AMERICAN UNIVERSITY OF BEIRUT

ASSESSING THE ANTI-CANCER ACTIVITIES OF NOVEL THERAPEUTICS ON HUMAN MEDULLOBLASTOMA CANCER CELLS

by
ROUBA JAMAL MEKAWI

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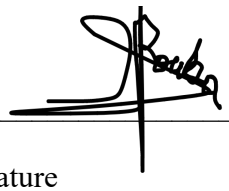

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ABSTRACT
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Title: Assessing the Anti-Cancer Activities of Novel Therapeutics on Human Medulloblastoma Cancer Cells

Medulloblastoma (MB) is the most common malignant intra-cranial solid tumor among children. Extensive research has been implicated in this field to look for novel therapeutic options that could help alleviate patients' symptoms or at least cease progression of the disease. Of late, new studies have indicated proof that a subpopulation of cells inside the tumor, namely cancer stem cells, is believed to be answerable for the protection from most therapeutics and radiation treatment, resulting in malignancy. Henceforth, it is critical to distinguish and to explicitly target those cancer stem cells. Therefore, assessing the effects of different novel therapeutics may contribute to the advancement of knowledge of the discrete mechanisms leading to pathological changes in the brain function and that can block the growth and spread of cancer by interfering with specific molecules. The overall aim of this proposal is to study the anti-neoplastic effect of thymoquinone which is abundantly present in seeds of Nigella sativa L. that is popularly known as black cumin or black seed, on human medulloblastoma cell lines (D556 Med and D283 Med).To begin to fill this void D556 Med and D283 Med will be cultured and maintained in DMEM High glucose media with 10% FBS and 1% P/S in a humidified incubator (37°C; 5% CO2). The cells will be treated with different concentrations of Thymoquinone reconstituted in methanol. MTT and Trypan blue exclusion assays will be used to assess the anti-proliferative effect of Thymoquinone in vitro. In addition, the ability of Thymoquinone to inhibit cell migration will be tested using the wound-healing migration assay. Finally, the 3D sphere-formation assay will be used to investigate the effect of Thymoquinone on the cancer stem/progenitor cells population in both cell lines. Using MTT and trypan blue exclusion assays, our preliminary data suggest that Thymoquinone reduced the proliferation of D556 Med and D283 Med cells in a time- and dose-dependent manners. We anticipate that Thymoquinone will inhibit cell migration by inhibiting wound closure compared to non-treated conditions. Furthermore, we expect that Thymoquinone will reduce sphere-formation ability of cells in a dose-dependent manner. In summary this will be one of the first studies to assess the effect of Thymoquinone on human medulloblastoma cells in vitro and based on our preliminary results we believe that the drug might have potential therapeutic value.

TABLE OF CONTENTS

ILLUSTRATIONS

Figure

TABLES

Table

ABBREVIATIONS

CNS: Central nervous system

MB: Medulloblastoma

WNT: Wingless

SHH: Sonic hedgehog

CSCs: Cancer stem cells

DNA: Deoxyribonucleic Acid

DMSO: Dimethyl sulfoxide

MRI: Magnetic Resonance Imaging

TQ: Thymoquinone

PNET: Primitive neuroectodermal tumors

MRI: Magnetic resonance imaging

CT: Computerized tomography

mRNA: Messenger RNA

CMB: Classic medulloblastoma

DMB: Desmoplastic medulloblastoma

CSF: Cerebral spinal fluid

ROS: Reactive oxygen species

EMT: Epithelial-mesenchymal transition

FBS: Fetal bovine serum

PBS: Phosphate buffered saline

MTT: Mean transit time

MBEN: Medulloblastoma with extensive nodularity

GY: Gray

NCI: National cancer institute

ELISA: Enzyme-linked immunoassay

TRAIL: TNF-related apoptosis-inducing ligand

ATCC: American Type Culture Collection

OD: Optical density

DMEM: Dulbecco's Modified Eagle Medium

NADH: Nicotinamide adenine dinucleotide

CO2: Carbone dioxide

Hrs: Hours

SFU: Spheres formation unit

FAK: Focal adhesion kinase

MAPK: Mitogen-activated protein kinase

NRP-1: Neuropilin-1

CHAPTER I

INTRODUCTION

A. Childhood cancer

Brain tumors are the most widely recognized solid tumors in children, and numerous subtypes keep on having an imperfect long-term outcome (Pollack, Agnihotri, & Broniscer, 2019). These tumors occur exclusively in the posterior fossa and have the potential for leptomeningeal spread (Millard & De Braganca, 2016). The infratentorial compartment will be the primary site for 60% to 70% of these tumors, including astrocytoma, medulloblastomas, and ependymomas (Golpayegani et al., 2018). During the last years, striking advances in the comprehension of the molecular underpinnings of these tumors have happened because of high-resolution genomic, epigenetic, and transcriptomic profiling, which have provided bits of knowledge to improved tumor characterization and molecularly coordinated treatments and therapies (Pollack et al., 2019). Despite the fact that the treatment of childhood cancer has advanced fundamentally in ongoing decades, forceful central nervous system (CNS) tumors are as yet a main source of morbidity and mortality in this population (Abdullah, Qaddoumi, & Bouffet, 2008).

The management of pediatric brain cancer, which comprise a wide range of histological subtypes, keeps on being a challenge. Outcome, estimated by survival rates as well as by the impacts of disease and treatment on the quality of life, has improved in the course of recent decades for some tumor types, most eminently medulloblastomas. For

other people, in any case, there has been little advancement, and quality of life for long-term survivors remains problematic (Packer & Vezina, 2008).

B. Medulloblastoma

Medulloblastoma (MB) includes a naturally heterogeneous group of embryonal tumors of the cerebellum and categorized as an embryonal neuroepithelial tumor of the cerebellum (**Figure 1**) (Northcott et al., 2019). MB is considered the most well-known threatening cerebellum tumor in kids between the ages of 0 and 9, comprising about 20 to 25% of all pediatric central nervous system neoplasms (Thomas & Noel, 2019). Despite the fact that the danger of death was diminished by 30% over the most recent years, yet MB is still deadly (Massimino et al., 2016). MB is multifactorial, owing to age at diagnosis, the nearness of metastases at analysis, the histologic variation of medulloblastoma, ability to spread via the cerebrospinal fluid, and thus treatment-related cognitive, neurologic, and endocrinologic impacts stay a crippling concern and a driving force for the quest for additional helpful therapeutic modalities (Mahapatra & Amsbaugh, 2020a).

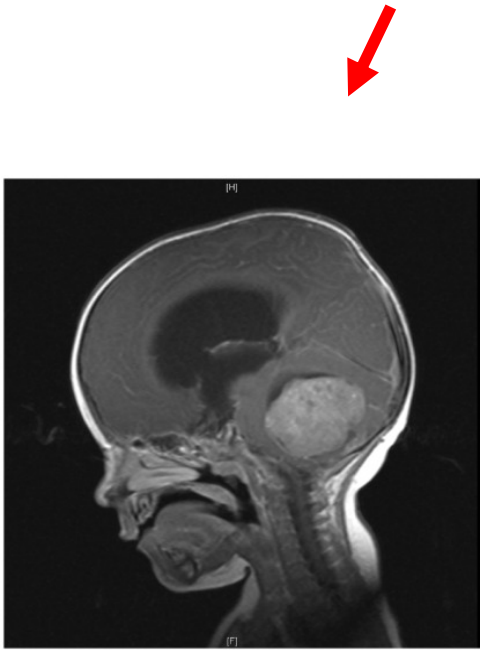

Figure 1: Sagittal view shows a midline posterior fossa medulloblastoma (red arrow) with intermediate signal intensity [Adapted and modified from (DeSouza, Jones, Lowis, & Kurian, 2014)].

1. History of Medulloblastoma

The name "Medulloblastoma" was first presented in 1925 (Bailey & Cushing, 1925). Drs. Cushing and Bailey had at first utilized the expression "spongioblastoma cerebelli" to depict the back fossa tumor seen in pre-adolescents (Raffel, 2004). In the early 1980's, based on histological similarity between medulloblastomas and other small round blue cell tumors arising in areas outside of the posterior fossa, it was proposed that these tumors be classified together under the umbrella group of primitive neuroectodermal tumors (PNETs) (Sharma et al., 2017). They changed the name to medulloblastoma to separate it from a distinct glial tumor contemporaneously depicted as a spongioblastoma by Globus and Strauss (Globus & Strauss, 1925). This new classification mirrored medulloblastoma's apparent determination from one of the five pluripotent stem cells thought to populate the primitive neural tube, despite the fact that it has since been

perceived that there is no embryonal cell that can be distinguished as a medulloblast (Millard & De Braganca, 2016).

2. Medulloblastoma Epidemiology

Numerous sources have expressed that medulloblastoma is the most widely recognized malignant brain tumor in children. Based on the Surveillance, Epidemiology, and End-Results (SEER) database from1973 through 2007, the annual incidence for medulloblastoma was reported at six per million children, approximately 450 new pediatric cases per year. Children age 4 to 9 years old had the highest incidence at 44%, followed by adolescents (10 to 16 years old) at 23%, and only a 12% incidence in infants (0 to 3 years old) (Mahapatra & Amsbaugh, 2020b) . The rate of medulloblastoma is evaluated to be 0.7 per 100000 children per year with a male predominance (Golpayegani et al., 2018).

3. Development of Medulloblastoma and types of cells

Four distinct subgroups were distinguished, in 2010, depending on transcriptional profiling studies: wingless (Wnt), sonic hedgehog (Shh), Group 3, and Group 4 (**Table 1**). Every subgroup was described by a special arrangement of segment and clinical highlights, genetics, and gene expression (Kool et al., 2012). Identification of molecular subgroup more precisely predicts result and clinical conduct than does histopathology or clinical staging. It is speculated that these diverse medulloblastoma subgroups emerge from distinct cells of origin (Northcott et al., 2012). This is to some degree upheld by the observation that medulloblastoma maintains its subgroup affiliation at the time of both recurrence and

metastasis, a finding that separates medulloblastoma from different malignant tumors which show a change in molecular subclass at recurrence or metastasis (Remke, Ramaswamy, & Taylor, 2013). Strikingly, an ongoing study by Perreault et al showed that molecular subgroup could be effectively predicted by neuroimaging in 65% of ninety-nine reflectively reviewed medulloblastoma cases depending on tumor location and enhancement pattern. In terms of location, Wnt pathway tumors were well on the way to arise in the cerebellopontine angle cistern or cerebellar peduncle, while Shh pathways tumors were found in the cerebellar ventricle. Group 3 and Group 4 tumors were the essential subgroups encountered in the midline fourth ventricle (Juraschka & Taylor, 2019).

Table 1: Table showing the four molecular subgroups of medulloblastoma and their clinical characteristics (age at diagnosis, gender ratio male to female, anatomic location of the tumor, histology, and the percentage of metastasis at diagnosis, LCA = large cell/anaplastic [Adapted and modified from (Juraschka & Taylor, 2019)].

	Subgroup	WNT	SHH	Group 3	Group 4
Clinical Characteristics	% of Cases	10	30	25	35
	Age at Diagnosis				
	Gender Ratio (M:F)	1:1	1:1	2:1	3:1
	Anatomic Location				
	Histology	Classic, Rarely LCA	Desmoplastic, Classic, LCA	Classic, LCA	Classic, LCA
	Metastasis at Diagnosis (%)	5-10	15-20	40-45	35-40

4. Medulloblastoma Diagnosis and prognosis

MB have particular imaging attributes on both computed tomography (CT) and magnetic resonance imaging (MRI). Seventy five percent of medulloblastomas emerge from the cerebellar vermis and will in general distend into the fourth ventricle, in spite of the fact that the site of origin in adults is more frequently the cerebellar hemisphere rather than the vermis. As opposed to ependymomas, medulloblastomas don't commonly reach out into the basal cisterns (Miranda Kuzan-Fischer, Juraschka, & Taylor, 2018). On CT, they are most observed as a hyperdense mass emerging from the vermis with cyst formation or necrosis more frequently seen in older patients. Effacement of the fourth ventricle and ventricular dilatation secondary to obstructive hydrocephalus are regularly observed. Conspicuous difference enhancement is present in 90% of cases with calcification found in 10-20% (Meshkini, Vahedi, Meshkini, Alikhah, & Naghavi-Behzad, 2014).

On MRI, medulloblastomas are hypointense to grey matter on T1-weighted imaging with heterogeneous gadolinium improvement in 90%. They are commonly iso-to hyperintense to grey matter on T2-weighted imaging and generally seem heterogeneous because of cyst formation, calcification and necrosis. Diffusion weighted imaging shows limited dispersion and medulloblastomas are hyperintense to encompassing brain on fluid attenuated inversion recovery(FLAIR) sequences (Zimny, Neska-Matuszewska, Bladowska, & Sasiadek, 2015). The current prognosis classification partitions medulloblastomas into "Low risk ", "Standard Risk" and "High Risk" and "Very high risk " depending on age, presence of metastases, degree of postsurgical residual disease and histology (Thomas & Noel, 2019). MB can be either classified based on histological or

molecular features according to the World Health Organization (WHO), Histologically, MB can be divided into four distinct subsets (Louis et al., 2007): classic MB (CMB), desmoplastic/nodular MB (DMB), MB with extensive nodularity (MBEN), and large cell/anaplastic with subgroups of melanotic MB which are extremely rare (**Figure 2**) (Crawford, MacDonald, & Packer, 2007). The most common histological subtype of MB is represented by CMB and is characterized by sheets of densely packed basophilic small round cells with high nuclear-to-cytoplasmic ratio, and showing a mitotic and apoptotic activity (Louis et al., 2007). MBEN shows small round tumor cells but is associated with reticulin-free islands within a reticulin-rich stroma (Giangaspero et al., 1999; Lamont, McManamy, Pearson, Clifford, & Ellison, 2004). And what differentiates the DMB from CMB is the presence of desmoplasia and marked tendency for neuronal differentiation (Kleihues et al., 2002). As for the large cell/anaplastic MB, it is located in the cerebellar vermis and characterized by highly aggressive behavior (Kleihues et al., 2002).

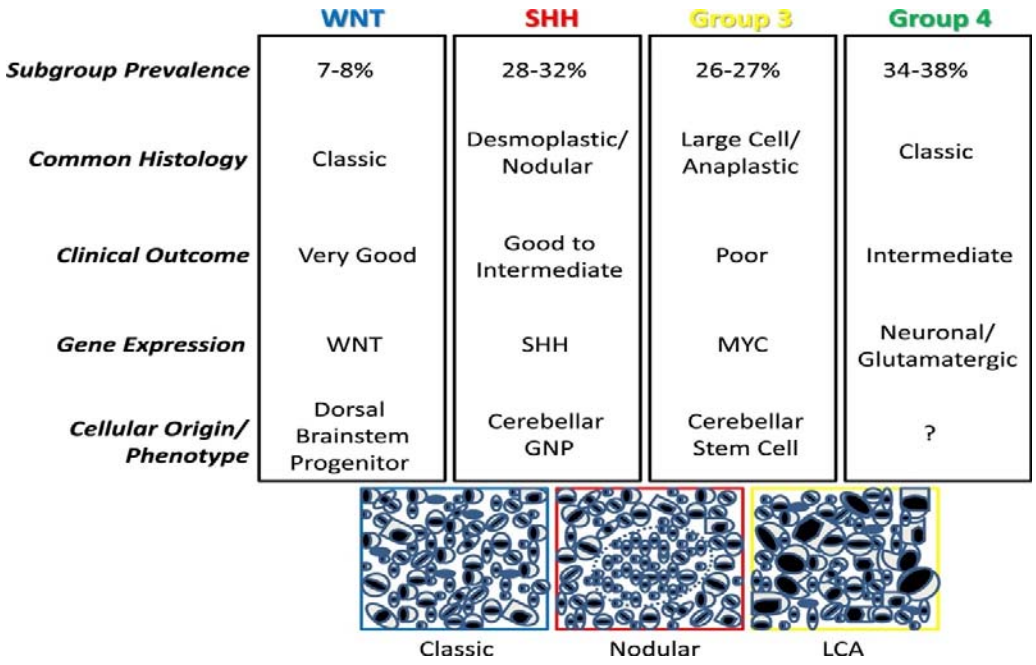

Figure 2: Medulloblastoma subgroups classification based on the percentage of prevalence, common histology (Classic, Demoplastic/Nodular, Large Cell/Anaplastic), Clinical outcome (Good to intermediate, very good , intermediate , poor), Gene expression of each subgroup WNT, SHH, MYC, Neuronal/Glutamatergic respectively for WNT, SHH, Group 3, Group 4 and their cellular origin and phenotype Dorsal brain stem progenitor in WNT, Cerebellar GNP in SHH, Cerebellar stem cell in Group 3, and still not clearly known in Group 4).[Adapted from (Eberhart, 2012)].

C. Medulloblastoma cancer stem cells

Cancer stem cells (CSCs) were first identified by John Dick in acute myeloid leukemia in the late 1990s (Lapidot et al., 1994). As a stem cell biologist, Dick proved important role of CSCs. CSCs are cancer cells that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. CSCs are therefore tumorigenic (tumor-forming) and may generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. Such cells are proposed to persist in tumors as a distinct population and cause relapse and

metastasis by giving rise to new tumors (Moharil, Dive, Khandekar, & Bodhade, 2017). CSCs can reside within the tumor bulk with their self-renewal ability are considered to be responsible for the resistance and recurrence properties of tumors against most chemotherapeutic agents and radiation therapy. That's why understanding their molecular signatures and genetic aberrations is essential in order to characterize those CSCs to develop new therapies that explicitly target them (Bahmad & Poppiti, 2020). Medulloblastoma are tumors in which the majority of cells have an undifferentiated stem- or progenitor-like appearance (Hemmati et al., 2003). Their differentiation into neurons, glia, and other cell types can be detected, on the other hand, providing support for the concept that medulloblastoma have properties of multipotent stem cells. Indeed, their primitive appearance led long ago to the suggestion that medulloblastoma arise from CNS stem cells. Recent studies have sought to more precisely define stem-cell subtypes in the cerebellum and their relationship to medulloblastoma (Fan & Eberhart, 2008).

D. Medulloblastoma treatments

Despite medulloblastomas heterogeneity, they are treated uniformly. Patients get medical procedure like surgery, radiation, and adjuvant chemotherapy, and albeit remedial at about 70%, this routine leaves survivors with crippling side effects and fails to cure all patients (Martin, Raabe, Eberhart, & Cohen, 2014). Medulloblastoma is classified as one of the most radiation therapy sensitive childhood brain tumors. The radiotherapy is a critical strategy for treatment for these tumors, yet the surgery is the primary treatment decision for medulloblastoma patients (Rutkowski et al., 2009).

The most recent clinical trials are focusing on using novel drugs, based on targeting signaling pathways and molecules which have risen as key oncogenic drivers of these tumors (Morrissy et al., 2017). The aim of the continuously evolving specific treatments and medicines is to maximize survival and minimize long term sequelae of treatment (De Braganca & Packer, 2013).

1. Surgical approach

The survival of medulloblastoma patients after surgery is correlated with the amount of residual disease. Maximal surgical resection of tumor ought to be done. Patients should have a staging work-up to survey the degree of the disease. This incorporates postoperative magnetic resonance imaging (MRI) of the brain, MRI of the whole spine and lumbar cerebrospinal fluid (CSF) inspecting for cytological examination, if considered safe (Thompson et al., 2016). In order to render a definitive diagnosis, the tissue must be obtained from neurosurgery, the majority of medulloblastoma tumors are amenable to total resection, if they do not penetrate the cerebellum and brainstem (Srinivasan et al., 2016). The surgical outcome is immediately influenced by peculiar factors like the massive tumor size, advanced clinical presentation and potential risk of enhanced morbidity and mortality. In addition in most centers posterior fossa surgery is performed in prone position or supine position with head turned to opposite side (Muzumdar et al., 2011).

2. Chemotherapy

The objective of chemotherapy in medulloblastoma is to aid in the local control of tumor and the management of micrometastatic disease. Likewise with most chemotherapeutics, these drugs influence rapidly dividing cells including those of the gastrointestinal tract, hair follicles, and bone marrow (Call et al., 2014). In the modern treatment of medulloblastoma, chemotherapy plays a critical role, by allowing the reduction of radiation dose and field in average-risk patients, and enhance the survival of patients with high-risk disease (Khatua, Song, Citla Sridhar, & Mack, 2018). The alternative regimens fusing additional chemotherapeutic agents or using various dosages and frequencies are regularly utilized in patients who are high-risk, children or adult (Gottardo & Gajjar, 2008). In adults, chemotherapy is less well tolerated and it has not yielded the clearly beneficial outcomes that it has in children (Suzuki et al., 2018). Current chemotherapy regimens are primarily determined by the patient's age, suitability for radiotherapy, and risk category (Packer & Vezina, 2008).

3. Radiation therapy

Radiation therapy should start ~30 days following definitive surgery. Generally, the entirety of the cerebellum is irradiated. Progressively, the tumor bed with a suitable margin is irradiated as supported by published evidence showing that a conformal boost to the tumor bed can accomplish similar local control and reduce exposure of the eighth cranial nerves (Rutkowski et al., 2009). Therapy is delivered in daily fractions of 1.8 Gy to a final dose of 54 Gy - 59.4 Gy. Ignoring infants, there are no children with MB for whom

radiation therapy is contraindicated (Martin et al., 2014). The combination of radiation with surgical resection improves the survival of the patients with medulloblastoma (De Braganca & Packer, 2013). Radiation therapy is required to the entire central nervous system, with a greater boost given to the region of the primary site or any massive residual disease for older children. Regardless of the presence of disease evidence, adjuvant chemotherapy must be given after radiation therapy, as the risk of relapse is substantial after radiation alone (Kline et al., 2017). Advances of radiation oncology are making it more feasible to combine radiation treatment with targeted drugs. Limiting toxicity to normal tissues starts with treatment planning and optimized dose distributions (Moding, Kastan, & Kirsch, 2013).

4. Plant Extracts

The anticancer properties of plants have been recognized for centuries. The National Cancer Institute (NCI) has screened approximately 35,000 plant species for potential anticancer activities, among them, about 3,000 plant species have demonstrated reproducible anticancer activity. Many studies have focused on the chemoprotective properties of plants such as anticarcinogenic properties of Anacardium occidentale (cashew extract) in hepatoma (Okonkwo, Okorie, Okonta, & Okonkwo, 2010), Asparagus racemosa (asparagus extract) in human epidermoid carcinomac (Verma, Tripathi, & Das, 2014), Boswellia serrata in human epidermal carcinoma of the nasopharynx (Roy et al., 2016), Gynandropis pentaphylla in hepatoma (Sivanesan & Begum, 2007), and Nigella sativa (black seed extract) in Lewis lung carcinoma (Desai et al., 2008). Treating cancer

cells with chemotherapeutic drugs sometimes creates toxicity and unfavorable side effects. Plants have enormous potential to provide newer drugs due to their natural chemicals that help in providing chemoprotective potential against cancer with minimal undesirable effects (Greenwell & Rahman, 2015) .

a. Thymoquinone:

In ancient civilizations and recent times, researchers have been attracted by Nigella sativa. Traditionally, it has been used in many different forms to treat several diseases like hypertension, asthma, inflammation, diabetes, headache, bronchitis, cough, dizziness, eczema and influenza. It has been shown experimentally that the Nigella sativa extracts and the main constituent of the volatile oil, Thymoquinone-TQ (commonly found in black cumin) (**Figure 3**), have anti-inflammatory, antioxidant and hepatoprotective, in addition to its cytotoxicity and anti-cancer properties, with specific mechanisms of action, which provide support to consider this compound as an emerging drug (Glass et al., 2018). People in different societies used Nigella sativa as condiment and different traditional medicinal system such as Ayurvedic and Unani systems consider Nigella sativa for the treatment of various maladies (Majdalawieh & Fayyad, 2016). The pharmacological investigations of TQ are almost as old as its isolation from Nigella Sativa in 1963 by El-Dakhakhny (Ahmad et al., 2013). Since then, numerous preclinical studies have been performed including those to determine the anticancer effects of TQ (Chowdhury, Hossain, Mostofa, Akbor, & Bin Sayeed, 2018). Ongoing research studies have shown that Nigella sativa, is very commonly used in certain Muslim countries by cancer patients as dietary supplement in

complementary and alternative medicine alongside with chemotherapy (Yimer, Tuem, Karim, Ur-Rehman, & Anwar, 2019).

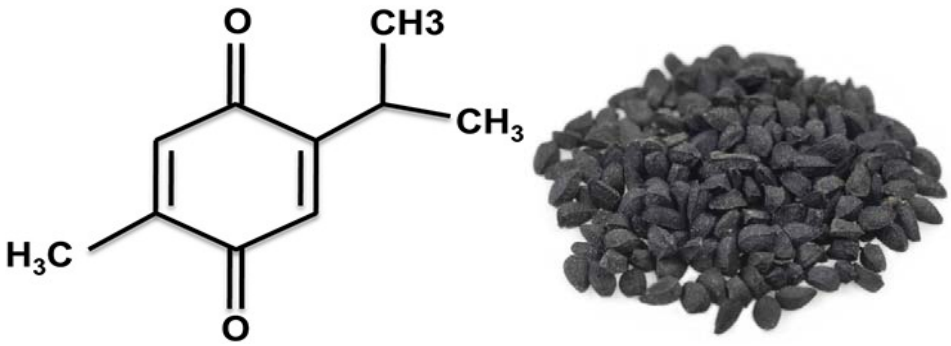

Figure 3: The molecular structure of thymoquinone, showing a benzene ring with 2 oxygen bonds and 5 methyl groups, (chemical name : 2-isopropyl-5methylbenzo-1,4quinone), and the black cumin seeds.[Adapted and modified from (Khan, Tania, Fu, & Fu, 2017)].

i. TQ on cancer cells

TQ exhibits anticancer activity via numerous mechanisms of action, specifically by showing selective antioxidant activity by interfering with DNA structure and synthesis, proliferation, and viability of cancerous cells. It targets cellular copper, which is present in the chromatin and is closely associated with DNA base guanine and causes oxidative breakage to DNA and consequent cancer cell death. In addition, it inhibits telomerase enzyme and cancer's proliferation (Khan et al., 2017). In prostate cancer, TQ targets carcinogenic signaling pathways by downregulation of androgen receptors, decrease the expression of antioxidant enzymes, regulates G1/S phase cell cycle transition, inhibits and

reduce phosphorylation and increase early apoptosis through the generation of ROS and the up-regulation and down-regulation of specific genes (Khan & Younus, 2018).

The immunomodulatory activity of TQ is another important mechanism of its anticancer activity, by suppressing tumor necrosis factors, down-regulation of expression of anti-apoptotic and angiogenic gene products and inducing both intrinsic and extrinsic pathways of apoptosis, it enhances the survival and activity of antigen-specific CD8-positive T cell, plays a role in inducing autophagy in glioblastoma cells (**Figure 4**) (Shaterzadeh-Yazdi, Noorbakhsh, Hayati, Samarghandian, & Farkhondeh, 2018). In vitro activity of TQ has been further implicated in animal models of cancer; however, no clinical application has been proven yet. This is the optimum time to focus on clinical trials for developing TQ as a future drug in cancer (Lei et al., 2012). TQ exhibited antineoplastic effect against different types of cancer including prostate cancer, breast cancer, colon cancer, bladder cancer, lung cancer, bone cancer, and gastric cancer. The anticancer activity of TQ is mediated through different modes of action that acts on various biological pathways that are incriminated in proliferation, cell cycle, regulation, apoptosis, angiogenesis, carcinogenesis and cancer metastasis (Mahmoud & Abdelrazek, 2019).

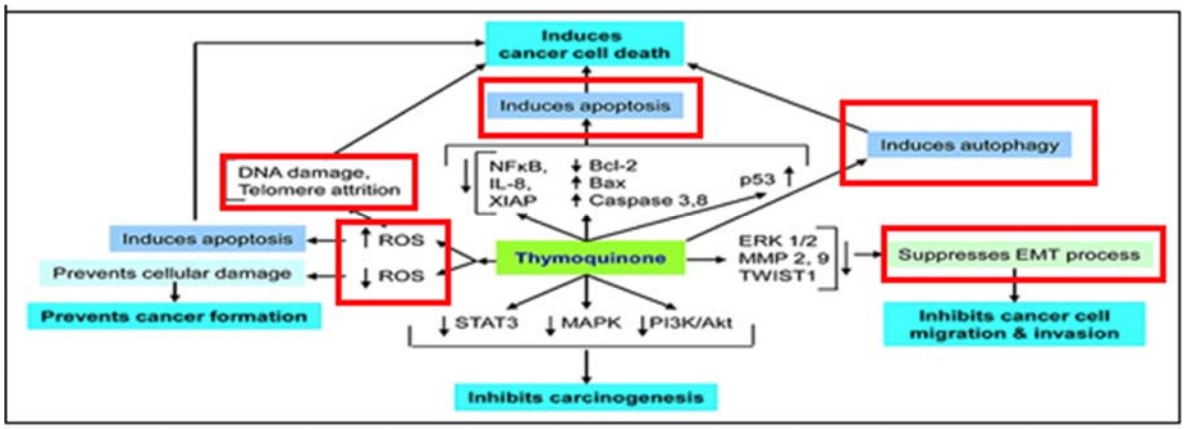

Figure 4: **Important mechanisms of thymoquinone's anticancer action**. TQ induces apoptosis in cancer cells via generating reactive oxygen species (ROS), DNA damage, telomeric attrition, immunomodulation, regulating signaling pathways and autophagy induction, it also regulates epithelial to mesenchymal transition (EMT) and inhibits cancer metastasis. In non-cancer cells, TQ shows antioxidant activities and chemopreventive activity [Adapted and modified from (Khan et al., 2017)].

ii. TQ on medulloblastoma cell lines

TQ has been shown to exhibit a wide spectrum of favorable biological activities such as anti-inflammatory, immune enhancing, antioxidative, and antimutagenic activities and potent antitumor properties against MB cells. TQ exhibits a significant dose-dependent cytotoxic activity against MB cells, which is mechanistically mediated via altered cell cycle progression and the activation of the apoptotic pathways (Khan et al., 2017). TQ has showed effects on MB cells by suppressing growth of these cells in a dose- and time-dependent manner, by causing G2M cell cycle arrest, and generation of reactive oxygen species (ROS), as well TQ induce the pro-apoptotic caspase-8, caspase-9, TRAIL-R1, Bcl-xS, and cytochrome c, It also up regulate the executioner caspase-3 and caspase-7 (Imran et al., 2018). In addition, TQ was assessed on two non-aggressive medulloblastoma cell lines Daoy and UW228-2, and showed a decrease in cell viability in dose and time dependent

manner, and the influence of TQ on cell cycle progression was determined by flow cytometry and the production of reactive oxygen species was measured based on the intracellular peroxide-dependent oxidation. Nevertheless the effect of TQ on MB cell migration and cancer stem cells was not assessed, in addition a low concentration of TQ was not tested on MB cells, so we aim to assess the anticancer effect of TQ on aggressive medulloblastoma cell lines using low concentrations of the drug, and testing it effect on MB cell lines migration and cancer stem cells formation.

E. Aims of the Study

MB is among the most common malignant childhood brain tumors, comprising about 20 to 25% of all pediatric central nervous system neoplasms, the available chemotherapeutics shrink the tumor bulk by eliminating the highly proliferating chemo-sensitive clones but therapy failure occurs due to chemo-resistant CSCs that retain the ability of recurrence and differentiation into highly proliferative cells which replenish the tumor and due to adverse side effects associated with the conventional treatment that limits the quality of life in children with medulloblastoma. Henceforth, it is essential to assess new approaches for the treatment of MB using “safe”, “cost-effective molecules”, prominently from plant extract. The plant derived TQ was shown to regulate cell death mechanism, inhibit cell growth, and interfere with several stages of MB carcinogenesis, however TQ effect on CSCs and aggressive MB cell lines was poorly reported. In this study, we aim to assess the therapeutic effect of TQ on two human medulloblastoma cells lines using D556 Med cell line having MYC amplification which is linked to aggressiveness of the tumor, were D283 Med does not which make it less aggressive, with

both of the cell lines belonging to the same MB subtype, Group 3. To investigate TQ's anti-cancer activity, MB cells where treated with low TQ concentrations for different time intervals using MTT and trypan blue exclusion assays. Using wound healing assay, we investigated the anti-proliferative effect of TQ on both cell lines followed by sphere forming assays to assess its effect on MB CSCs and their sphere-forming potential.

CHAPTER II

MATERIALS AND METHODS

A. Cell lines

Two human medulloblastoma cancer cell lines were used: D283 Med (**Figure 5**) (derived from metastatic site: peritoneum of a 6 years old Caucasian male child), and D556 Med (**Figure 6**) a MYC amplified cell line, (derived from brain/cerebellum of a 4 years old Caucasian male child). All cells were obtained from the American Tissue Culture Collection (ATCC). Cells were cultured and maintained in DMEM High glucose (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% Penicillin/Streptomycin (Sigma-Aldrich), and 0.2% plasmocin prophylactic (InVivogen). Cells were incubated at 37°C in a humidified incubator containing 5% CO2.

B. Preparation of TQ

TQ (Sigma-Aldrich: CAS: 490-91-5; 99.5% purity) was reconstituted in methanol, separated into aliquots of concentration 10mM and stored at -20 °C.

C. MTT cell growth assay

MTT ([3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) cell growth assay was used to measure the anti-proliferative effect of TQ in vitro. MTT reacts with NADH through specific mitochondrial enzymes of viable cells and receives electrons therefore it is converted from a yellow colored agent into a purple colored formazan

product (Riss et al.,2004). Human medulloblastoma cell lines D283 Med and D556 Med were seeded at a density respectively of 2x 10^3 cells/well and 4x10^3cells/well, respectively, in 100µl complete growth medium in three different 96-well culture plates, one plate per time point (24hrs, 48hrs and 72hrs). Cells were incubated overnight at 37°C in a humidified incubator containing 5% CO2. Cells were then treated in triplicates with 0.1% DMSO (vehicle condition) or different TQ concentrations for 24, 48, and 72hrs. Four concentrations of TQ were used: 5, 10, 25, and 50µM. At each time point, media was removed and replaced with fresh media along with 10µl/well of 5mg/ml (in 1x PBS) MTT yellow dye and incubated at 37°C, 5% CO2 for 4hrs, after which 100µl/well of the solubilizing agent was added. After overnight incubation, the reduced MTT optical density (OD) was measured by the microplate ELISA reader (Multiscan EX) at a wavelength of 595nm. The percentage of cell proliferation was measured according to the following formula: $\%\ cell\ proliferation = (OD\ of\ treated\ cells - OD\ of\ blank)\ x100$

Data are reported s means ± Standard Error of the Mean (SEM).

D. Trypan Blue Exclusion Assay

Cell viability was assessed 24, 48, and 72 hrs after treatment. Briefly, human medulloblastoma cell lines D283 Med and D556 Med were seeded in 24-well plates and incubated at 37°C in a humidified incubator containing 5% CO2. After reaching 40-60% confluency, both cell lines were treated with four concentrations of TQ (1, 5, 10, and 25µM of TQ). After 24hrs, cells were trypsinized and counted by Trypan Blue method using a hemocytometer. Cell viability was calculated by the following formula: $Cell\ Survival$ (%) =Number of living cells counted/ $Total\ number\ of\ cells$ ×100

Same procedure was repeated after 48 and 72 hrs. Data are reported as mean ±SEM.

E. Wound Healing Assay

Directional cell migration in vitro was assessed using the scratch or wound healing assay, Briefly, D556 Med and D283 Med cells were seeded in 12-well plates at a density of $10x10^4$cells/well and $12x10^4$ cells/well, respectively. Cells were incubated at 37°C in the humidified incubator containing 5% CO2 until reaching 90-100% confluence. Cells were then treated with 10mg/mL of Mitomycin C (Sigma, USA) for 1hr at 37°C to inhibit cellular proliferation. Later on, Mitomycin C was removed and two uniform scratches of almost the same width were made using a 200µL micropipette tip. Cells were washed twice with PBS to eliminate the detached cells. Remaining cells were cultured in complete growth media with or without treatment (TQ vs. control). Finally, bright field images were taken at different time points (0, 4, 18, and 24 hrs.) until the wound closes completely in the untreated group (control).

The distance of the wound was measured using Zen Software (Zeiss) and data represent an average of three independent experiments and reported as mean ±SEM.

F. Three-Dimensional (3D) Culture and spheres-Formation Assay

Using 96-well plates, $1x10^3$ cells/well were suspended in cold growth factor-reduced Matrigel™/serum-free medium (1:1) in a total volume of 10µl (10µl mix: 5µl cold cell suspension + 5µl cold Matrigel™). The mix of cells and Matrigel™ was kept on ice and pipetted up and down to keep the cells uniformly suspended before plating. The

solution was then plated gently around the bottom rim of individual wells uniformly and bubble-free in a circular manner and allowed to solidify for 45-60 minutes at 37°C in a humidified incubator containing 5% CO2. After solidification, 500µL of DMEM High glucose medium with 5% FBS, with or without TQ, was added gently to the center of each well and the media was replenished every 2-3 days. Spheres were counted at day 7 and the sphere forming unit was calculated using the following formula:

$SFU\ (\%) = Number\ of\ spheres\ counted / Number\ of$ cells s$eeded$ i$nitially\ x$ 100

G. Statistical Analysis

Statistical analysis was achieved using GraphPad Prism 7 software. The significance of the data was determined using proper statistical tests, including the student t-test. P-values of $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) were classified as significant, highly significant and very highly significant, respectively.

CHAPTER III

RESULTS

A. Cell lines morphology

For our study we used two human medulloblastoma cell lines D283 Med and D556 Med showing a morphologically mixed cellular population, containing from polygonal cells to fibroblastic cells. D283 Med (**Figure 5**) have a majority of cells with a polygonal shape while D556 Med (**Figure 6**) have most of the cells with fibroblastic shape.

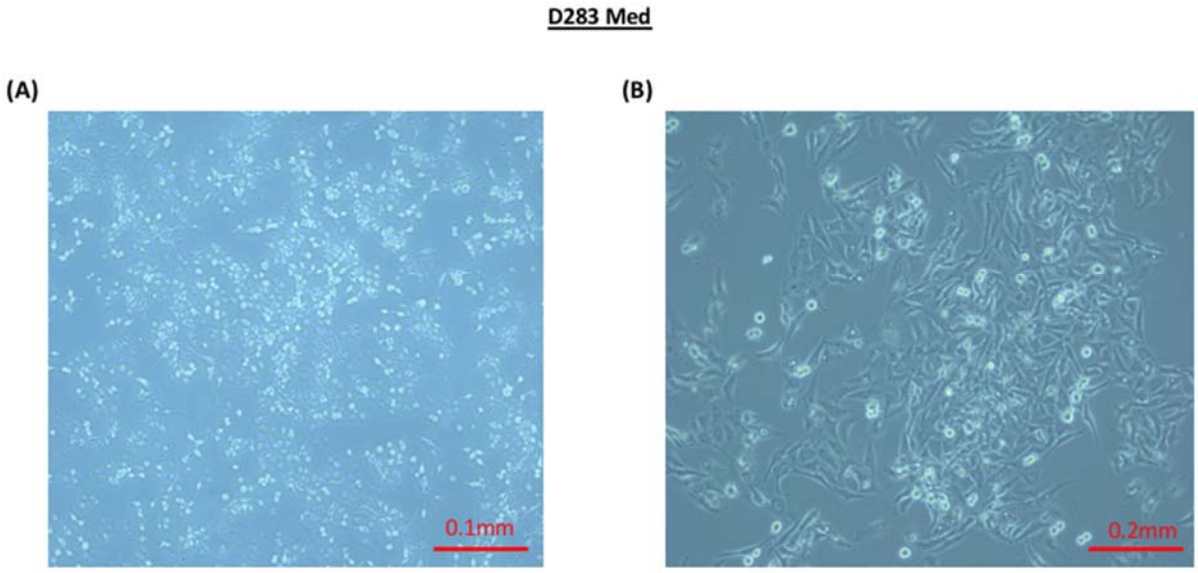

Figure 5: Representative bright field images of D283 Med. Images were acquired using Zeiss Axiovert light microscope, showing the morphology of D283 Med cell line, on low magnification (5x) **(A)** and high magnification (10x) **(B)**.

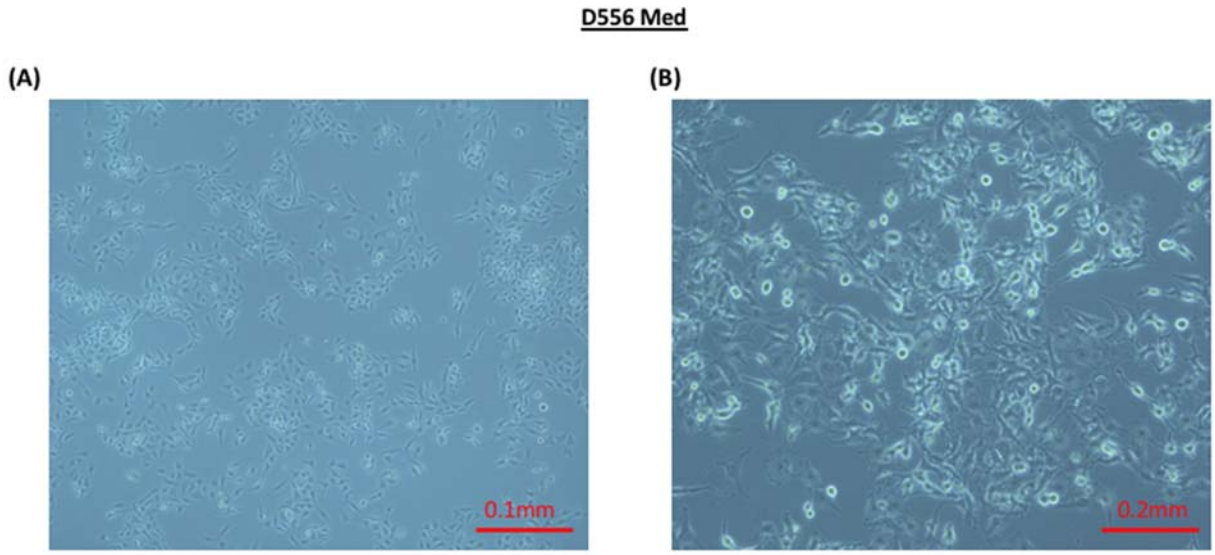

Figure 6: Representative bright field images of D556 Med. Images were acquired using Zeiss Axiovert light microscope, showing the morphology of D556 Med cell line, on low magnification (5x) **(A)** and high magnification (10x) **(B)**.

B. Thymoquinone inhibits Medulloblastoma Cell proliferation in vitro in a Dose-and Time Dependent Manner

First, we assessed the in vitro anti-proliferative effect of increasing concentrations of TQ on D283 Med and D556 Med cells using the MTT assay. TQ had significant anti-proliferative effects on both cell lines (**Figure 7**), reaching around 68% after 48 hours of treatment and 64% after 72 hrs ($p=0.0332$) at 10µM of TQ for D283 Med and by 52% after 48 hrs and 53% after 72hrs ($p=0.0016$) for D556 Med. Increasing the concentration of TQ were able to significantly inhibit cell proliferation by approximately 60% at 25µM of TQ for D283 Med after 48 hrs ($p=0.0332$) and by 51% after 72 hrs ($p=0.0002$), and by almost 42% for D556 Med after 48 hrs ($p=0.0016$) and 45% after 72 hrs ($p=0.0002$), the further increase in TQ concentration to 50µM showed the maximum percentage of reduction in proliferation by almost 53% at 48hrs ($p=0.0002$) and 42% at 72hrs ($p=0.0002$) for D283

Med and by approximately 42% at 48hrs (p=0.0002) and 38% at 72 hrs (p=0.0002). Upon using a very low concentration of TQ (5μM), the proliferation wasn't significantly decreased in both cell lines as compared to the control at the three time points indicated.

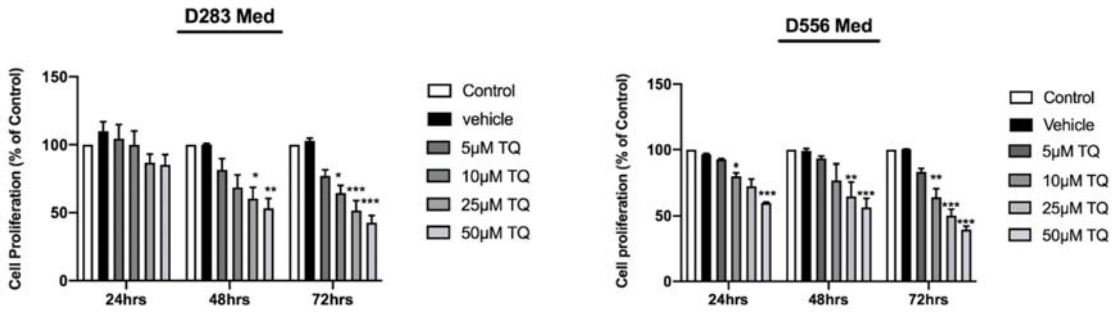

Figure 7: Thymoquinone reduces human medulloblastoma cell lines D556 Med and D283 Med cell lines proliferation in dose- and time-dependent manner. After incubation of the two cell lines for 24, 48 and 72hrs with and without TQ, cell proliferation was determined using MTT assay. Results are expressed as a percentage of the treated group compared to its control. Data represent an average of three independent experiments and are expressed as mean ± SEM (error bars) (*p<0.05, **p<0.01, ***p<0.001).

C. TQ Reduces Medulloblastoma Cell Viability in vitro in a Dose- and Time-Dependent Manner

The Trypan blue test is used to determine the number of viable cells found in a cell suspension. It is based on the principle that living cells have intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, in the other hand dead cells do not. So in this test in order to visually examine whether cells take up or exclude the dye the cell suspension is mixed with the trypan blue dye, so a viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm (Lukic, Simec, Zatezalo, Jurenec, & Radic-Kristo, 2017).

Trypan blue results showed a time- and dose-dependent reduction in cells viability in response to TQ in both D283 Med and D556 Med (**Figure 8**), the inhibitory effect of TQ commenced at a concentration of 1µM at 24hrs, decreasing cell viability by 48% in D283 Med (**Figure 6A**), and by almost 66% in D556 Med (**Figure 6B**). The maximum percentage of reduction in viability was shown when both cell lines were treated with 25 µM of TQ at 72hrs after treatment in D283 Med was 96% compared to 94% in D556 Med. These results were consistent with MTT assay.

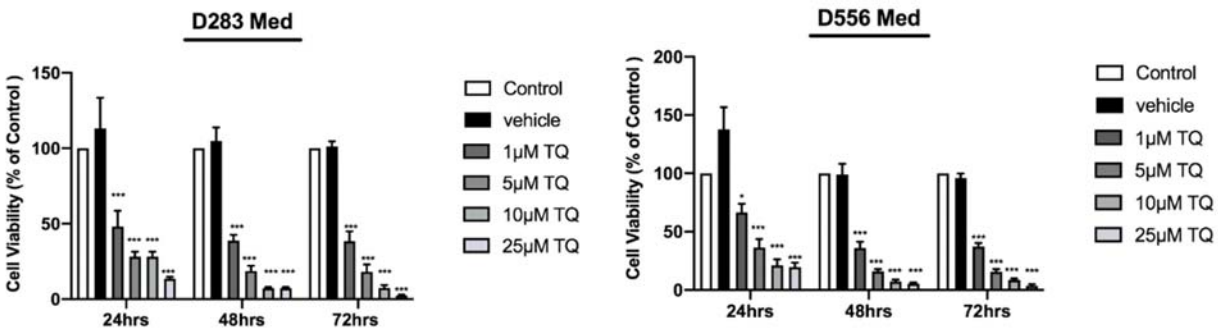

Figure 8: Thymoquinone decreases human medulloblastoma cell lines D283 Med and D556 Med viability in dose- and time-dependent manners.
After incubation of D283 Med and D556 Med cell lines for 24 hrs, 48 hrs, 72 hrs with or without treatment (TQ), cell viability was determined using trypan blue exclusion assay. Results are expressed as a percentage of the treated group compared to its control. Data represent an average of three independent experiments and are expressed as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).

D. Thymoquinone Inhibits Medulloblastoma Cell Migration in vitro

The wound healing assay is used in a range of disciplines to study the coordinated movement of a cell population (Jonkman et al., 2014). Studying cell migration is vital to many physiological and pathological processes including tissue development, repair, and

regeneration, cancer metastasis, and inflammatory responses (Cormier, Yeo, Fiorentino, & Paxson, 2015). In this assay we are interested in quantifying the migratory capacity of human medulloblastoma cell lines D283 Med and D556 Med, and to assess how migratory potential may be altered by TQ treatment.

We assessed cell migration under different experimental conditions, and we investigated the effect of TQ on cell migration of human medulloblastoma cell lines D283 Med and D556 Med. Hence, we used a wound-healing/ scratch assay where the cells were divided into 5 groups: untreated (control), vehicle, and treated with 1μM, 5μM, and 10μM of TQ. The main advantage of this assay is that it mimics migration of tumor cells in vivo and allows studying the cell–cell interactions and communication between cancer cells and their extracellular matrix (ECM) in order to regulate the cell migration process (Liang et al., 2007). Our results showed that the wound was almost completely healed after 24hrs in the control and vehicle for both D283 Med (**Figure 9B**) and D556 Med (**Figure 10B**), and the migration of both cell lines was significantly decreased after the treatment with 5μM and 10μM of TQ. Nevertheless, TQ showed a reduction in the rate of wound closure after 18 hrs post treatment with 5μM and 10μM of TQ as compared to the untreated groups (control & vehicle) in both D283 Med and D556 Med. While the wound was completely healed after 24hrs in D283 Med (**Figure 9A**) and D556 Med (**Figure 10A**) in control and vehicle conditions. A 10μM of TQ was interestingly able to significantly suppress the migration and closure of the treated cells by almost 72% in D283 Med and 74% in D556 Med 18hrs post-treatment.

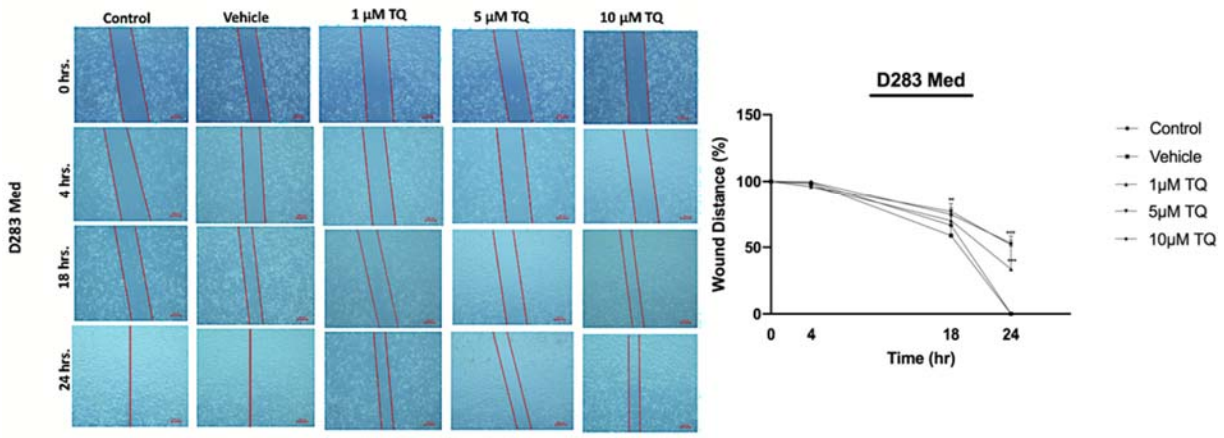

Figure 9: Thymoquinone reduces human medulloblastoma cell line D283 Med cell migration in dose- and time-dependent manners. (A) After incubation of D283 MB cell lines for 24 hrs with or without treatment (TQ), cell migration was tested by scratch assay, and showing a complete wound healing after 24 hrs in the control and vehicle and suppression of the wound closure after 18 hrs and significantly after 24 hrs with 1µM, 5µM and 10µM of TQ, and TQ as represented in (**B)** was able to reduces the migratory potentials of medulloblastoma cancer cell lines. Results indicates the ability of TQ to inhibit the cells from migration of D283 Med at different time points (4,18, and 24hrs), while the untreated wound shows a complete closure of the cell monolayer at t24 hrs D283 Med, At 24 hrs 5µM of TQ reduced cell migration by almost 65%. Data are reported as mean ±SEM (error) bars) (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

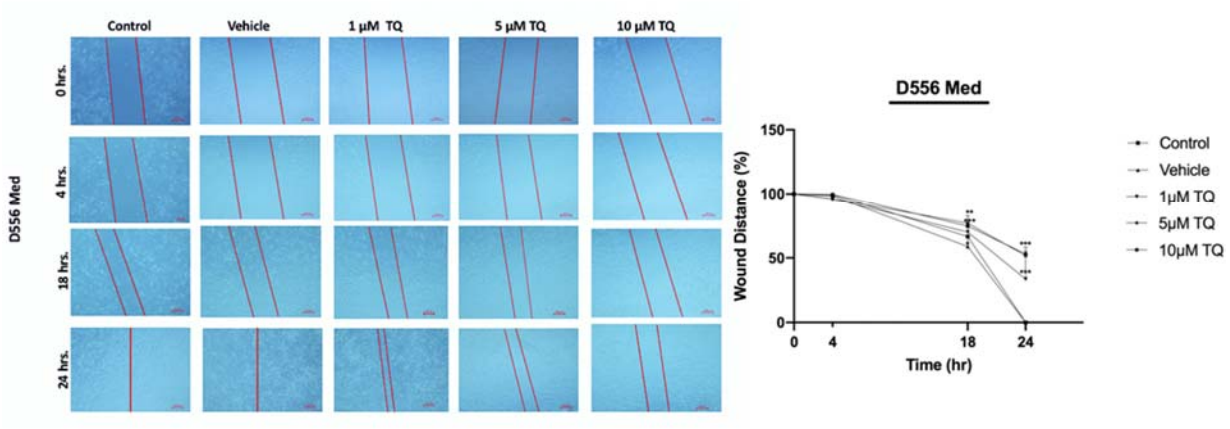

Figure 10: (A)Thymoquinone decreases human medulloblastoma cell line D556 Med cell migration in dose- and time-dependent manners. After incubation of D556 Med cell line for 24 hrs with or without treatment (TQ), cell migration was tested by scratch assay, and showing a complete wound healing after 24 hrs in the control and vehicle and suppression of the wound closure after 18 hrs and significantly after 24 hrs with 5µM and 10µM of TQ and as results indicates in **(B)** TQ was also able to inhibit the cells from migration of D556 Med at different time points (4,18, and 24hrs), while the untreated wound shows a complete closure of the cell monolayer at 24 hrs for D556 Med, At 24 hrs 5µM of TQ reduced cell migration by almost 53%. Data are reported as mean ±SEM (error bars) (*p<0.05, **p<0.01, ***p<0.001).

E. Three-Dimensional (3D) Culture and spheres Formation Assay

Having established TQ's inhibitory effect on both cell lines in 2D, we next aimed at investigating its potential inhibitory effect on targeting the self-renewal capacity of MB CSCs enriched from D283 Med and D556 Med cell lines in 3D cultures using sphere formation and propagation assays. 50 µl of DMEM High glucose with 5% FBS, with or without TQ was added gently to the center of each well after solidification and the media was replenished every 2-3 days. Spheres were counted at day 7 for both human medulloblastoma cell lines D283 Med and D556 Med after plating, and the spheres

formation efficiency or sphere formation unit (SFU) was calculated based on the following formula: SFU = (number of spheres counted ÷ number of input cells) × 100.

TQ had significantly decreased the area of spheres in both D283 Med (**Figure 11**) and D556 Med (**Figure 12**), in addition to a remarkable decrease in the sphere formation unit in both cell lines (**Figure 13**), when treating with increasing concentration of TQ (1µM, 5µM, 10µM).

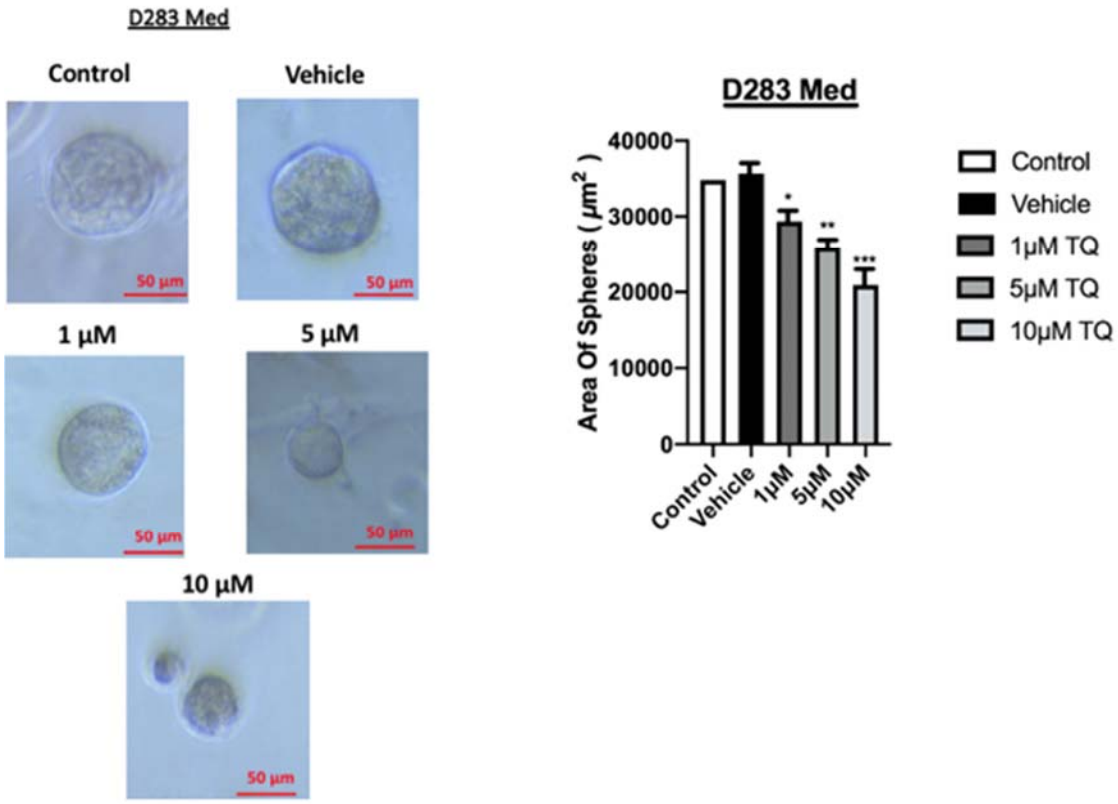

Figure 11: Thymoquinone diminishes the sizes of human medulloblastoma cell line D283 Med cultured spheres in a dose-dependent manner.
Images of cultured spheres were visualized and taken by Zeiss Axiovert inverted microscope and analyzed by Carl Zeiss Zen 2012 images software. We measured the diameter and calculated the area of 30 fully grown spheres. The average size of D283 Med spheres (μm^2) decreased significantly with higher TQ concentrations. The concentration of 10µM has decreased the area of the spheres by 40 %, and 5µM of TQ was able to decrease

the spheres area by 26 %. Data are represented by mean ± SEM (error bars) (*p<0.05, **p<0.01, ***p<0.001).

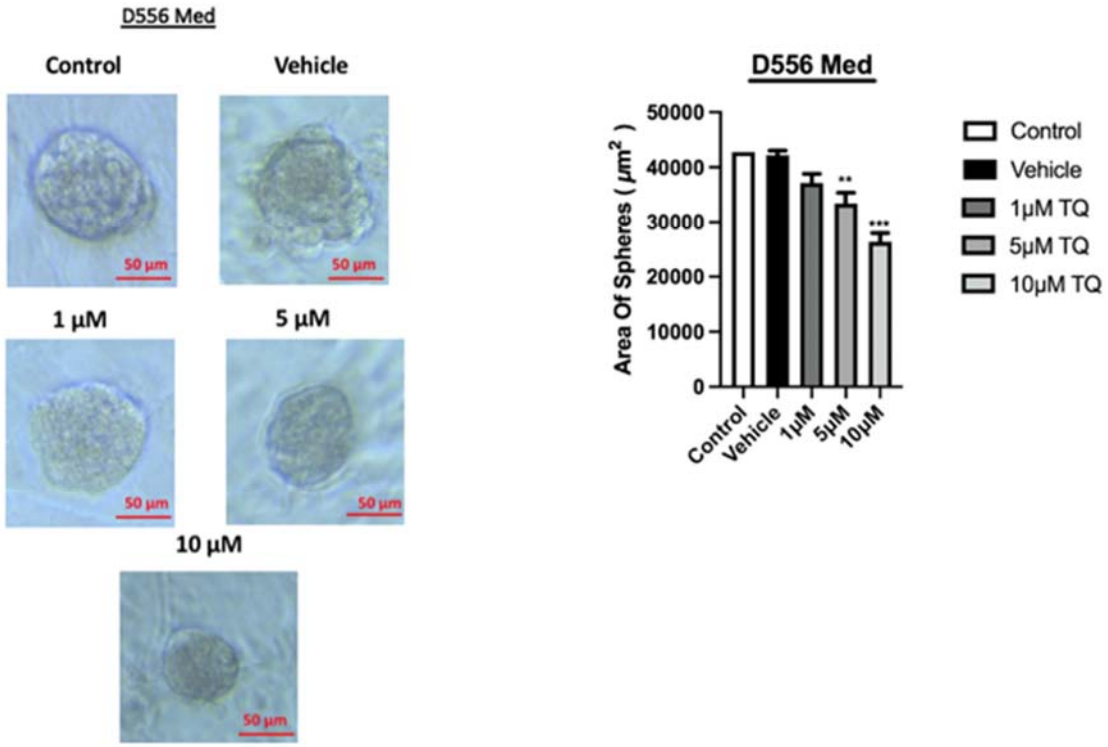

Figure 12: Thymoquinone diminishes the sizes of human medulloblastoma cell line D556 Med cultured spheres in a dose-dependent manner. Images of cultured spheres were visualized and taken by Zeiss Axiovert inverted microscope and analyzed by Carl Zeiss Zen 2012 images software, we measured the diameter and calculated the area of 30 fully grown spheres.
The average size of D556 Med spheres(μm^2) decreased significantly when the TQ concentration increase. The concentration of 10µM has decreased the area of the spheres by 39%, and 5µM of TQ was able to decrease the spheres area by 22%. Data are represented by mean ± SEM (error bars) (**p<0.01, ***p<0.001).

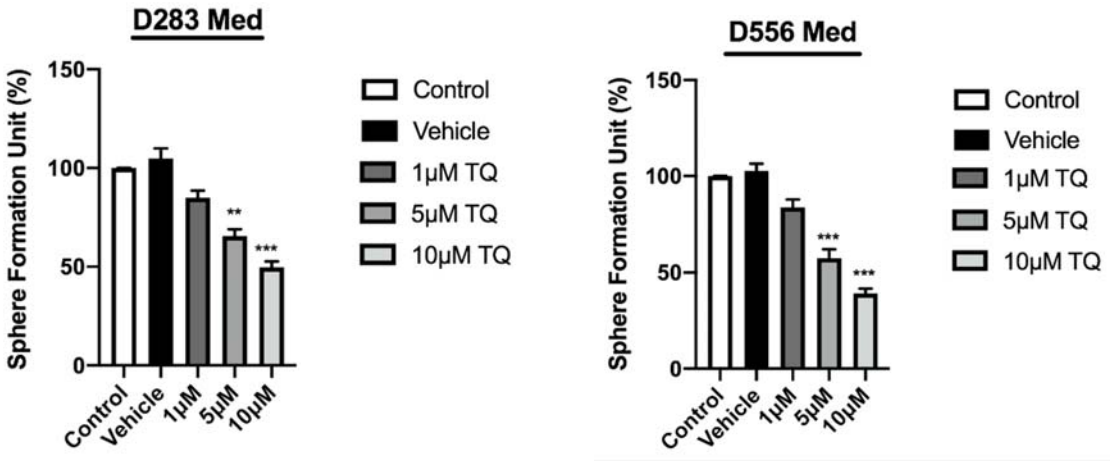

Figure 13: Thymoquinone reduces the sphere forming units of both human medulloblastoma cell lines D283 Med and D556 Med cultured spheres in a dose-dependent manner. Spheres formed in each well, referred to as generation 1 spheres (G1), were counted at day7 post- plating. A 5μM of TQ was able to decrease the SFU by 35% ($p=0.0013$) for D283 Med and by 43 % ($p=0.282$) for D556 Med, while a dose of 10μM TQ significantly reduced the SFU by 58% ($p<0.0001$) and by 61% ($p<0.0001$) respectively for D283 Med and D556 Med .
Results are represented as mean ±SEM (**$p<0.01$, ***$p<0.001$).

CHAPTER IV

DISCUSSION

MB remains a major cause of cancer-related mortality in children, accounting for 20% of childhood brain tumors (Miranda Kuzan-Fischer et al., 2018). CSCs, a subpopulation residing within the tumor bulk, are thought to be largely responsible for tumor initiation, maintenance, dissemination, and relapse. Henceforth, knowing their vital roles, targeting CSCs in MB therapy can increase treatment efficiency (Huang et al., 2016). Chemotherapy, radiation and surgical resection represent the main MB therapeutic strategies, however, such treatments are accompanied with several side effects and hence long-term morbidities (Thomas & Noel, 2019). In the process of discovery and development of new potential anticancer agents, growing interest is heading towards safe, cost-effective and widely available molecules, prominently extracted from plants. Therefore, we were interested in studying a plant extract agent TQ, which has been used to treat different conditions such as hypertension, diabetes, influenza and much more (Enayatfard, Mohebbati, Nizamand, Hosseini, & Shafei, 2018;AbudKhader, 2012;Umar et al., 2020).

In this study, we showed the anti-cancer effect of TQ on human medulloblastoma cell lines D283 Med and D556 Med, where TQ was able to decrease cell proliferation and viability in time and dose-dependent manner. These data are in accordance with Ashour et al., were TQ was able to decrease the proliferation and viability of human medulloblastoma cell lines Daoy and uw228-2 (Ashour et al., 2014). Yet in our study, we used much lower doses of TQ, as compared to those used in Ashour et al., study. In addition, we used Group

3 human MB cell lines which have lowest survival, poor prognosis, and highest rate of metastasis, while in the Ashour study they used SHH medulloblastoma cell lines with high survival and low metastasis rate. Interestingly, one of the cell lines we sued in this study, D556 Med cell line, has MYC amplification which is related to aggressiveness, and our data has showed encouraging results targeting those cells. Other studies have shown anti-proliferative effects of TQ in different tumor models like human chronic myeloid leukemia (Sethi, Ahn, & Aggarwal, 2008), hepatic cancer cells (Nagi, Al-Shabanah, Hafez, & Sayed-Ahmed, 2011), human pulmonary epithelial cells (Woo, Hsu, Kumar, Sethi, & Tan, 2013), and colon cancer cells (Ballout et al., 2020). Therefore, it seems that TQ might be a potential anti-proliferative agent for targeting cancer cells.

One of the anticancer mechanisms of TQ is upregulating ABIN-1 and ABIN-2 mRNAs, NF- κB pathway and ROS (reactive oxygen species). ABINs are intracellular negative feedback regulators of NF-κB that induces various target genes, such as pro-proliferative and anti-apoptotic genes. NF-κb pathway and ROS affect many signaling pathways, including those involving STAT3, AP1, interferon regulatory factors, NRF2, Notch, WNT–β-catenin and p53 (Ashour et al., 2014;Nagi, Al-Shabanah, Hafez, & Sayed-Ahmed, 2011). Furthermore, studies are needed to decipher the mode of action of TQ in the context of cell viability, proliferation and apoptosis on the cell line models we are using.

Metastasis remains the greatest challenge in the clinical management of cancer. Cell migration is a fundamental and ancient cellular behavior that contributes to metastasis. Therefore, it is important to elucidate the effect of TQ on cell migration. Interestingly, our data showed that low doses of TQ ranging from (1μM-10μM) were able to reduce cell migration in both MB cell lines in dose and time dependent manners. This is consistent

with previous studies, that showed that TQ inhibits cell migration and invasion in pancreatic cancer cell lines (Torres et al., 2010), breast cancer cells (Kabil et al., 2018), renal carcinoma (Zhang et al., 2018), and colon cancer cells (Ballout et al., 2020). One possible mechanism of TQ inhibiting cell migration can be via targeting the epithelial-to-mesenchymal transition program (EMT program). Khan et al., showed that TQ reduced the transcriptional activity of TWIST, a transcription factor expressed in mesenchymal precursor populations in metastatic cancer cells that promotes cell proliferation and migration (Khan et al., 2015). Other groups showed that TQ decreased the expression of TWIST1, ZEB1, and increased the expression of E-Cadherin at both mRNA and protein levels (Dehghani, Hashemi, Entezari, & Mohsenifar, 2015). Henceforth, it remains important to further investigate the mechanisms behind TQ-inhibiting cell migration on our MB cell models.

As we mentioned earlier, it is important to potentially target CSCs that account for the tumor regeneration, recurrence, and metastasis (Huang et al., 2016). This is the first study to tackle the effect of TQ on CSCs of human MB cell lines, were we showed that low doses of TQ reduced both the size and the number of tumorspheres.This is consistent with other studies that investigated the effect of TQ on CSCs in other cancer cells models like colon cancer cells (Ballout et al., 2020), and human breast cancer (Bashmail et al., 2020). It remains important to know the mechanism that lead to the effect of TQ on medulloblastoma. In fact, Garg et al., showed that the enrichment of CD133 in Group 3 medulloblastoma cells is associated with increased rate of metastasis and poor clinical outcome, and promote tumorigenesis through regulation of c-MYC, a key genetic driver of Group 3 MB cells (Garg et al., 2017). In addition, the stem cell marker CD44 has been

showed to be overexpressed in MB cells, where CD44 is crucial in tumor initiation, and known to have a multifunctional role in many cellular processes, like survival, growth, and differentiation, and may regulate stemness in CSCs (Qi, Wang, Fonkem, Huang, & Wu, 2019; Thapa & Wilson, 2016). In future studies, it remains important to investigate the effect of TQ on self -renewal ability in our MB cell models and to check the expression of stem cell markers

In conclusion, our study investigated the effect of TQ against human medulloblastoma cell lines where we showed that TQ inhibits cell viability, cell proliferation, and cell migration in time and dose dependent manners. Moreover, this study is the first to show the inhibitory effect of low doses of TQ against Group 3 medulloblastoma cell lines, with and without MYC amplification. Furthermore, we provide initial evidence that TQ might targets MB CSCs subpopulation. And with further studies, TQ might prove to be a potential therapeutic for the management of human medulloblastoma especially that TQ has low cytotoxicity, low adverse side effect, and low cost.

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