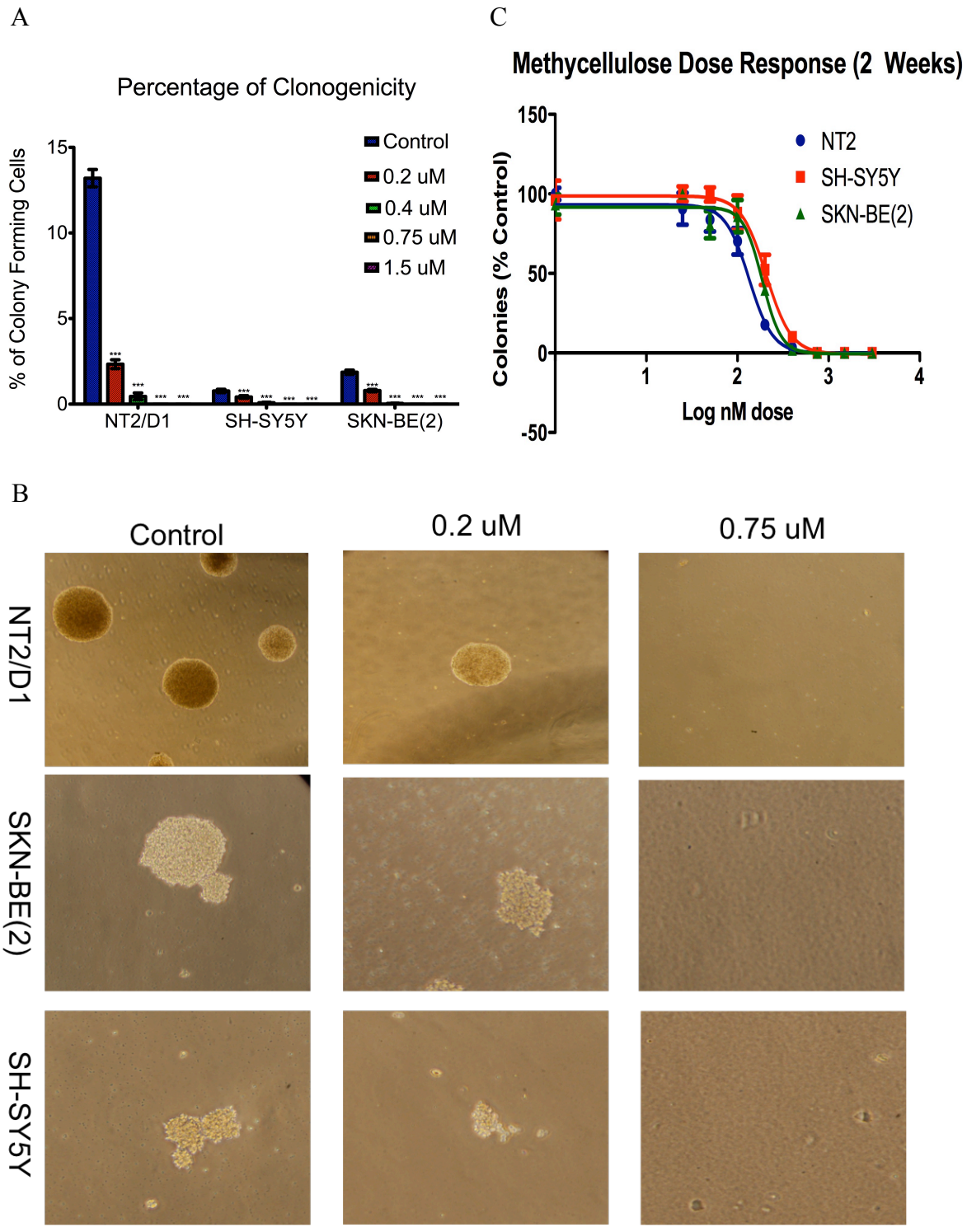
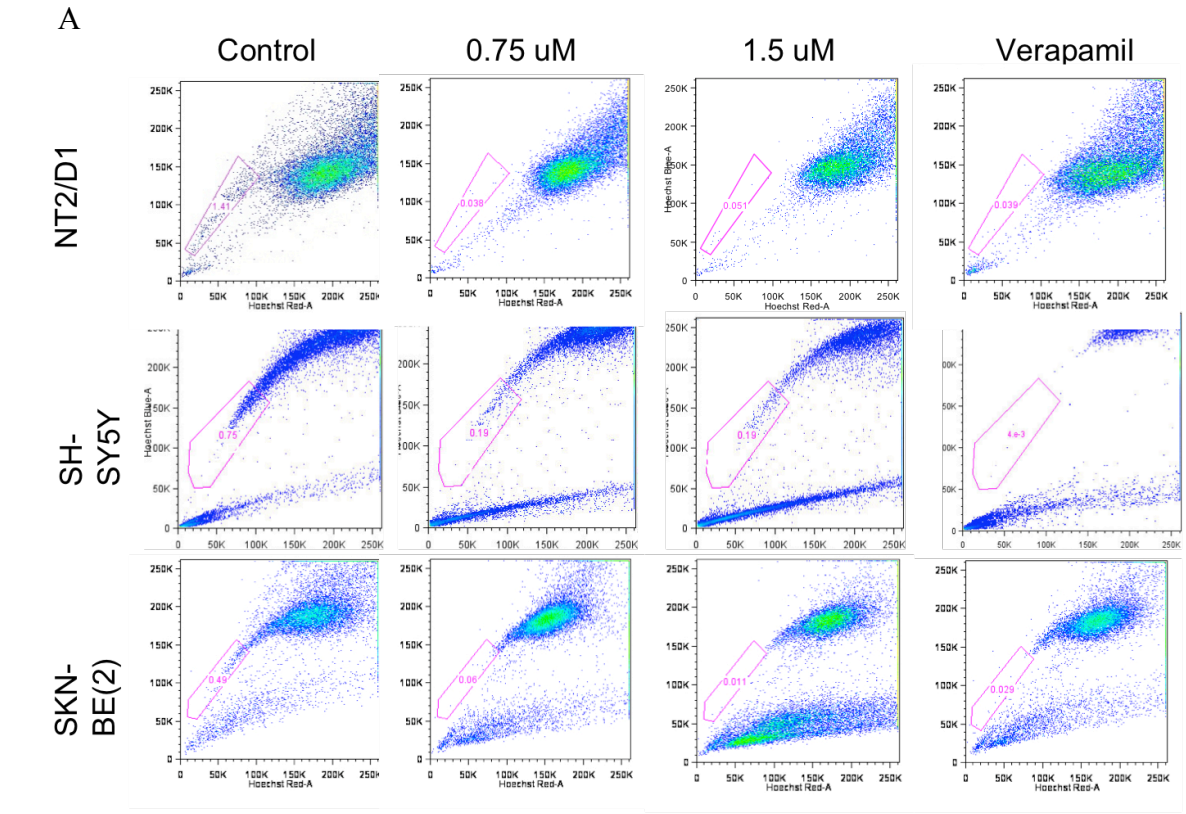
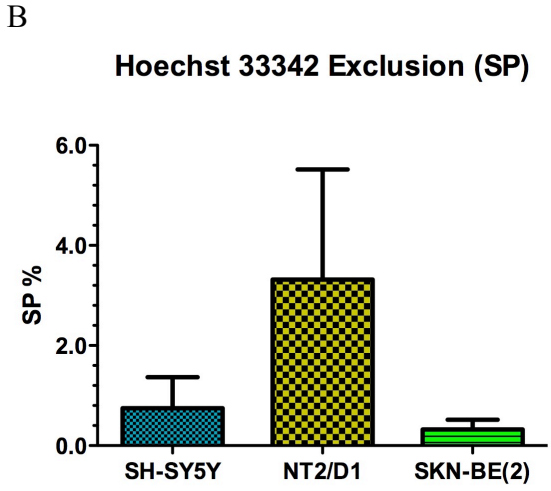
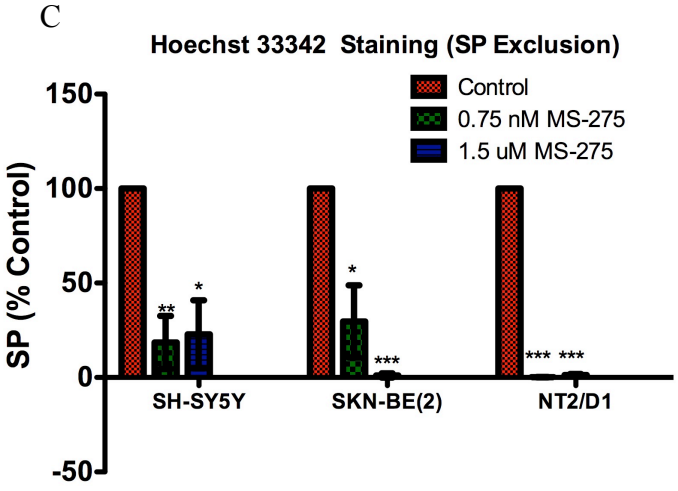


Figure 7: Clonogenic potential of teratocarcinoma NT2/D1 and NB SH-SY5Y and SKN-BE(2) cell lines was investigated by a 2 week methycellulose colony assay. **A)** NT2/D1, SH-SY5Y and SKN-BE(2) has colony forming potential of 13.2±0.507%, 0.797±0.037% and 1.863±0.124% respectively. MS-275 treatment starting at 0.2 uM was able to have a significant impact on colony formation of all three cell lines. Percentage of colonies remaining at LD50 dose determined in 7C is 2.33±0.260, 0.417±0.075 and 0.791±0.069 respectively for NT2/D1, SH-SY5Y and NT2/D1. Starting at 0.75 uM and above no colonies formed. All dose values 200 nM and above in all three cell lines had a $p < 0.005$ as compared to control. **B)** 20X phase contrast magnification of colonies in all three cell lines at control, 0.2 uM and 0.75 uM doses. At 0.2uM, there is a reduction in colony number and size. **C)** Following normalization of colony numbers to control, all three cell lines demonstrated similar dose response to MS-275 with LD50 number being 0.1342, 0.2071, and 0.1875 uM for NT2/D1, SH-SY5Y and SKN-BE(2) respectively. Clonogenic assays were repeated in triplicates and unpaired 2-tailed t-tests were performed. Astericks, *, ** and *** are used to denote significance of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively.

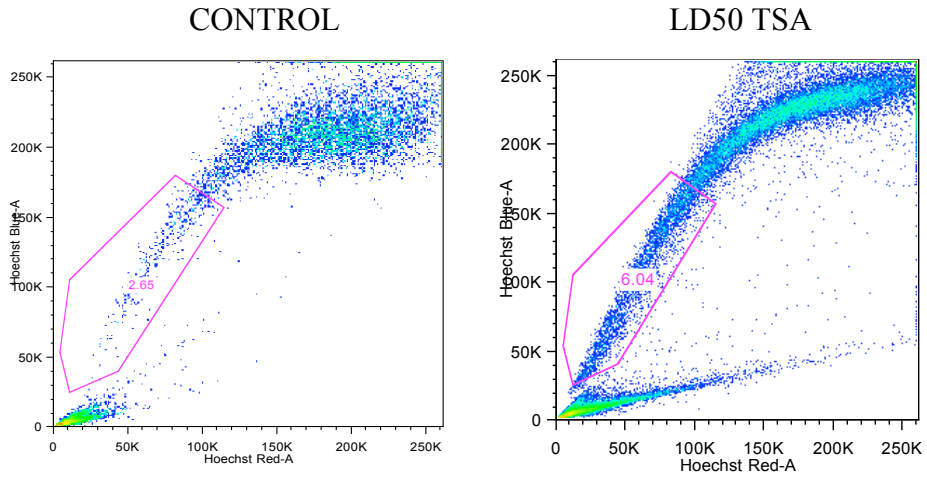
Figure 8: SP population of NB and teratocarcinoma cell lines is reduced by MS-275 treatment.







D


CONTROL
LD50 TSA
Hoechst Blue-A
Hoechst Red-A
2.65
6.04
0
50K
100K
150K
200K
250K

E

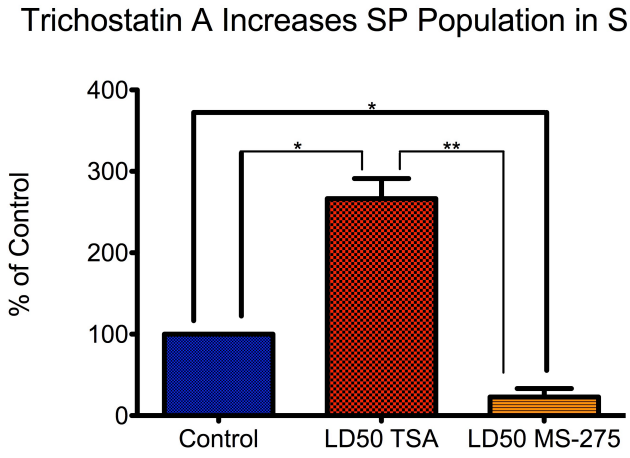

Trichostatin A Increases SP Population in SH-SY5Y
% of Control
400
300
200
100
0
*
*
**
Control
LD50 TSA
LD50 MS-275

Figure 8: The SP percentage of NB SH-SY5Y and SKN-BE(2), and teratocarcinoma NT2/D1 was determined by Hoechst exclusion and the effect MS-275 has on this population was analyzed. **A)** Representative profile of FACs analysis for all three cell lines. Gating was determined by a verapamil negative control. **B)** Percentage of SP in each cell line was analyzed with NT2/D1, SH-SY5Y and SKN-BE(2) containing 3.320±1.268%, 0.7433±0.3580% and 0.3233±0.1122% respectively. **C)** SP analysis following MS-275 treatment was quantified and normalized to percentage of control. A 48 hr treatment of 0.75 uM of MS-275 was able to reduce the SP population in NT2/D1, SH-SY5Y and SKN-BE(2) to 0.165±0.091, 18.547±8.131 and 29.705±11.013% of control respectively ($p < 0.0001$, $p = 0.0098$ and $p = 0.0237$). Similar treatments with 1.5 uM MS-275 reduced the SP population in NT2/D1, SH-SY5Y and SKN-BE(2) to 1.269±0.361, 22.884±10.403 and 1.082±0.650% respectively ($p < 0.0001$, $p = 0.0177$ and $p < 0.0001$). The reduction in SP percentage between 0.75 and 1.5 uM doses was not significant in any of the cell lines. **D)** Treatment of a equivalent LD50 dose of TSA (100 nM) induced potent SP population increase in SH-SY5Y. Representative FACs profile of control SH-SY5Y and LD50 TSA treated cells. **E)** Normalizing to control values, LD50 dose of TSA increase the percentage of Hoechst 33342 negative cells to 266.6±24.63% of control ($p = 0.0212$). This is as compared to a reduction to a 22.884±10.403% of control by the MS-275 LD50 dosage ($p = 0.0177$). The difference between the LD50 MS-275 and TSA dose was also significant of each other at $p = 0.0089$. FACs analysis were performed in triplicates and significance was calculated by paired two-tail t-tests. Astericks, *, ** and *** are used to denote significance of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively.

Figure 9: NB and teratocarcinoma cell lines express an ABCG2 positive population that decreases following MS-275 treatment.

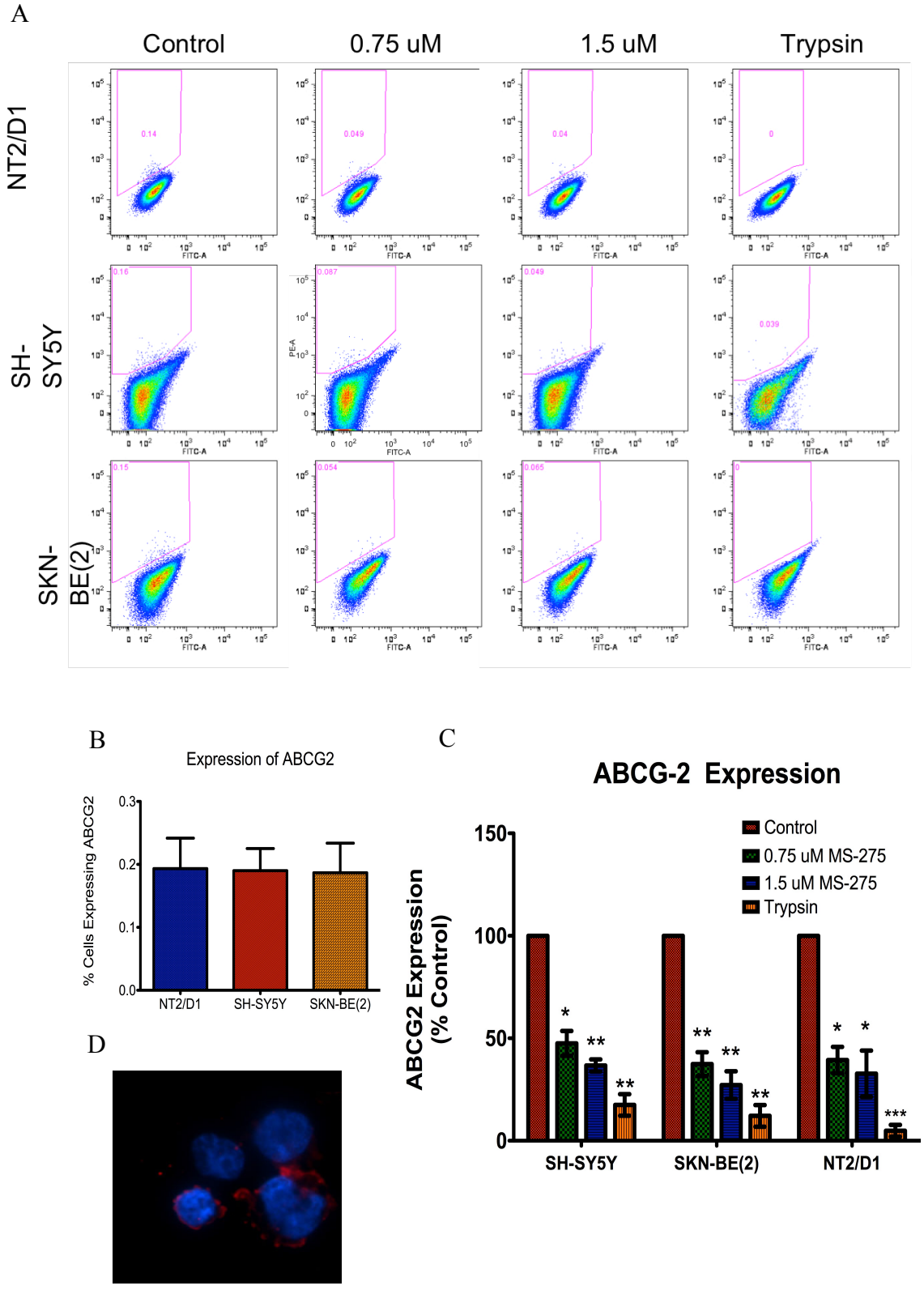
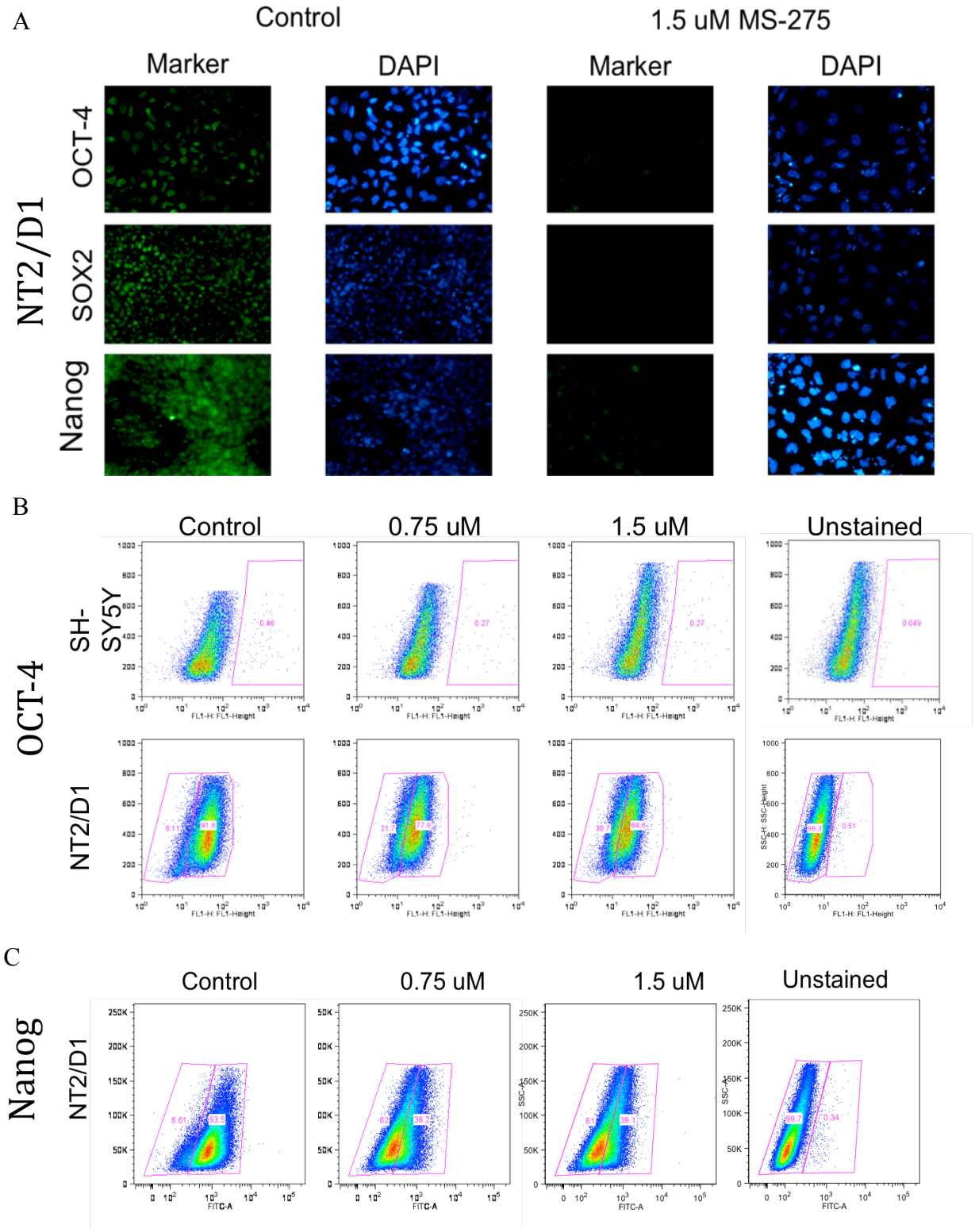
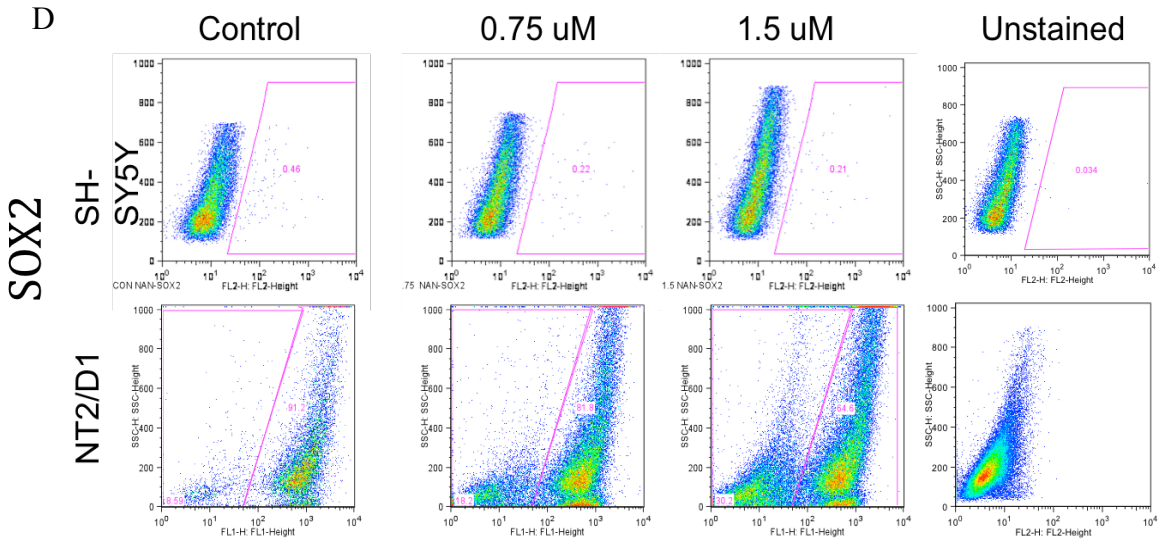
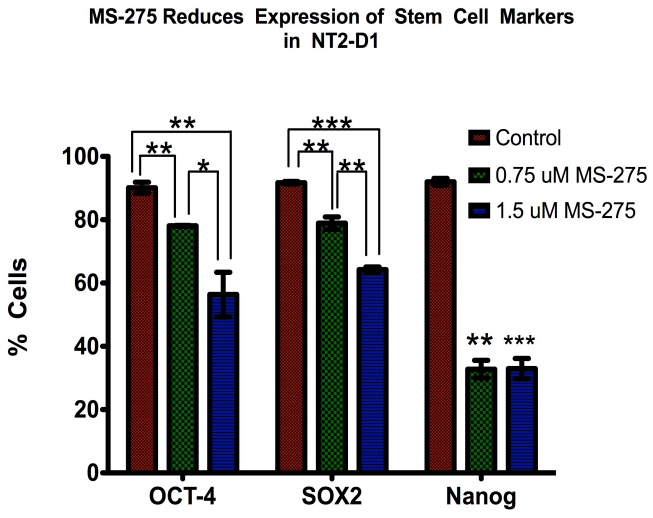


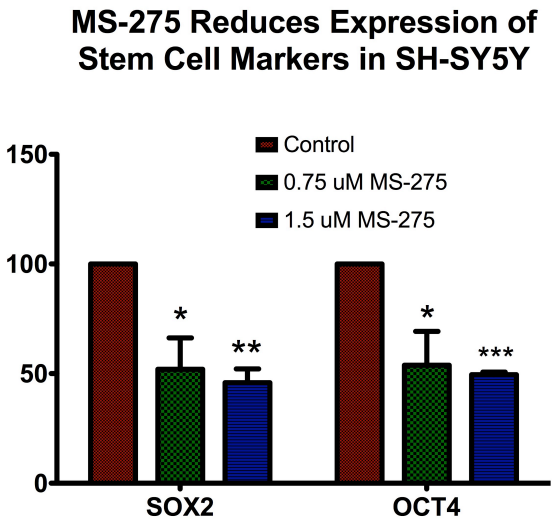
Figure 9: FACs analysis demonstrated that a 48 hr dosing of MS-275 decreases the percentage of cells expressing ABCG2 on the extracellular surface. **A)** Representative FACs profile for NT2/D1, SH-SY5Y and SKN-BE(2) of ABCG2 staining in control, 0.75 and 1.5 uM treated cells and in the trypsin negative control. Trypsin is used as a negative control because it can cleave the extracellular motif of ABCG2 that the antibody recognizes. Gating was compared to unstained cells and only viable cells (unstained with 7-AAD) were analyzed. **B)** Teratocarcinoma NT2/D1 and NB cell lines SH-SY5Y and SKN-BE(2) have 0.1933±0.04842, 0.1900±0.03512 and 0.1867±0.04702 % of cells expressing ABCG2. **C)** The percentage of ABCG2 expressing cells compared to control in NT2/D1, SH-SY5Y and SKN-BE(2) decreases following 0.75 uM MS-275 treatment to 39.345±6.430, 47.475±6.065 and 37.375±5.831% of control respectively ($p = 0.0111$, $p = 0.0131$ and $p = 0.0086$); and with 1.5 uM MS-275 to 32.686±11.299, 36.722±2.966 and 27.156±6.739 of control respectively ($p = 0.027$, $p = 0.0022$ and $p = 0.0084$). Trypsinization of control cells reduced the percentage of cells expressing ABCG2 to 4.861±2.864, 17.439±5.262 and 12.124±5.293 for NT2/D1, SH-SY5Y and SKN-BE(2) ($p = 0.0006$, $p = 0.0040$ and $p = 0.0061$ respectively). **D)** Surface staining of ABCG2 on fixed NT2/D1 cells demonstrates the specificity of the ABCG2 antibody (red = ABCG2, blue = DAPI; 400X magnification). FACs analysis were performed in triplicates and significance was calculated by paired two-tail t-tests. Astericks, *, ** and *** are used to denote significance of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively.

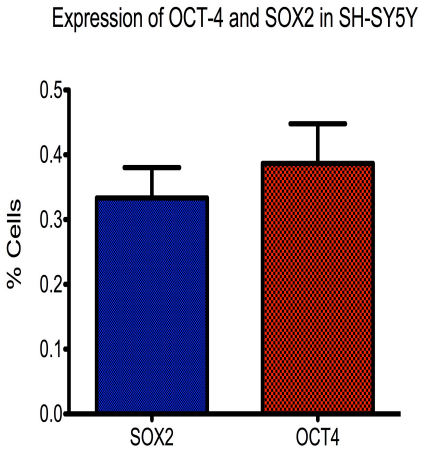
Figure 10: MS-275 reduces the percentage of cells expressing and expression levels of makers associated with self-renewal.

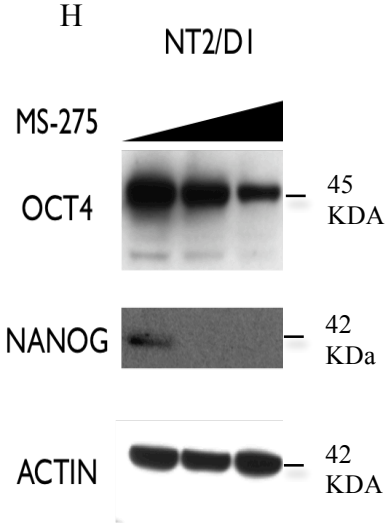



D
Control
0.75 uM
1.5 uM
Unstained
SOX2
SH-SY5Y
NT2/D1


E
MS-275 Reduces Expression of Stem Cell Markers in NT2-D1
% Cells
Control
0.75 uM MS-275
1.5 uM MS-275
OCT-4
SOX2
Nanog


G
MS-275 Reduces Expression of Stem Cell Markers in SH-SY5Y
Control
0.75 uM MS-275
1.5 uM MS-275
SOX2
OCT4


F
Expression of OCT-4 and SOX2 in SH-SY5Y
% Cells
SOX2
OCT4


H
NT2/D1
MS-275
OCT4
45 KDA
NANOG
42 KDa
ACTIN
42 KDA

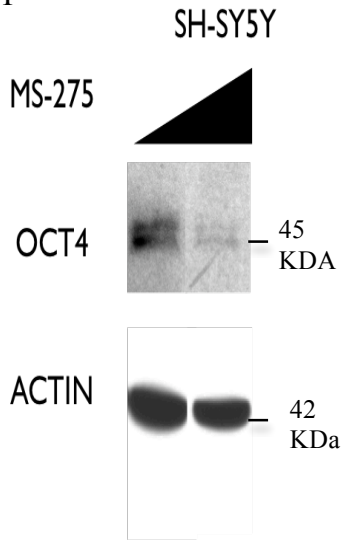

I
SH-SY5Y
MS-275
OCT4
45 KDA
ACTIN
42 KDa

Figure 10: MS-275 treatment reduces expression of stemness markers in both NB SH-SY5Y and teratocarcinoma NT2/D1. **A)** Immunofluorescence at 200X magnification of NT2/D1 for OCT-4, Nanog and SOX2. Nearly every cell expresses OCT-4, Nanog and SOX2 in the nucleus. Following treatment with 1.5 uM MS-275 for 48 hrs, there is almost a complete loss of expression. DAPI staining is shown side by side with antibody staining. **B)** Representative FACS profiles of NT2/D1 and SH-SY5Y cells treated with MS-275 and stained for OCT-4. Unstained denotes for negative control stained only with secondary antibody. This accounts for autofluorescence that occurs due to non-specific secondary antibody binding. **C)** Representative FACS profiles of NT2/D1 and SH-SY5Y cells treated with MS-275 and stained for Nanog. **D)** Representative FACS profiles of NT2/D1 and SH-SY5Y cells treated with MS-275 and stained for SOX2. **E)** Graphical representation of data derived from figures 10B, 10C and 10D. NT2/D1 cells contains 90.10±1.756, 91.96±1.073 and 91.70±0.404% of cells that express OCT-4, Nanog and SOX2 respectively. Following 0.75 uM MS-275 treatment, percentage of cells expressing OCT-4, Nanog and SOX2 dropped to 78.067±0.145, 32.77±2.790, and 78.933±1.954 respectively ($p = 0.0024$, $p < 0.0001$ and $p = 0.0031$). Treament with 1.5 uM MS-275 reduced the percentage of cells expressing OCT-4, Nanog and SOX2 to 56.367±7.015, 32.967±3.203 and 64.233±0.829% respectively ($p = 0.0096$, $p < 0.0001$, and $p = 0.0023$). The percentage difference for OCT-4 and SOX2 between low and high dose treatment was significant as well at $p = 0.0365$ and $p = 0.0023$ respectively. **F)** Graphical representation showing that SH-SY5Y cells contain 0.387±0.061% OCT-4 and 0.333±0.047% SOX2 expressing cells. **G)** MS-275 significantly reduces the percentage of cells expressing OCT-4 and SOX2 in SH-SY5Y following 0.75 uM treatment to 53.802±15.447 and 51.961±14.291% of control ($p = 0.0480$ and $p = 0.0391$). Treatment with 1.5 uM MS-275 similarly significantly reduced expression of OCT-4 and SOX2 to 49.458±1.149 and 45.778±6.306% of control ($p = 0.0003$ and $p = 0.0066$). The percentage difference between the doses was not significant. **H)** Western blot analysis of lysates from NT2/D1 cells treated for 48 hours with 0.75 and 1.5 uM MS-275 confirms the decrease in expression of OCT-4 (45 KDA) and Nanog (42 KDA). **I)** Western blot analysis of lysates from SH-SY5Y treated with 1.5 uM MS-275 confirms that

expressional decrease of OCT-4 (45 KDA). Two bands corresponding to OCT-4a and OCT-4b can be observed. FACs analysis were performed in triplicates and significance was calculated by paired two-tail t-tests. Astericks, *, ** and *** are used to denote significance of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively. Western blots were performed once, due to a lack of MS-275 reagent.

Figure 11: Percentage of cells expressing the TSC indicators OCT-4, SOX2, ABCG2, clonogenic capacity and SP may not necessarily correlate to each other.

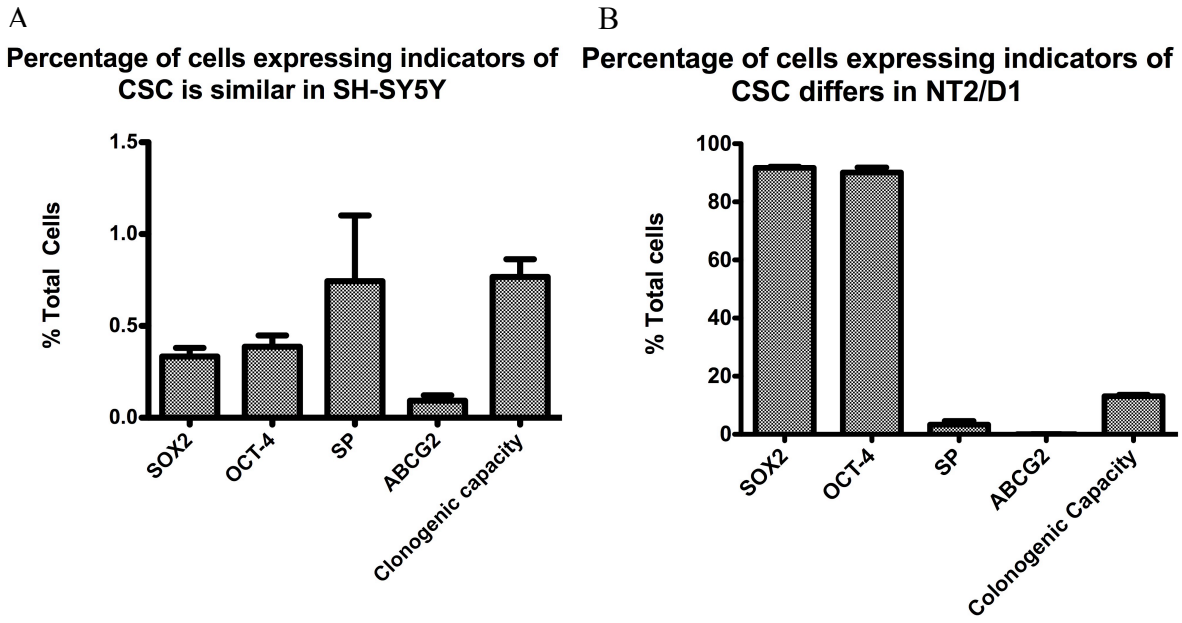


Figure 11: While both NB and teratocarcinoma have been previously found to contain a highly tumourigenic TSC-like population, they differ in that in NB SH-SY5Y, similar percentages of cells express the different indicators of TSCs, while in NT2/D1, the percentages of cells that express these indicators vary tremendously. **A)** In SH-SY5Y, the percentage of cells that express OCT-4, SOX2, SP, ABCG2 and clonogenic capacity is 0.387±0.061, 0.333±0.047, 0.743±0.358, 0.093±0.030 and 0.767±0.097% respectively. These values are all similar to each other and do not differ significantly from each other ($p>0.05$), with the exception of ABCG2. **B)** in NT2/D1, the percentage of cells that express OCT-4, SOX2, SP, ABCG2 and clonogenic capacity is 90.10±1.756, 91.7±0.404, 3.320±1.268, 0.076±0.032 and 13.10±0.550. With the exception of OCT-4 and SOX-2 all these values differ significantly from each other ($p < 0.05$). Significance was calculated using paired two-tailed t-tests.

3.1.3.7 Summary of results

The results in this chapter confirm that NB cell lines SH-SY5Y and SKN-BE(2), and teratocarcinoma NT2/D1 harbour a rare subpopulation of Hoechst 33342 excluding SP cells and that MS-275 can reduce this population significantly. MS-275 also dramatically reduced the clonogenicity of both NB cell lines and teratocarcinoma using a methycellulose based clonogenic assay. Taken together, these results suggests that the TSC-like cells of NT2/D1 and MYCN non-amplified NB SH-SY5Y and MYCN amplified SKN-BE(2) can be targeted by MS-275.

The reduction in the population of SP cells corresponds to a decrease in the percentage of cells expressing the ABCG2 drug pump on the surface. The reductions in clonogenicity and the SP phenotype were also correlated to a decrease in expression of, and in the number, of cells expressing stem cell markers OCT-4, SOX2 and Nanog. These stem cell markers are well-established regulators of self-renewal. Furthermore, TSA, an HDACi commonly used to study the effects of epigenetic modulation was found not to be able to reduce the percentage of SP. Rather, it potently increased the SP population. Finally, the percentage of SP cells was compared to the percentage of cells exhibiting clonogenic capacity and ABCG2, OCT-4 and SOX2 expression. It was found that in NB SH-SY5Y the percentage of cells expressing these TSC prognostic markers was similar, whereas in NT2/D1 SP the percentage was much lower than the percentage of cells expressing stem cell marker or clonogenic capacity.

3.1.4 Discussion

The results presented in this chapter demonstrate that HDACi MS-275 has a potential to target TSC-like cells found in NB cell lines and to target pathways and functional properties associated with TSCs.

MS-275 reduces clonogenicity

Clonogenicity, the ability of single cells to form suspension colonies in agar, methycellulose or other viscous media has been associated with highly tumorigenic cells that have high self-renewal ability [102, 131, 136, 152, 198, 199]. In this study we found that MS-275, was able to reduce the formation of colonies from N-type MYCN amplified SKN-BE(2) and non-amplified SH-SY5Y cell lines and the NT2/D1 teratocarcinoma cell line. Other HDACi such as HC-toxin have previously been shown to be able to reduce clonogenicity of NB cell lines [43] and MS-275 has been shown to reduce clonogenicity of PC3 prostate carcinoma, TC71 and TC32 Ewings sarcoma, RCC1-5 renal cell carcinoma and U937 leukemic cell lines from 40 to 100% relative to control [82, Wang, 2005 #279, 201]. However, while clonogenicity is an indicator of self-renewal capacity, a key property of TSC, it is also proposed and documented that transit amplifying cells slightly removed from TSCs also have clonogenic capacity [202]. In the self-renewal assay, colonies formed in methycellulose assays are digested to single cells and replated to form new clononies and a self-renewal index was calculated[199]. However, in our study we found that treatment with MS-275 at the LD20 and LD50 doses completely eliminate colony formation, rendering a self-renewal assay unnecessary. Furthermore, whereas the 0.75 uM dose in the methycellulose assay produced a complete loss of colonies, the same concentration in monolayer only reduced cell viability by 20%. This decrease in colony formation and cell viability suggests 1) that MS-275 can target cells with high self-renewal capacity; 2) sensitivity to MS-275 is highly dependent on culture format and organization. Furthermore, while NB cell lines have a low clonogenic potential and NT2/D1, as expected, has a high clonogenic potential, MS-275 was able to reduce the colony forming capacity to a similar level. In fact the methycellulose dose response curves, when normalized to percentage of control, were nearly identical. This

further suggests that the mechanism by which MS-275 targets the clonogenic/self-renewing cells is not simply a non-specific cytotoxic one that targets all cells. However, from this assay, it was not possible to determine whether the clonogenic/self-renewing cells have undergone cell death, or have simply lost their potential to self-renew.

MS-275 reduces the percentage of cells expressing self-renewal proteins OCT-4, SOX2 and Nanog

The ability of stem cells to self-renew has been documented to be due to their expression of stemness proteins OCT-4, SOX2 and to a lesser extent Nanog [166, 203]. While many other proteins have also been identified, these three are the core and critical ones. TSCs have also been noted to express high levels of OCT-4, SOX2 and Nanog [130, 131, 136, 162, 163]. NB SP and TIC cells have also been noted to express higher levels of these proteins and genes associated with their transcriptional regulation [130, 136, 138]. Exogenous expression of these proteins has also been used to create iPS (induced pulripotent stem) cells [204]. HDACi such as TSA have been shown to be able to modulate stem cells by potentiating self-renewal and expansion of human stem cells. HDACi TSA and valproate have shown an ability to expand and maintain a primitive hematopoietic stem cell population by forcing them into cell cycle replication [205-207]. VPA has also been shown to be able to enhance the induction of stem cells by the reprogramming of mouse embryonic fibroblasts[204]. KLF4, another self-renewal related protein expressed in neural crest stem cells, is upregulated in colon carcinoma cells by butyrate and SAHA[208, 209]. In contrast, it has been noted that HDACi such as TSA can target, modulate, and impair myeloid stem cell differentiation, potentially resulting in an impairment of blood cell development[57, 210]. MS-275 and SAHA, two HDACi furthest into clinical trials (SAHA is already approved to treat CML), have also been document to promote apoptosis and senescence of human mesenchymal stem cells through the activation of the intrinsic pathway of apoptosis and cell cycle inhibition via p16 and p27[181]. As such, it is critical to determine whether the stemness self-renewal pathways of TSCs are targeted.

Following MS-275 treatment of NB SH-SY5Y, both the OCT-4 and SOX2 expressing population was significantly decreased by 50%. This result suggests the idea that MS-275 can target the self-renewal pathway in NB TSCs. This perhaps is the reason for the loss of clonogenicity in the methylcellulose assay. The results of this study strengthen the notion that MS-275 can target the self-renewal machinery of stem and stem cell like cells. Furthermore OCT-4 and SOX2 has been noted to be co-expressed and work upstream of Nanog[203]. In this study we found that NB SH-SY5Y has a population of cells that express OCT-4 and a population that expresses SOX2. While their co-expression was not investigated, the percentage of cells expressing OCT-4 or SOX2 was nearly identical. Interestingly, OCT-4 and SOX2 populations were reduced to a nearly identical percentage. This may suggest, but by no mean conclusively, that they are coexpressed. Because OCT-4 and SOX2 has been repeatedly documented in TSCs and their expression correlates with the TSC phenotype, our results suggest that MS-275 is able to target the NB TSC-like population. Whether MS-275 induces TSC cell death, differentiation, or just loss of self-renewal capacity is not known and needs to be investigated.

To further elaborate on the potential of MS-275 to target TSCs we exploited the NT2/D1, a cell line where greater than 90% of cells express (albeit non-functional forms) of the self-renewal proteins OCT-4, SOX2 and Nanog at high levels[166]. We found that MS-275 significantly reduced the percentage of cells and overall expression of OCT-4, SOX2 and Nanog. Again we noted that OCT-4 and SOX2 percentages were nearly identical before and after treatment, further strengthening the notion that they are co-expressed and affected similarly by MS-275. Nanog expression was decreased substantially greater than OCT-4 or SOX2, perhaps due to the fact that in the pathway it is downstream. While in NB the percentage reduction in cells expressing OCT-4 or SOX2 may indicate a targeting of the TSC-like population, this may not be concluded with NT2/D1. Furthermore, it has previously been noted that two isoforms of OCT-4 exist. OCT-4a is more commonly associated with the stem cell phenotype then OCT-4b which can be found in non-pluripotent cell types[211]. In our western analysis of NB, both isoforms could be seen, and both were decreased following MS-275 treatment. Therefore, we surmise that MS-275 affected the TSC like fraction in NB.

SP, a model of TSC is significantly reduced by MS-275

The ability of cells to exclude Hoechst 33342 has been well established to be a property of stem cells and stem cell like cells[125, 126]. While there are exceptions to the case, it is well accepted that these SP cells are stem cell like. In primary cancers, SP cells isolated have been characterized as TSCs[107]. In-vitro, numerous cancer cell lines have been found to contain a SP population that is highly tumorigenic, having high self-renewal capacity, have increased dissemination capabilities, resistance to therapeutics and have a TSC molecular signature[123, 125]. As such SP cells from cell lines are TSC-like and can serve as a model to study TSC behaviour and biology.

While SP is well accepted as a model to study TSCs, there are also a number of caveats to their usage[123]. Hoechst 33342 binds to DNA and is well known to be toxic. Therefore experiments that sort SP and compare the tumorigenicity and/or biology of the SP and non-SP population have drawn criticism[123]. Furthermore, Hoechst 33342 SP assay, being based on the exclusion of the dye by ATP-dependent pumps such as ABCG2, is very sensitive to drug concentration, dose time and handling. Different cell lines and cell type have been shown to require different concentrations and dosings to effectively isolate a SP population. Because the expression of drug pumps define the SP population, there is the argument that they reflect only a drug resistant phenotype, and not a true TSC population. Finally, because SP is analyzed by FACs, instrument gating (signal paramaters) is critical. While verapamil, a drug pump inhibitor is used as a negative control to define the gating, many authors have shown that the SP tail can be separated into 3 distinct populations, where the true dye excluding cells are in the bottom tip of the tail[126]. However, a flow cytometer with a powerful UV laser is required to identify such a population.

Despite of all these caveats associated with SP, NB SP cells isolated from in-vitro cell lines have demonstrated a a highly tumourigenic population where only 100 cells were able to form a xenograft in-vivo[124, 130]. This strengthens the concept that NB SP is enriched in TSC-like cells that behave similar to the putative ultimate TSC that can form a tumor from a single cell. As such, in this thesis work SP was used and treated as a

population that is enriched in TSC-like cells. Previously, our lab has documented that not only do NB cell lines have a SP population that is enriched in TSC-like cells in that they express stemness proteins, the ABCG2 pump, have increased self-renewal capacity and tumorigenicity, but they are also insensitive to conventional therapeutics such as cisplatin[130, 136]. Others have also shown that mitoxantrone, a topoisomerase I inhibitor, is unable to target the SP population of NB despite inducing cytotoxicity in the non-SP population [135]. The inability of conventional therapeutics to target TSC has been associated with these cells being quiescent and having high drug pumping capabilities. Hirschmann-Jax et al., also demonstrated in their work that mitoxantrone actually increased the SP population of NB cell lines[135]. Though they attributed this to normal variance of the SP population, Tsuchida et al., have similarly shown that cisplatin can expand the SP population by way of activating a VEGF/FLT1 autocrine loop [136]. As such, while MS-275 has shown an ability to reduce clonogenicity and reduce expression of and percentage of cells expressing stemness markers, it was important to determine whether MS-275 could target the SP population of NB. Our results demonstrate that the SP populations of MYCN and non MYCN amplified NB and NT2/D1 was significantly reduced by MS-275. This suggests that the SP cells are sensitive to MS-275. While HDACi has been theorized to be able to target TSC because of the key roles HDAC play in transcriptional control of self-renewal [172], this is the first evidence that a HDACi can indeed target them. However, as a control, SH-SY5Y, which contains ~1% in SP cells were treated with trichostatin A, a natural potent hydroxamic acid HDACi that predominantly inhibits type 1 HDACs [31]. Because it is effective at low nM concentrations, it has been thoroughly investigated as an anti-cancer agent in numerous neoplasms[31]. SH-SY5Y cells treated with TSA saw a greater than 2.5 fold expansion of the SP population.

Furthermore, because NB SP is defined by the expression of ABCG2 drug pumps, it will be important to determine whether there is a transient upregulation of ABCG2 in cells, and whether this translates to increased tumorigenicity.

<u>MS-275 reduces the percentage of cells expressing ABCG2</u>

Importantly, the mechanism by which MS-275 targets the NB SP needs to be explored. Clonogenic data and expression analysis of stemness genes suggested that the self-renewal pathway was being targeted, but the change in expression of self-renewal proteins could be a secondary effect due to induction of cell death or differentiation. NB SP analysis is based on expression and function of the ABCG2 ATP-dependent drug pump. In NB, it has previously been reported that ABCG2 plays the predominant role in defining the SP phenotype [135]. Decreased expression of ABCG2 might equate to a loss of SP in NB. It is also critical to assess whether ABCG2 and other drug pumps associated with Hoechst 33342 exclusion are upregulated, especially with respect to HDACi. Because HDACi have the potential to upregulate transcription of up to 10% of the genome, an upregulation of drug pumps would lead to an increase in SP phenotype and drug resistant cells [32]. Indeed Hauswald et. al, have recently demonstrated in acute myeloid leukemia lines that phenylbutyrate, valproate, TSA, suberoylanilide hydroxamic acid (SAHA, Vorinostat), induced an overall drug resistance phenotype that increased drug efflux by inducing expression of P-glycoprotein and ABCG2 by the hyperacetylation of the histones associated with their promoter regions [34]. This was also observed with the HDACi apicidin in HeLa cells, where P-glycoprotein was induced, resulting in an increase in the rhodamine 123 negative population (similar to Hoechst 33342 exclusion) [212]. Evidently, while HDACi can induce cell death and differentiation, they can also induce a more drug resistant phenotype. Of note, a reduction or increase in SP cells due to an increase/decrease expression of ABCG2 may not necessarily translate into changes in tumorigenicity. ABCG2 positive and negative cells have been documented in some instances to be similarly tumorigenic [127].

In contrast, in this study NB cell lines SKN-BE(2) and SH-SY5Y, and teratocarcinoma NT2/D1, when treated with MS-275, the percentage of ABCG2 expressing cells decreased. This decrease correlated with the decrease in SP percentage and also the decrease in expression of stemness protein expressing cells. Curiously, however, the actual ABCG2 expressing population as determined by FACs analysis was substantially lower than the percentage of SP. This has previously been noted, as ABCG2 is not the only pump associated with SP, and also that not all SP cells may be truly TSCs, but may include transit amplifying cells that are just shortly removed in ability to self-renew as

compared to the TSC [124-126]. Furthermore, this decrease may also be a result of the detection limit of the antibody employed and the flow cytometer, or the gating that was chosen. Furthermore, our results do not address what is happening to the ABCG2 protein. While FACs analysis shows that the percentage of cells expressing it on the cell surface has decreased, it may be that the protein itself has been internalized or perhaps ABCG2 transcription has been upregulated. Further studies need to be carried out to characterize the decrease in ABCG2 expressing cell after treatment of MS-275.

Identification and characterization of the NB TSC

ABCG2 expression, SP, stemness protein expression and clonogenic capacity have all been linked to TSCs and have been proposed as a way to identify them [94]. In this study we noted that in NB SH-SY5Y, the percentage of cells expressing these markers was similar, suggesting, but not concluding, that each of these identifies a similar population. However, the data on NT2/D1 reinforces the idea that no one test should be used alone in trying to identify TSC or TSC-like cells. In NT2/D1, the ABCG2 expressing population was dramatically lower than the SP, the clonogenic potential and the expression of stemness markers, indicating that non-functional proteins, or a large transit amplifying population may skew TSC identification when using any one technique. This highlights the need to keep developing assays and discovering markers that may be able to indentify NB TSCs. CD133, Nestin, CD117, and the aldefluor assay have all been proposed or investigated to identify NB TSCs [117, 135]. However, none of these have been well substantiated in multiple NB lines as a putative NB TSC marker.

Walton et al., have proposed that the I-type NB represent NB TSCs. While these I-type cells are developmentally more primitive, have increased tumorigenicity, are associated with prognostically poor NB and have a high self-renewal capacity, they can hardly be called TSCs [5]. Ross et al., deduction of I-type as NB TSC was based on the fact that injection of 5 million cells into immuno deprived mice formed xenografts at higher frequencies than other NB phenotypes. In addition, they assayed clonogenicity and claimed self-renewal capacity. A truly tumorigenic TSC population would form tumors at very low numbers reflecting a single cell tumor formation capacity. While their

conclusion that I-type cells in culture represent NB TSCs is ambitious, they proposed an method of identification of NB TSCs, in that more primitive NB cells express markers of both neuronal and Schwannian lineage. It will be of interest to assay whether NB SP cells that are enriched in TSCs express an elevated level of such markers and whether such an identification method could help with the identification of the true NB TSCs.

Chapter 4:
Conclusions and Future Directions

4.1 Conclusions

Summary

In this thesis we demonstrated that HDACi MS-275 has the potential to target NB in-vitro by decreasing viability of the overall cancer cell line population and further, by targeting its TSC like component. In SH-SY5Y, a MYCN non-amplified NB cell line, MS-275 forced cells into a G1 cell cycle arrest through the upregulation of p16 cell cycle inhibitor, and the decreased expression of the CDK4/CyclinD1 complex. The decrease in Ki67 expression suggests cells are exiting cell cycle as well. Early and late apoptosis was induced as well through the intrinsic pathway of apoptosis, characterized by a decrease in expression of the anti-apoptotic proteins BCL2 and survivin and an increase in pro-apoptotic protein BAX. Cleaved caspase 3 was also found to be increased. It was also shown that MS-275 induced morphological changes to NB that reflect a more Schwannian phenotype. This was confirmed by the increase of S100beta expression detected by western blotting. A loss of neuronal processes of SH-SY5Y did not correspond to a decrease in neurofilament expression. Instead, mature neurofilament isoform NF200 was found to be expressed following MS-275 treatment despite a lack of observable neurites. Nestin, a neural progenitor marker was induced despite expression of mature NF isoforms. As a whole this evidence suggests that HDACi MS-275 inhibits NB viability by modulating cell cycle, apoptosis and differentiation.

With the discovery of TSCs, it is becoming evident that to completely treat a tumor, its TSC population must be targeted. As such, MS-275 was investigated for its ability to target the TSC population of MYCN amplified and non-amplified NB cell lines SH-SY5Y and SKN-BE(2). Teratocarcinoma NT2/D1, a reference embryonic cell line, was also investigated. Our results demonstrated that NB and NT2/D1 teratocarcinoma cell lines all contain a SP population that has previously been shown to possess tumorigenicity and to be enriched in TSCs. MS-275 was able to significantly reduce this population. To characterize the effect on the TSC-like population, we obtained evidence that MS-275 reduces clonogenicity completely. This loss of clonogenicity corresponded with a decrease in the expression of stemness proteins involved with self-renewal.

Furthermore, MS-275 was discovered to reduce the percentage of cells expressing ABCG2, the key pump that defines the SP population.

Together the data in this thesis suggests that MS-275 is a potent anti-NB therapeutic agent which can target NB by inducing growth arrest by classical mechanisms attributed to HDACi, and importantly by reducing its TSC population.

Insights toward the mechanism of action of MS-275

The ability of MS-275 to induce a G1 cell cycle arrest and apoptosis argues in favor for evaluating its therapeutic potential in pre-clinical models and in usage for clinical trials alone or in combination with other therapeutics to classically target the proliferating tumor cell population to reduce tumor growth. Indeed, Jaboin et al, have shown in their study that in preclinical mouse models of NB that MS-275 can reduce growth of NB cell line KCNR xenografts when injected to the periadrenal fat pad [54]. Inhibition of NB xenograft growth and tumor size has been noted for other HDACi such as CBHA, 4-phenylbutyrate, TSA and BL1521 [41, 45-47, 54, 67]. Whether this anti-tumor effect in-vivo is due to the targeting of only transit amplifying tumor cells or the TSC component is not known. Because the SP of NB cell lines have been demonstrated to be the population that contains the highly tumorigenic TSC component, a critical reduction in this component would translate to reduced tumorigenic potential, aggressiveness, minimal residual disease and metastasis of a tumor.

While our results suggest that the SP is being reduced by the loss of ABCG2 and loss of stemness self-renewal associated proteins the question remains as to which signaling pathway is being affected. While this thesis did not directly address this issue, we offer a number of candidates for consideration and future study. Previously, in NB, it has been found that rapamycin a drug that targets the mTOR survival pathway preferentially targets NB TICs [116]. In leukemia, TSCs were found to have nonfunctional PTEN, which normally suppresses the mTOR survival pathway [213]. Furthermore, in these leukemic lines rapamycin was shown to be able to target these cells [214]. Similar studies have also shown the efficacy of rapamycin and other drugs that target the mTOR pathway in combating pancreatic and breast TSCs [156, 215]. Our lab previously

showed that a VEGF/FLT1 autocrine loop induces the expansion and survival of SP in NB [136]. Therefore it is relevant that VEGF production and signaling is partly dependent on mTOR induced-expression of HIF-1alpha [216]. HIF-1alpha is also found to be upegulated during hypoxic conditions, which has been shown to expand stem cells and tumor stem cells [130, 139, 140, 217]. Taken together, it appears that the mTOR/HIF/VEGF pathway may play an important role in TSC biology. It has recently been reported that MS-275 can target the mTOR signaling pathway and cooperate with rapamycin to induce enhanced cell death and differentiation in acute myeloid leukemia [218]. Furthermore, HDACi have been shown to be able to induce acetylation of HIF-1alpha and inhibit its transcriptional role [219, 220]. HDACi treatment also inhibits HDAC7, preventing it from binding to HIF-1alpha and activating it transcriptionally [221]. MS-275 and other HDACi also have well documented ability to inhibit angiogensis by modulating the expression of VEGF, VEGF-R [39, 222, 223]. VEGF-R2 transcription is also shown to be affected by HDACi, as their transcription complexes such as HOX9A contain HDAC that can be enzymatically inhibited by HDACi [224]. VEGF-R3 was shown undergo ubiquitination to protein degradation by MS-275 [223], while VEGF is similarly targeted for ubiquitination or prevented from secretion by TSA, sodium butyrate and SAHA [39, 225, 226]. Decrease in VEGF by HDACi also leads to a decrease in expression of VEGF receptors [39]. In addition, cytosolic thioredoxin-1 which stabilizes HIF-1alpha and induce increase translation of HIF-1alpha via the mTOR pathway [227] can be modulated by HDACi such as SAHA and MS-275 [50, 51]. Thioredoxin-1 has also been demonstrated to stabilize OCT-4 DNA binding and induce its transcriptional activity [228]. In NB, in TIC preclinical mouse xenograft models, it has been shown that rapamycin acts synergistically with a tyrosine kinase inhibitor, sunitinib, to target VEGFR expression and thereby effectively reduce tumor load [142]. Given the above findings, MS-275 and other HDACi may prove effective in synergizing with sunitinib and rapamycin to target NB TSC and TIC. Furthermore, in conjunction with the other cytotoxic and differentiating effects exhibited by MS-275, MS-275 may prove to be a powerful drug that can target tumours at both the TSC and transit amplifying levels. The proposed mechanisms by which MS-275 and other HDACi may affect TSC is summarized in figure 12.

In this study we also demonstrated that TSA, a potent hydroxamic acid HDACi that targets class one HDACs was able to dramatically increase the SP following treatment. We have yet to determine whether this would correlate with increased clonogenicity, increased tumorigenicity and expression of TSC markers. However, it may be possible that the increase in the detectable SP phenotype is due to upregulation of ABCG2, and therefore, it may not translate to an increase in the TSC compartment. Importantly, this result raises a cautionary flag and suggests that despite the potential therapeutic benefits that HDACi may have in inducing cell cycle arrest, apoptosis and differentiation, their clinical application needs to be thoroughly investigated whether drug resistance or stemness is modified. As discussed earlier, both drug resistance and stemness have been shown to be induced or potentiated by HDACi in AML and hematopoetic stem cells respectively [34, 206]. In addition, the effect TSA has on SP has also been observed with chemotherapeutics such as cisplatin and mitoxantrone [135, 136]. In cisplatin, an increase in the SP phenotype correlated to an increase in tumorigenicity. This then raises an interesting and clinically relevant questions regarding HDACi differences.

As both MS-275 and TSA are type 1 HDAC inhibitors that have been documented to induce cell death, cell cycle arrest and differentiation in NB, the question arises as to why they appear to have different effects against the SP population. Experimentally it has been found that HDACi have differential effects and efficacy against different HDACs [172]. This implies that perhaps a differential inhibition of different HDACs may be the reason for the observed differences between two HDACi that inhibit nearly identical HDACs. TSA is also known to be a powerful HDACi that inhibits a broad spectrum of type 1 HDACs [31]. Because MS-275 is more selective in that it can only inhibit HDAC 1 and 3, it may operate more selectively than TSA. We observed (data not shown) that MS-275 at lower doses had no effect on the SP population. Perhaps this suggests that because HDAC3 is inhibited at uM levels, and HDAC1 is inhibited at low nM doses, it is the HDAC3 targeting that induces the change in SP.

Our results with the clonogenic assay and reduction in OCT-4 and SOX2 expressing cells in NB by MS-275 also suggest that self-renewal is targeted. Previously, Tsuchida et al., reported that a VEGF/FLT1 autocrine loop in NB promotes the survival of SP cells and may regulate OCT-4 expression. Furthermore, HIF-2alpha overexpression by hypoxia has been linked to OCT-4 expression as well [164]. Because HDACi can modulate the hypoxia inducible factor pathway, the VEGF pathway and the mTOR pathway which links them, it stands to reason that HDACi may be able to target the self-renewal pathway. Recently, it has also been described that SIRT1, a type 3 HDAC, regulates HIF-2alpha to regulate OCT-4 [229]. Also, a combinatorial usage of rapamycin and HDACi LBH589 has shown to be able to target HIF-1alpha potently to reduce angiogenesis in prostate and renal carcinoma cell lines PC3 and C2 [230]. In addition, as stated earlier, thioredoxin-1 has been found to associate with and increase OCT-4 DNA binding and transcription[228]. Thioredoxin-1 has also been found to be downregulated by HDACi SAHA and MS-275 directly or by upregulation of thioredoxin binding protein[51]. Therefore, while there is no direct evidence to show HDACi can modulate self-renewal, multiple lines of evidence suggests that such a link exists.

The self-renewal proteins are classically documented in embryonic stem cells, adult stem cells, and have been noted in numerous TSCs. Their ability to regulate self-renewal and prevent differentiation in stem cells has been documented to be due to their association with HDAC repression complexes such as Nurd and Sin3 in what is coined the NODE complex [200]. The HDACs associated with these repression complexes have been noted to be type 1 HDACs. As MS-275 can enzymatically inhibit type 1 HDACs, it is also plausible that HDACi can interfere directly with OCT-4 expression and its differentiation repression ability and also in conjunction to its control in modulating HIF expression. In NB, MYCN amplification has been documented to maintain and regulate the expression of KLF4, which is also a protein of pluripotency commonly found in neural crest stem cells and one of the 4 proteins found to be necessary to induce pluripotency in fibroblast cells [137]. Numerous HDACi, including MS-275 have been documented to be able to reduce amplified MYCN expression, suggesting that in tumors where MYCN is amplified, HDACi may target self-renewal by targeting MYCN expression [36, 38, 66, 160]. Interestingly, while KLF4 along with c-MYC, OCT-4 and SOX2, is one of the 4

critical genes discovered needed to induce pluripotency, KLF4 and c-MYC were found to be able to be substituted by HDACi valproate, strengthening the idea that HDACi can induce pluripotency[204]. Finally, while our results suggest that HDACi MS-275 can target the TSC population, it must be noted that HDACi have been shown to induce proteins involved with self-renewal as well. In colon cancer lines, HDACi butyrate and SAHA induced the expression of KLF4[208, 209]. While the authors did not note an increase in self-renwal capacity, they showed that an induction of KLF-4 induced resistance to apoptosis and cell cycle arrest that is commonly associated with HDACi treatment.

It has been recently discovered that HDAC8 plays a critical role in NB tumor behaviour [70]. Oehme et al., have previously documented that HDAC8 is associated with advanced disease, metastasis, poor prognostic markers, and decreased overall survival. Knockdown and inhibition of HDAC8 inhibited proliferation, clonogenicity, cell survival, p21 induced cell cycle arrest and induced neuritic differentiation of SKN-BE(C) I type NB cells (a selected metastatic subclone of SK-N-BE(2)). Overexpression of HDAC8 similarly prevented retinoid induced differentiation. These observations parallel the overall trends that we have documented with MS-275 in SH-SY5Y and SKN-BE(2) in this study. It is therefore critical to ask whether MS-275 in NB is able to target HDAC8. However, MS-275 is known to inhibit HDAC8 only marginally at extreme high doses (~ 100uM) that are beyond the physiological pharmacological range[31]. These pharmacokenetic studies by Hu et al., demonstrated that MS-275 has only a low ability to enzymatically inhibit HDAC8 by assaying the ability of MS-275 to inhibit the capacity of HDAC8 to deacetylate histones in vitro using biochemical assays. As such it may be possible that in NB MS-275 can target HDAC8 by mechanisms not involved in histone deacetylation. For example, the direct binding of HDACi may inhibit their association with other protein complexes. Perhaps such a direct binding of MS-275 can deregulate HDAC8 involved pathways necessary in regulating NB phenotype. Furthermore, the HDAC8 used in the study of Hu et al., was recombinantly made. It is also possible that the recombinant HDAC8 is correctly formed and reflects a form found in-vivo. As such Hu et al.'s assessment of HDAC8 enzymatic inhibition by MS-275 may not be sufficient in assessing the true ability of MS-275 to modify HDAC8 activity and signaling.

Clinical implications for application of MS-275

NB metastasic disease has been linked to TSCs as they have increased homing capacity and metastatic capacity [124, 130]. It has also been noted that NB cells isolated from bone marrow possess a TSC like phenotype[116]. Indeed, Hansford et al., isolated a NB TIC from patient bone marrow samples and noted that a greater population of more primitive NB cells reside in the bone marrow of metastatic disease[4]. Recently, it has been proposed that retinoids can be used to target metastatic NB disease, as they can induce differentiation[16]. The differentiation of a metastatic TSC would prevent the formation of metastases. In this thesis, we noted that not only can MS-275 target the NB TSC-like population, but it is possible it can generally induce differentiation of NB. While neurite extension was not noted, we obtained evidence for both neuronal and Schwannian type differentiation. MS-275 has been noted previously in the prostate cancer lines PC3 to be able to induce differentiation alone or in conjunction with retinoic acid to induce a more benign phenotype[191, 192]. Furthermore, it maybe that the loss of SP documented here is due to a differentiating effect imposed by MS-275. Recently, it has also been documented that leukemia TSC and metastatic NB SP have high expression of CXCR4 and that the CXCR4/SDF1alpha chemokine pathway plays a critical role in their metastasis[130, 231]. Indeed, inhibition of CXCR4 or SDF1alpha has led to decreased homing, invasion and metastasis in in-vivo mouse models. HDACi, LBH589 have been previously described to be able to reduce CXCR4 expression [222]. However, it must also be noted that in hematopoetic stem cells that HDACi TSA and valproic acid have been documented to increase expression of CXCR4 in melanoma cells [232]. Nevertheless, it can be surmised that MS-275 has the potential to impact NB metastatic disease by targeting the TSC-like component and inducing differentiation.

Minimal residual disease has also been linked to TSCs since these cells are chemoresistant and are able to reform tumors from just one cell. TSCs have been documented to be resistant to chemotherapy because of their quiescent state, increased expression of drug pumps and also expression of strong anti-oxidant mechanisms that are similar to that in normal stem cells [121, 123, 125, 157, 158]. As such a drug regimen that does not target this population may lead to relapse. In this thesis, we have

documented that HDACi MS-275 can target the TSC-like population. This may perhaps translate to it being able to prevent minimal residual disease. As MS-275 also appears to induce a neuroblastic like SH-SY5Y cell line into a Schwannian phenotype in culture, it may be possible that MS-275 has the potential to transdifferentiate NB cells. S-type NB cells have previously been documented to be markedly less tumorigenic than N- or I-type NB and have lower self-renewal potential [5]. In NB, tumors possessing more differentiated ganglionic cells and Scwhannian stroma are less aggressive and have a favorable outcome [2]. This is further reflected in the observation that NB, the most undifferentiated and least stromal containing neuroblastic tumor is the most aggressive. GNB and GN which are more differentiated or completely differentiated with high amounts of stroma are less aggressive and fare prognostically better. In fact GN is considered benign. It has also been shown that both the neuroblast and Schwannian component of NB tumors can arise from a similar cellular origin[7]. This suggests that an agent that can transdifferentiate tumorigenic N- and I- type NB cells into a S-type cell produce a more benign phenotype. MS-275 may have such a potential. In addition to its cell cycle inhibiting, apoptotic inducing and TSC targeting capability, this may further support the clinical potential of MS-275 for treatment of NB.

Figure 12: Possible Anti-TSC mechanism of HDACi

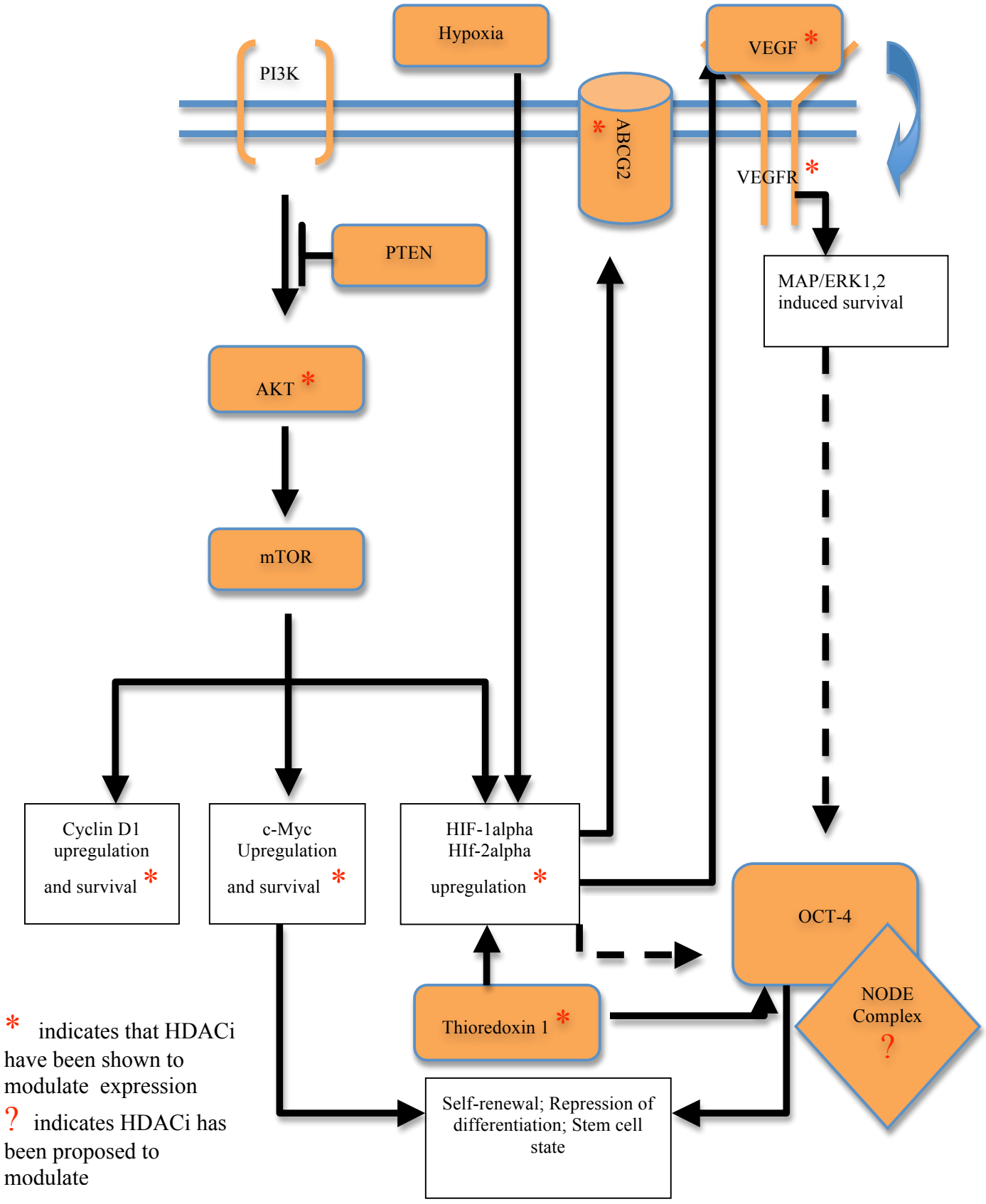


Figure 12: Possible mechanisms of TSC self-renewal targeted by HDACi. mTOR pathway is proposed to play a key role in survival of TSC population[213, 215, 233]. mTOR induces expression of cyclin D1, c-MYC and HIF-1alpha and HIF-2alpha[215, 216]. HIF upregulation is also directly induced by hypoxia [234]. HIF-2alpha induction has been associated with maintenance of OCT-4 expression, self-renewal and the stem cell state [164]. HIF induction upregulated production of ABCG2, an ATP-dependent drug pump that has been associated with TSC drug resistant phenotype [235]. VEGF and VEGF-R expression is also induced by HIF expression, and is a well known process in angiogenesis [164]. VEGF/FLT1 autocrine signaling has been associated with survival and expansion of stem cells [130, 136]. While the mechanism by which a VEGF/FLT1 autocrine loop regulates the stem cell state has not yet been elucidated, it has been shown that hypoxia induction of HIF is associated with maintenance and induction of the TSC. [130, 139, 164] The HDAC repression complex NODE associates with OCT-4 to repress transcription of differentiation programs [200]. c-MYC modulation has been associated with maintenance of a stem cell state in hematopoietic stem cells [236]. Thioredoxin 1 has been associated with stabilization and induction of HIF-1alpha and regulation of OCT-4 transcription[51, 227].

HDACi are able to modulate expression of the above proteins and pathways. However, the exact mechanism is not fully known. HDACi MS-275 has been shown to be able to block AKT activation of mTOR and survival in AML[218]. Cyclin D1 expression is decreased resulting in a G1 cell cycle block. This block has been demonstrated in this study and numerous others in various cancers with assorted HDACi[20]. HIF-1alpha and HIF-2alpha have been documented to be modulated by HDACi[219-221]. VEGF and VEGF-R expression is downregulated by HDACi[39, 221, 224-226]. The modulation of type 1 HDACs in the NODE differentiation repression complex has been previously proposed for HDACi[200]. ABCG2 downregulation is demonstrated in this study. Thioredoxin has previously been demonstrated to be downregulated by HDACi MS-275 and SAHA[50, 51]. Taken together, it appears the targeting of the mTOR pathway may play an important role in reducing TSC growth, survival and self-renewal capacity. c-MYC expression has also been previously documented to be reduced by HDACi SAHA, TSA and FK228 in colon, renal and prostate carcinomas, in-vitro and in in-vivo mice

xenograft models [237-239]. Dashed lines denote proposed models that have not yet been shown conclusively. Red asterics denote proteins and mechanisms modulated by HDACi.

4.2 Future Directions

In this thesis, we have shown that HDACi MS-275 can target the normal cancer cell line and also the TSC population in these cell lines. Along with the observation that HDACi have low toxicity against normal cells, HDACi MS-275 appears to be a potent anti NB therapeutic. However, it has also been noted that HDACi work best in combination. MS-275 has been used in combination with retinoids, demethylating agents, CDK inhibitors and IL-2 to treat a whole host of cancers including neuroblastoma, prostate cancer, hepatic cancers, leukemia and renal cell carcinoma[52, 83, 90, 91, 191]. Here the potential that MS-275 has to target TSC is suggested and this argues for its combinatorial usage with rapamycin to determine whether stronger anti-TSC effects can be induced in preclinical NB mouse models, as it has been shown for leukemia [218]. Perhaps MS-275 may work synergistically with rapamycin as sunitinib has been shown to, due to the ability of MS-275 to affect the VEGF pathway of angiogenesis as well as the mTOR pathway[142].

The potential of MS-275 to target metastasis and TSCs may both be due to similar mechanisms of inhibiting VEGF, mTOR and HIF pathways. Therefore there exists an opportunity to study a drug that can modulate all three pathways in concert and to elucidate whether it affects the self-renewal pathway. Previously, NB SP cells have been shown in to be expanded by hypoxia. Das et. al, proposed that a hypoxic niche maintains and expands the TSC population [130]. Hypoxia and reoxygenation induced a tumorigenic SP population into a phenotype that was more tumorigenic. If MS-275 is indeed able to modulate the activity of HIF1alpha or HIF2alpha by acetylation or modulation of HDAC associated with HIF1alpha, it would prevent the expansion of this population. Tsuchida et al., also demonstrated that a VEGF/FLT1 autocrine loop maintained the survival and perhaps expansion of NB SP cells. It will be critical to see whether this autocrine loop is affected by MS-275[136].

We have observed that while HDACi MS-275 reduced the SP population significantly, TSA in contrast expanded it. It will be necessary to characterize the loss of SP cells treated by such drugs. The loss of SP induced by MS-275 may be due to cytotoxic effects on the SP, or conversion of SP into non-SP. If there is a cytotoxic effect, this needs to be determined by sorting out SP cells and treating them with MS-275. In contrast, if the effect is due to a conversion of SP into non-SP, it must be determined whether this loss results in a loss of tumorigenicty as well, or whether there is just a loss of expression of cell surface drug pumps. While our results suggests that the latter is not the case, due to a reduction in self-renewal, it must also be determined whether the loss in SP induced by MS-275 is transient, in that cells can revert back to their original SP phenotype. Similarly for TSA, the increase in SP must be characterized in terms of effects on clonogenicity and tumorigenicity.

I have shown in this thesis that the molecular factors involved in maintenance of self-renewal of NB TSCs is affected. As HDACi have been shown to induce the expression of KLF4 in cancer, it will be necessary to assay the expression and functionality of KLF4 following treament, before further claiming that self-renewal is completely inhibited[208]. If KLF4 is induced it could represent a push of NB cells into a more primitive neural crest state that may or may not be tumorigenic [240]. Futhermore, self-renewal must be ascertained. In this study, only clonogenicity was assayed because no colonies were formed following MS-275 treatment. Colonies that remain following a lower dose treatment of MS-275 should be dissociated and replated in methycellulose to assess the true effect on assay self-renewal.

Differentiation of NB SH-SY5Y in this study must also be characterized in depth. It was demonstrated that MS-275 may perhaps have potentiated the induction of a Schwannian phenotype based on morphology and increased in expression of S100beta. Further molecular markers such as calcyclin are needed to further substantiate this[241]. Induction of mature neuronal markers may also indicate a potentiation of the neuronal pathway. Combinatorial treatment of MS-275 with retinoic acid will demonstrate whether extensive axonal networks predicted by elevated expression of NF200 can be produced. The induction of nestin also needs to be characterized. The induction of

nestin may signify a more primitive state. Previously, it has been noted by Mahller et. al, that TICs isolated from NB lines strongly express nestin[117]. Acosta et. al, found contrasting results showing that nestin is expressed at low levels in numerous NB cell lines[241]. Furthermore, Ross et. al, have also demonstrated that I-type NB cells which are more primitive and can give rise to both N- and S- types, express both neuronal and Schwannian properties. In effect, our results might suggest that MS-275 is inducing a more primitive progenitor phenotype in NB SH-SY5Y that may be further directed into neuronal or Schwannian lineages. Nestin is also found in myogenic cells, of which neural crest cells can give rise to[195]. Therefore a continuing experiment is to place NB SH-SY5Y cells treated in culturing conditions that stimulate myogenic, neurogenic or Schwannian lineage differentiation and to characterize the changes that occur. For example, it has been previously documented that BrDU can induce Schwannian differentiation in SH-SY5Y cells[242].

Given that HDAC8 plays a key role in NB and its downregulation and inhibition reduces tumorigenicity and clonogenicity, it is tempting to speculate that MS-275 may act on the HDAC8 pathway in NB[70]. Therefore, we would have to address whether HDAC8 is targeted, reduced or modified in NB following MS-275 treatment. If so, it offers another mechanistic level by which MS-275 may exert its effect on NB and in particular NB TSC.

Finally, in further studies of MS-275 against NB TSCs it is necessary to determine if MS-275 induces similar effects on normal stem cells. Since any therapeutic that can target TSC may also target normal stem cells, this is of crucial importance in the treatment of childhood cancers such as NB. This is because this patient population is highly dependent on a large pool of undifferentiated stem cells for development. To examine this possibility, MS-275 can be tested against SKPs (skin derived pluripotent cells) as they represent normal stem cell progenitors of the neural crest and exhibit similar differentiated phenotypes after induction [141].

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