Targeting Rhabdomyosarcoma with Temozolomide: How Autophagy Regulates TMZ-Induced Apoptosis in Rhabdomyosarcoma Cells

By

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

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Abstract

Rhabdomyosarcoma (RMS) is a muscle-derived tumor and is the most common pediatric soft tissue sarcoma representing 5% of all childhood cancers. Statistically, RMS is a major clinical problem in pediatric oncology. Treatment of RMS with the oral alkylating agent temozolomide (TMZ), alone or in combination with other drugs, has recently received considerable interest. However, the mechanism of action of TMZ remains unclear. The aim of this investigation was to determine if autophagy modulates TMZ-induced cell death in an RMS cell line (RH30 cells), and determine if the cellular response to TMZ is different in RMS cells compared to non-transformed mouse myoblast cell line (C2C12 cells). We show that TMZ decreased the viability of RMS cells in a dose- and time-dependent manner and induced accumulation of sub-G1 cell population, representing apoptotic cells. Interestingly, TMZ induced apoptosis by 17-fold in the RH30 cells, (2.11% vs 36.94%; $p<0.05$), but only by 3-fold in C2C12 cells (10.95% vs 29.64%; not significant). In RH30 cells, TMZ decreased the expression of antiapoptotic proteins BCL-XL and MCL-1. Moreover, we show that TMZ induced biochemical markers of autophagy, such as LC3 lipidation and P62 degradation (Immunoblotting), and induced morphological evidence autophagy, including accumulation of autophagosomes and autophagolysosome in both cell lines, determined by transmission electron microscopy. Treatment of RMS cells with the autophagy inhibitor Bafilomycin A1 significantly increased TMZ-induced cell death in RMZ cell line. We have demonstrated that autophagy inhibition increased TMZ induced apoptosis. Our investigation showed that TMZ induced simultaneous autophagy and apoptosis in both RH30 and C2C12s; however, the regulation of apoptotic cell death induction by TMZ appears to be dependent on autophagy processes.

ACKNOWLEDGEMENTS

DEDICATION

I would like to dedicate my thesis to all patients with cancer.

ABBREVIATION LIST

3-MA: 3-methyladenine

AGT: alkylguanine DNA alkyltransferase

AIC: 5-aminoimidazole-4-carboxamide

AMPK: adenosine monophosphate-activated protein kinase

AMPK: adenosine monophosphate-activated protein kinase

AP-1: AP-1 transcription factor

ARMS: alveolar rhabdomyosarcoma

ARMS: alveolar RMS

ATG: autophagy-related genes

AZQ: diaziquone

BCL-2: BCL-2 apoptosis regulator

BER: base excision repair

Cl: confidence interval

CMA: chaperone-mediated autophagy

CTOS: Spanish Group for Research on Sarcomas

EORTC: European Organization for Research and Treatment of Cancer

ERK: extracellular signal-regulated kinase

ERK: extracellular signal-regulated kinase

ERMS: embryonal rhabdomyosarcoma

ERMS: embryonal RMS

GIST: gastrointestinal stromal tumors

GISTs: gastrointestinal stromal tumors

GISTs: gastrointestinal stromal tumors

GST: glutathione-S-transferase

HMGB1: high mobility group box 1

ITT: temsirolimus

JNK: c-Jun N-terminal kinase

MAP1LC3: LC3 microtubule-associated protein 1 light chain 3

MGMT: methylguanine DNA methyltransferase

MMR: DNA mismatch repair

MSS: musculoskeletal sarcomas

MTIC: 5-(3-methyltriazen-1-yl)-imidazole-4- carboxamide

MTOR: mechanistic target of rapamycin

MYOD1: myogenic differentiation 1

N3-MeA: N3-methyladenine

N7-MeG: N7-methylguanine

NCOA2: nuclear receptor co-activator 2

O6-MeG: O6-methylguanine

PARP: poly(ADP-ribose) polymerase

PE: phosphatidylethanolamine

RMS: rhabdomyosarcoma

RMS: rhabdomyosarcoma

ROS: reactive oxygen species

ROS: reactive oxygen species

SD: stable disease

siRNA: small interfering RNA

SIRT1: sirtuin 1

SIRT2: sirtuin 2

SSB: DNA single strand break

TMZ: temozolomide

UVRAG: UV radiation resistance associated

PDOX: patient-derived orthotopic xenograft

VOIT: Vincristine, oral irinotecan, and temozolomide

TOTEM: topotecan in combination with temozolomide

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Chapter 1: Introduction

1.1. Sarcoma

Sarcomas are a heterogeneous group of rare malignant cancers that arise predominantly from soft tissues and bone [1, 2]. Sarcomas affect the full age spectrum, ranging from children to adults and can occur anywhere in the body [3]. Based on the tissue of origin and the location of the tumor, there is a nomenclature for naming sarcomas; including bone sarcomas (osteosarcomas and chondrosarcomas), fat tissue tumours (liposarcomas), muscle tissue tumours (leiomyosarcomas), skeletal muscle sarcomas (rhabdomyosarcomas), and fibrous tissue tumours (fibrosarcoma) [4]. Assessments conducted by the National Institutes of Health (NIH) identified that sarcomas occur in 12,000 to 15,000 cases annually in the United States [5, 6]. For example, in 2014 there were approximately 12,000 patients diagnosed with soft tissue sarcomas, while 3,000 patients were diagnosed or living with bone sarcomas [7]. Over the past 35 years, the incidence of both soft tissue and bone sarcomas have increased and the frequency of patients with soft tissue sarcomas remains more common than bone sarcomas [8-10]. The 5-year survival rate for both bone and soft tissue sarcoma is approximately 65 percent, and has not changed for the past several decades [11, 12]. Since most sarcomas do not present early with major signs or symptoms, they can become very large before diagnosis [13]. Each type of sarcoma has specific characteristics, which have been briefly discussed in the following sections.

1.1.1. Osteosarcoma

Osteosarcoma is a malignant cancer that represents less than 1% of all cancers, however it represents nearly 20% of all bone cancers, and occurs predominantly in the distal femur, proximal tibia, and proximal humerus [14-17]. In the United States, approximately 2,000 cases

of osteosarcoma are diagnosed each year, with most patients being children and young adults [18]. Previous reports also indicate that osteosarcoma affects males 1.4 times more than female [19]. The prognosis for the patients with non-metastatic osteosarcoma is 60%-70% survival in 5 years; however it is important to note that the survival rate drops considerably in metastatic disease [20, 21]. The molecular profile of osteosarcoma is heterogeneous and there is no consistent pattern among patients [22, 23]. However, scientists have attempted to clarify the important signaling pathways in osteosarcoma to be used as the basis of new therapeutic approaches to treatment [24-26].

1.1.2. Fibrosarcoma

Fibrosarcoma only represents about 15% of all soft tissue sarcomas and only makes up around 1% of all malignant tumours of the head and neck region [27]. This form of sarcoma often involves the deep soft tissues of the extremities, trunk, head and neck [28]. Fibrosarcoma tends to occur in middle-aged and older adults (greater than 50 years of age) and disproportionately affects males more than females [28]. The fibrosarcoma known as a rapidly growing tumor which depends on its different type can act differently [29].

Low-grade fibromyxoid sarcoma (LGFMS) is a type of fibrosarcoma which predominant mostly in young adults; however, affects also ~20% of children [30-32]. LGFMS arises in any organ contains the viscera which superficially located tumours are considered to be predominant in children [33, 34]. The metastatic risk for this type of fibrosarcoma in young adults accounts around 15%, but it was found that LGFMS enter to the metastasis phase in a very late stage (>20 years after diagnosis) [30-32]. Sclerosing epithelioid fibrosarcoma (SEF) is mostly occur in extremities in patients a wide age range [35-37]. There are reports showing that SEF is an aggressive and metastatic tumor; however, in some cases it was explained as a non-metastatic

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tumor [38, 39]. Dermatofibrosarcoma protuberans (DFSP) is another type of fibroblast which is most common in the trunk and proximal extremities, occurs in middle-aged young adults, but might be observed in patients of any age [39-41].

1.1.3. Chondrosarcoma

Chondrosarcoma is an uncommon heterogeneous group of malignant tumor characterized by the production of cartilage matrix by tumor cells [42]. It is the second most common malignant bone tumor which is comprised about 15% of all bone tumors [43]. Chondrosarcomas are mainly non-metastatic and are likely diagnosed in ages between 40 and 60 [44]. The overall incidence of all chondrosarcomas is 1 in 20,000 [45]. Interestingly, unlike osteosarcoma and fibrosarcoma, there is no known gender predominance for chondrosarcoma, which mainly occur in the head and neck area [46]. However, chondrosarcoma's can also arise in the axial skeleton, pelvic girdle, femur humorous, vertebra, shoulder, sternum and ribs [46]. Conventional, central (intramedullary) chondrosarcomas are the most common subtype of this form of cancer, and approximately 90% of these have a low metastatic potential [47].

1.1.4. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is described as a rare, aggressive, soft-tissue malignant tumor that occurs in both children and adults [48]. In the United States, the incidence of RMS is around 600 cases per year, and is more common in children (59%), compared to adults (41%) [49]. The incidence rate is greater in the White population (4:3 ratio) compared with the African-American population [50]. Between 2006 and 2010, the estimated number of new RMS cases and average annual age-standardized incidence rates (ASIR) in Canadian children (0–14 years) was 145 and 5.1, respectively [51]. Statistics like these indicate that RMS is a major

clinical problem in the field of pediatric oncology. It is reported that soft tissue sarcomas account for approximately 1% of all cancers and 10% of all childhood cancers, while RMS comprises 3% of all soft tissue sarcomas and 50% of all childhood soft tissue sarcomas [52, 53]. Since RMS survivors, especially children, need to continue medications and treatment for a long time in order to decrease the risk of cancer relapse, significant long-term morbidities exist in the survivors and they are forced to endure difficult conditions [54]. Clinically, survival among metastatic RMS patients has not improved appreciably in the past years, emphasizing an urgent need for uncovering the underlying signaling mechanisms of RMS in order to develop new strategies to treat and prevent this disease [55].

According to the World Health Organization (WHO), four subgroups of RMS have been described based on histological and genetic criteria, and clinical characteristics [56].

Embryonal RMS (ERMS) is the most frequent subtype and commonly detected in young children less than 10 years [57]. This type of RMS is distinguished by small round blue appearance [58]. ERMS is considered a heterogeneous disease that mostly occurs in the head, neck, and genitourinary system and, in general, ERMS entails a good prognosis [56, 59].

Pleomorphic RMS (PRMS) metastasizes within 5 years of diagnosis is an aggressive tumor of adults between ages 60-70 and often involves invasion of the deep soft tissues [60]. Histologically, multiple cell types are present in PRMS making it difficult to distinguish from other pleomorphic sarcomas [58]. Although this type of tumor is defined as complex karyotypes, no recurrent structural changes have been identified [61, 62].

Spindle cell/sclerosing RMS has been recognized as another type of RMS which is found in both child and adult populations [63]. This RMS contains spindle cells with eosinophilic and

fibrillar cytoplasm [64]. While this type of tumor, in the majority of childhood cases, is located in paratesticular region, in adults it affects the deep soft tissues of the head and neck. This neoplasm shares different molecular genetics and clinical features; however, the prognosis is satisfactory [56]. Clinically, congenital and infantile spindle cell RMS involving a specific fusion gene involving the nuclear receptor co-activator 2 (NCOA2) gene carries a good prognosis, while spindle cell/sclerosing RMS involving recurrent mutations in myogenic differentiation 1 (MYOD1) is associated with a poor outcome [65-67].

Alveolar RMS (ARMS) is another aggressive subtype of RMS suffered by adolescents and young adults [56]. This type of RMS is composed of small, oval or round cell tumor aggregates with loss of cellular cohesion, creating "alveolar" appearance [68]. ARMS is correlated with t(2;13)(q35;q14) or t(1;13)(q36;q14) translocations that arise from fusion of PAX3 on chromosome 2 or PAX7 on chromosome 1 [69]. Both PAX3 on chromosome 2 or PAX7 on chromosome 1 fuse to FOXO1 resulting in generation of PAX3-FOXO1 or PAX7-FOXO1 fusion proteins which are present in about 60% and 20% of patients, respectively [70, 71]. There are several different PAX3 gene fusion partners, however; their exact roles are unclear and under debate. It is also shown that fusion gene negative ARMS and ERMS have similar prognosis [71-73].

1.2. Site and Survival Rate of Rhabdomyosarcoma

Rhabdomyosarcoma may arise in any part of the body where there is skeletal muscle; however, some parts of the body were considered to be in high risk for this cancer [49]. It has been explained that head and neck, genitourinary tract, and extremities are the most common

sites of rhabdomyosarcoma [74]. Statistically, it was found that this cancer can affect head and neck region (36%), genitourinary tract (23%), extremities (19%) and others (22%). In the head and neck region, rhabdomyosarcoma can arise in different sites including orbit, parameningeal sites (i.e. paranasal sinuses, nasopharynx, nasal cavity, infratemporal fossa, and middle ear), or other nonparameningeal locations (i.e. pharynx, thyroid, parotid gland, scalp and parathyroid glands, and oral cavity) [74]. It was also discovered that in patients diagnosed by rhabdomyosarcoma almost 25% were metastatic and 75% non-metastatic. The lung was found as primary organ of metastases for this cancer (40–50%) [49]. However, RMS also might metastasize to other organs including bone marrow (20–30%), bone (10%) and lymph node (20%) [49]. The sites of occurrence for the primary RMS have been presented as favorable tumors and unfavorable tumor sites [75]. The orbit, biliary tract, genitourinary (non-bladder, non-prostate, non-kidney), and non-parameningeal head and neck have been considered as favorable tumor sites. While other sites including bladder, prostate, parameningeal, and extremity have been known as unfavorable tumor sites [75]. Studies showed that survival rate for childhood RMS is different and depends on the primary tumor site (figure 1) [76].
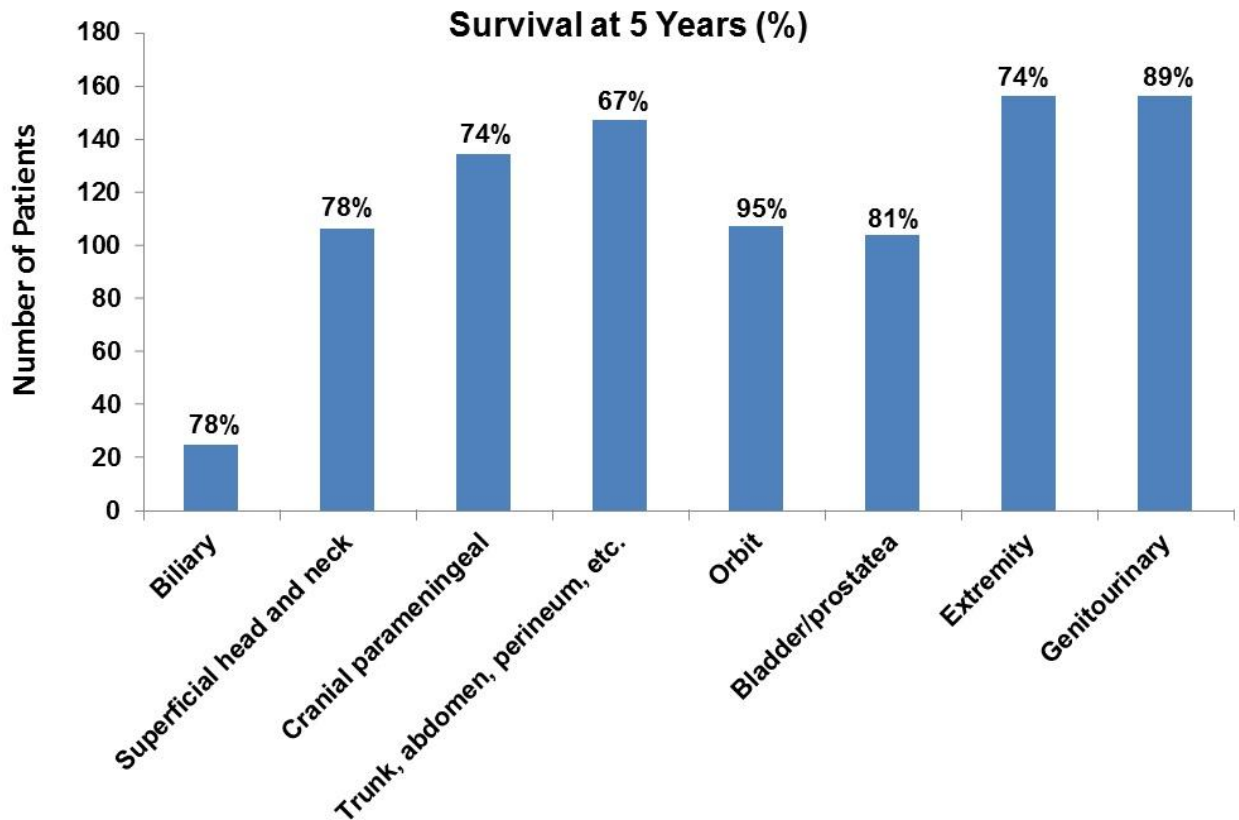
Figure 1. 5-Year Survival by Primary Site of Disease [76]. [a]Patients treated on Intergroup Rhabdomyosarcoma Study III [75]. [b]Patients treated on Intergroup Rhabdomyosarcoma Studies I–IV [77].

1.3. Treatment Option Overview for Children Rhabdomyosarcoma

At present, there are few therapeutic options available for patients with RMS since treatment for children RMS requires a multimodality approach [74]. Systemic chemotherapy in conjunction with local therapy (i.e. surgery and radiotherapy alone or in combination) are applied against all types of rhabdomyosarcoma in children [78, 79]. Prior to chemotherapy, surgery might be a primary approach if surgical resection will not result in organ dysfunction or disfigurement [74]. The strategy and management of these three treatment options (Surgery as a

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local control, radiotherapy a local control, and Chemotherapy) applied by the Children's Oncology Group (COG) and by groups in Europe (as exemplified by trials from the Soft Tissue Sarcoma Committee of the COG [COG-STS], the Intergroup Rhabdomyosarcoma Study Group [IRSG], and the International Society of Pediatric Oncology Malignant Mesenchymal Tumor [MMT] Group) was reported to be different [80]. Radiotherapy followed after the initial surgery or biopsy (but not in metastatic disease) has been used by COG-STS for patients with residual disease [79]. However, MMT group has been using chemotherapy as primary line therapy and then local control management as a second-line therapy when there were poor responses. Subsequent surgical resection is preferred over radiotherapy, which is used only after incomplete resection, documented regional lymph node involvement, or a poor clinical response to initial chemotherapy. This method is designed in order to reduce long-term side effects and major surgical procedures caused by radiotherapy [81]. The results obtained by the MMT study showed that the overall survival was 71%, whereas overall survival rate was found 84% in IRS-IV study. In addition, Event-free survival in 5 years in MMT89 study was reported 57% and that of for IRS-IV study was 78%. Interestingly, the outcome has been revealed to be considerably different in patients with extremity and head and neck nonparameningeal tumors. Failure-free survival was found in lower rate in bladder/prostate tumors patients which they did not receive radiotherapy as part of their initial treatment, but there was no difference in overall survival between the two methods for these patients [81]. It was concluded that using primary local therapy (radiotherapy) to increase the survival rate in RMS patients is preferred [76]. In the MMT trials, some patients have been spared aggressive local therapy, which may reduce the potential for morbidities associated with such therapy [78, 80].

1.4. Chemotherapy Treatment Options in Rhabdomyosarcoma

Chemotherapy is an essential therapeutic strategy against childhood rhabdomyosarcoma. The duration, timing, and intensity of the chemotherapy in patients with RMS is decided based on the tumor stage and site of tumor [82]. In clinical setting, patients with RMS are divided into three subgroups including low risk, intermediate, and high risk for treatment purposes [83, 84].

1.4.1. Low-risk tumor management

Statistically, 25% of diagnosed patients with RMS are low risk. The treatment for patients with low risk RMS is a two-drug or three-drug regimen [76]. The vincristine and dactinomycin (VA) with or without cyclophosphamide is utilized for low risk patients [85, 86]. In a study by Soft Tissue Sarcoma Committee of the Children's Oncology Group, 388 patients with low-risk embryonal rhabdomyosarcoma were separated into two subgroups and treated in different ways. Subgroup A (n = 264; Stage 1 Group I/IIA, Stage 2 Group I, and Stage 1 Group III orbit) were treated with a two-drug chemotherapy regimen that including VA for 48 weeks with or without radiotherapy. The subgroup B (n = 78; Stage 1 Group IIB/C, Stage I Group III nonorbit, Stage 2 Group II, and Stage 3 Group I/II disease) received vincristine, dactinomycin, and cyclophosphamide (VAC) chemotherapy. The results showed that overall survival rate for subgroup A patients in 5 years was 97%; however, that of for subgroup B patients was found 93% in 5 years [85]. A recent clinical trial study in 2017 confirmed that reducing total cumulative cyclophosphamide in treatment of low risk patients with RMS treated with VAC regime cannot significantly change the overall survival rate in patients with low risk RMS [86]. The survival rate after treatment with triple therapy was found at least 90% in patients with low risk RMS [76].

1.4.2. Intermediate-risk group

Around 50% of patients with RMS are diagnosed in the intermediate-risk level. VAC therapy as multi-agent chemotherapy is known to be standard treatment for this group [76]. The IRS-IV study tested VAC therapy compared with VA and ifosfamide as an alkylating agent (VAI) and vincristine, ifosfamide, and etoposide (VIE) in patients with intermediate-risk group [87]. In 3 years, the survival rates in intermediate-risk patients treated with VAC,VAI, VIE was between 84% to 88% [87]. Although, no significant differences were found between these three treatments (VAC, VAI, and VIE), the VAC therapy was easier to administer making it the prefered choice in RMS with intermediate-risk [87]. Another study has examined if topotecan and cyclophosphamide therapy can improve survival rate in patients with intermediate-risk rhabdomyosarcoma. In this preclinical study Patients who received topotecan and cyclophosphamide had no significant activity against RMS compared to VAC alone [88]. The potency of topotecan as a single-agent against RMS also has been reported in untreated children with rhabdomyosarcoma previously [89].

In pilot studies, a triple drug regime including vincristine, doxorubicin, cyclophosphamide (VDC) alternating with etoposide and ifosfamide (EI) was applied in order to evaluate the therapeutic effect against intermediate-risk RMS. However, it was emphasized that the efficiency of this regime should be evaluated further and compared with the standard approach [90]. In a clinical trial (SIOP-MMT-95) in Europe, 457 previously untreated patients with incompletely resected embryonal RMS, alveolar RMS, undifferentiated sarcoma, and soft tissue primitive neuroectodermal tumor were treated with ifosfamide, vincristine, and dactinomycin (IVA) therapy plus carboplatin, epirubicin, and etoposide for a period of 27 weeks. They showed that addition of carboplatin, epirubicin, and etoposide to the IVA therapy

provides no survival advantage and adds toxicity [91]. The survival rate for RMS intermediate-risk patients in 3 years was 82%; however, the overall survival for combination of IVA plus carboplatin, epirubicin, and etoposide was 80% [91].

1.4.3. High-risk group

The high-risk patients suffer from metastatic and aggressive RMS at the time of diagnosis [92]. The prognosis of disease in these patients is poor even with the current chemotherapy. The survival rate in 5 years for this group was reported ≤50% and emphasized there is urgent need to apply new approaches to treatment [75, 84]. The standard treatment for metastatic RMS has been known as triple therapy i.e. VAC [76]. Many efforts have been made to improve the outcome of treatment of patients with metastatic RMS by adding one or more chemotherapy drugs to the standard regime (VAC chemotherapy); however, to date, none of the new regimes have been shown to be more beneficial than the VAC regime [93]. A study group examined VAC therapy followed by pre-administration of ifosfamide/etoposide (IE) [94], vincristine/melphalan (VM) [94], and ifosfamide/doxorubicin (ID) [95] in high risk patients with RMS. They found that overall survival rate for patients after treatment with IE, ID and VM were 31%, 34%, and 22%, respectively [95]. In phase II clinical trial, topotecan plus cyclophosphamide was added to the VAC, but no change were observed in overall survival in children with metastatic RMS [88]. An up-front window trial of topotecan in patient with metastatic RMS also did not show any differences in survival rate [89]. Another group studied irinotecan alone and irinotecan with vincristine and demonstrated that irinotecan plus vincristine has a better response compared to irinotecan alone in metastatic RMS; however, overall survival in a preliminary analysis was not improved over previous experience [96].

1.5. Alkylating agents

Recently, oral alkylating agents, especially temozolomide (TMZ), have received considerable attention in RMS therapy [97]. Alkylating agents are a diverse class of reactive compounds that exhibit a wide range of biological, pharmacological, and chemoprotective properties [98-102]. Structurally, alkylating agents contain alkyl-sulfates, N-nitroso compounds, aliphatic epoxides, and haloalkanes, which each of these components contribute to their activity [99, 103, 104]. Together, these structural components allow alkylating agents to react with electron-rich atoms and transfer alkyl carbon groups onto a biological molecule [105]. Exposure to alkylating agents in the general population are often unavoidable, as they occur naturally in the air we breathe in, as well as in the food and water we consume [106, 107]. However, environmental pollution also plays an important role, with these agents being found in tobacco smoke, fuel combustion by products and other major sources of pollution [108, 109]. Importantly, alkylating agents can also exist in cells as byproducts of oxidative damage, and are naturally produced by cellular methyl donors, which occasionally attack DNA resulting in carcinogenic properties [110]. Accumulation of these alkylating agents in the body increases an individual's risk of alkylation damage, which is known to be cytotoxic, teratogenic, and carcinogenic [111]. Despite the toxic properties exhibited by alkylating agents in normal physiology, these agents have been shown to be beneficial in cancer therapy [112, 113]. Researchers have actively tried to use certain toxic alkylating agents as chemotherapeutic drugs to efficiently and safely kill cancer cells [114, 115]. This option exists because each individual alkylating agent can produce a distinct form of cellular damage, which will be mitigated by different cellular repair mechanisms or DNA damage response pathways. Together this means that the cellular response to each type alkylating agent will vary, allowing different

pharmacological regiments to target distinct cellular pathways, simply through altering the alkylating agent in use [116, 117].

1.6. Temozolomide

Temozolomide (TMZ), an alkylating agent, is a member of the triazine class of anticancer drugs. TMZ has a broad spectrum of antitumor activity, while being well-tolerated by the patient due to its relatively low toxicity [118-120]. Biochemically, TMZ is a small lipophilic molecule (194 Da) and is stable in acidic environment pH, while being less stable in alkaline environments (> pH 7) [121, 122]. These basic biochemical characteristics exhibited by TMZ allows for it to be administered orally [123]. Following absorption, TMZ enters cytosol and breaks down to form monomethyl triazene 5-(3-methyltriazen-1-yl)- imidazole-4-carboxamide (MTIC). MTIC reacts with water to form 5-aminoimidazole-4-carboxamide (AIC) and the highly reactive methyldiazonium cation [124]. This highly reactive species then transfers its methyl group to purine bases of DNA and methylate it in three different positions: (i) at N7 positions of guanine in guanine rich regions (N7-MeG; 70%), (ii) at N3 adenine (N3-MeA; 9%), and (iii) at O6 guanine residues (O6-MeG; 6%) [125, 126].

N7-meG is reported to be not mutagenic or toxic lesion, while it can enhance guanine depurination which has potential to form abasic sites. The formation of abasic sites may cause toxic and mutagenic properties and lead to single nucleotide polymorphisms, can act as a block transcription and replication [127]. N3-meA is a mutagenic and toxic lesion that can function as a block replication and cause A:T to T:A transversions [128]. Both N7-meG and N3-meA can be repaired by the base excision repair (BER) pathway. This repair system is activated by substrate specific glycosylases that recognize damaged bases [117]. Furthermore, Methylguanine-DNA methyltransferase (MGMT) is responsible for direct repair of O6-MeG, removing the methyl

adduct and restoring guanine. During DNA replication, O6-MeG mispairs with thymine but not with cytosine and alters DNA mismatch repair (MMR) [129, 130]. MMR recognizes and corrects mismatches thymine on the daughter strand generated during DNA replication and excises it. However, O6-MeG persists in the template strand. This process can lead to persistent DNA strand breaks, resulting in activation of apoptosis followed by triggering of G2/M cell cycle arrest and replication fork collapse [131-133]. Thus, the cytotoxicity of TMZ is primarily mediated at O6 guanine residues, causing both carcinogenic and mutagenic lesions [116, 134, 135]. It is known that functional MMR and low levels of MGMT are essential for a good response to TMZ [136].

1.6.1. Application of temozolomide in sarcomas

To date, the common treatments for sarcoma have yielded unsatisfactory responses from patients. Hence, new agents and combinations of available therapeutic agents are an active area of research. There is an increasing body of evidence supporting the different response of various types of sarcoma to temozolomide [137]. In 1999, the European Organization for Research and Treatment of Cancer (EORTC) conducted a phase II study in 31 patients with variable bone and soft-tissue sarcomas. This trial used orally administered TMZ at a dose of 750 mg/m2 divided over 5 days and repeated every 28 days. Of the 31 patients involved, only one individual with retroperitoneal leiomyosarcoma metastatic to breast, skin, and liver, demonstrated a partial response. In an additional 9 patients with varying histologies, TMZ treatement stablized their disease, while the remainder of patients had progressive disease to therapy, and one patient was excluded from the study [137]. Importantly however, no Grade 4 toxicity (ie. life-threatening toxicity) and no fatalities resulted from TMZ treatment [137]. Overall, the response rate in this

trial was 3.33, indicating that TMZ cannot be recommended for further study in advanced soft tissue sarcoma - at the same dose and schedule.

In another study looking at the effect of TMZ on different soft-tissue sarcomas, TMZ was administered to 25 patients, two times per day, for 5 days out of each month. Two patients exhibited partial responses (8%); three cases with stable disease for over 6 months (12%); two cases exhibited mixed responses (8%). Interestingly, all patients that responded to TMZ treatment had uterine or non-uterine leiomyosarcoma. This study also reported low toxicity at this relatively high dose, suggesting the possibility that using an increased dose may lead to an improved response [138]. The Spanish Group for Research on Sarcomas (CTOS) investigated another regimen in patients with varying soft tissue sarcomas. TMZ was administered orally at 75 mg/m2 dosage for 6 weeks, followed by a 3 week rest period. Among the 27 evaluated patients, 4 exhibited partial responses to TMZ treatment, two with and two without leiomyosarcoma. Further, the responses in the two with leiomyosarcoma lasted for 14 months while the non-leiomyosarcoma responses lasted only 10 months [139].

In Ewings sarcoma, treatment with TMZ or irinotecan alone has been shown to not singificantly impact this cancer's growth and development [140-143]. This was first observed in a phase II trial that showed when patients with advanced soft tissue sarcomas - including leiomyosarcoma, miscellaneous sarcoma, neurogenic sarcoma, unclassified sarcoma, liposarcoma, malignant fibrous histiocytoma, and synovial sarcoma – were treated with TMZ alone, it was an ineffective second-line therapy (96% certainty that the response rate is < 20%) [137]. Consistant with this result, a study by the EORTC Soft Tissue and Sarcoma Group showed that mitozolomide, a drug from the imidazotetrazine class, had no response on 25 patients with advanced soft tissue sarcoma [144]. Another report showed only modest activity of

TMZ against unresectable or metastatic leiomyosarcoma of both uterine and nonuterine origin [138]. This ineffectivity of TMZ also has been observed in musculoskeletal sarcomas, particularly in certain histological subtypes, leiomyosarcoma, solitary fibrous tumors and Ewing's sarcoma as prominent examples [139, 145-147].

Interestingly, combination therapies including both TMZ and irinotecan have been shown to be a well tolerated treatment against Ewing's sarcoma. This synergistic action of TMZ and irinotecan against Ewing's sarcoma was first reported in animal model, and was then further confirmed by phase I clinical trial that included 7 patients [148, 149]. In this trial, 1 patient was shown to have a complete response, with 2 additional patients having a partial or minimal response to treatment. These promising results demonstrate that combination therapies that include both TMZ and irinotecan may be useful in the treatment of Ewing's sarcoma [149].

Combination of vincristine, irinotecan, and temozolomide - with median follow-up of 3 months in 22 patients with relapsed and refractory Ewing sarcoma - indicated a complete response in 5 patients, partial response in 7 patients, stable disease in 3 patients, and progression disease in 7 patients. The overall response rate was 68.1%. This regimen was particularly satisfactory for relapsed Ewing's sarcoma, compared to patients who progressed to initial therapy [150].

It was hypothesized that azacytidine, a hypomethylating agent that particularly targets caspase-8 genes, can enhance the effect of TMZ in patients with unresectable, metastatic soft tissue sarcoma, as well as malignant mesothelioma. 15 patients died over a period of 2 to 59 months. In this trial an additional 10 patients remained with stable disease [151]. An additional phase 1 clinical trial showed that the combination of irinotecan, temozolomide, and temsirolimus

every 21 days in children, adolescents, and young adults suffering from solid tumors is well tolerated. This finding suggests a potential role for irinotecan, temozolomide, and temsirolimus (ITT) in a spectrum of relapsed or refractory childhood solid tumors. [152].

1.6.2. Chemotherapy Application of Temozolomide in Rhabdomyosarcoma (RMS)

At present, there are few therapeutic options available for patients with RMS. Numerous studies support that TMZ is an active agent against RMS [153-155]. The synergistic and additive actions of TMZ in combination therapy in RMS have also been demonstrated in several preclinical and phase I/II settings [156-158]. More recently, TMZ alone, or combined with other chemotherapy drugs, proved tolerable and effective in RMS in relapse settings [97, 156].

It has been shown that the combination of irinotecan and TMZ has synergistic antitumor activity against RMS in preclinical models, as well as clinical activity in pediatric sarcomas [159-162]. In another study, using a mouse xenograft model, the synergistic effect of TMZ with irinotecan was observed [163]. Recent findings also show that the combination of TMZ with irinotecan can result in tumor regression and a reduction of tumor volume, when using an adult pleomorphic rhabdomyosarcoma patient-derived orthotopic xenograft (PDOX) nude-mouse model [158]. In a phase I trial that used a combination of vincristine, irinotecan, TMZ, and antibiotic (cefpodoxime), known as VITA, resulted in partial response with 2 cycles and a complete radiographic response with 6 cycles in a single patient with RMS [164]. Furthermore, another research group showed that a combination regimen of VITA can be beneficial in the overall success of treatment in patients with relapsed ARMS [97].

Additionally, the combination of vincristine, oral irinotecan, and temozolomide, known as VOIT, was administered in a children's oncology group as a phase 1 consortium study. Daily

dosage for 5 day for 1 week was found to be well tolerated in greater dose intensity of irinotecan and temozolomide than VOIT administration daily for 5 days every 2 weeks for children with relapsed or refractory solid tumors. However, no objective responses were seen among the 6 patients with RMS treated with vincristine, oral irinotecan, and temozolomide [156]. In another study, prolonged stable disease was observed in 1 patient with RMS treated with topotecan in combination with temozolomide (TOTEM) [165]. However, when temozolomide (TMZ) and etoposide (VP) were combined in a trial with 5 RMS patients, treatment resulted in no considerable response [166].

1.7. Apoptosis

Apoptosis is an important physiological process, which plays role in aging, tissue homeostasis, and development, and regulates cell fate in various organisms [167]. The regulatory mechanisms of apoptosis are controlled through two different pathways including the extrinsic and the intrinsic mitochondrial pathways (Figure 2) [168]. The extrinsic signaling pathway can be activated via signals received from outside of the cell, inducing death receptors in the cell surface (FasR, DR4, DR5, TNF-R1) [168]. Cell death receptors are apoptosis-mediating receptors, which require ligation with their specific ligands including FasL, TNF-alpha, Apo3L and TRAIL for activation [169]. Activated death receptors trigger initiator caspase (ie. caspase -8, -10), and then effector caspase (ie. caspases -3,-6, and -7) activation, which in turn leads to apoptosis [170, 171]. Activated caspase-8 can also effect the function of mitochondria through truncated Bid. Bid is an important apoptotic target of caspase-8 and is the molecular linker bridging the extrinsic and intrinsic apoptotic pathways [172]. Bid is truncated after activation and then induces pro-apoptotic proteins (Bax), causing the release of cytochrome c from mitochondria that triggers activation of caspase 9 and apoptosis [173, 174].

The intrinsic apoptotic pathway can be activated by different extracellular stimuli such as nutrients, drugs, and radiation. Importantly, however, various intracellular stimuli including oncogene expression, oxidative stress, DNA damage, and endoplasmic reticulum (ER) stress can also activate the intrinsic pathway [175-178]. Stress signals activate Bcl2-family proteins including pro-apoptotic members (e.g. Bax, Bak) [168, 179] and anti-apoptotic proteins (e.g. Bcl-2 and Bcl-xl, and others) [180]. The balance between the pro-apoptotic proteins and anti-apoptotic proteins determines cell fate. Excess in expression of pro-apoptotic proteins can increase the sensitivity of cells to apoptosis, whereas higher expression of anti-apoptotic proteins can lead to cell resilience. When pro-apoptotic proteins (i.e. Bax [BCL-2-associated X protein) and BAK [BCL-2 antagonist/killer]) are stimulated, the mitochondrial outer membrane (MOM) is permeabilized. This promotes the release of cytochrome c from the mitochondrial intermembrane space to cytosol and the activation of apoptosis [181, 182]. Conversely, the anti-apoptotic proteins decrease MOM permeabilization and prevent mitochondria from releasing cytochrome c [183].
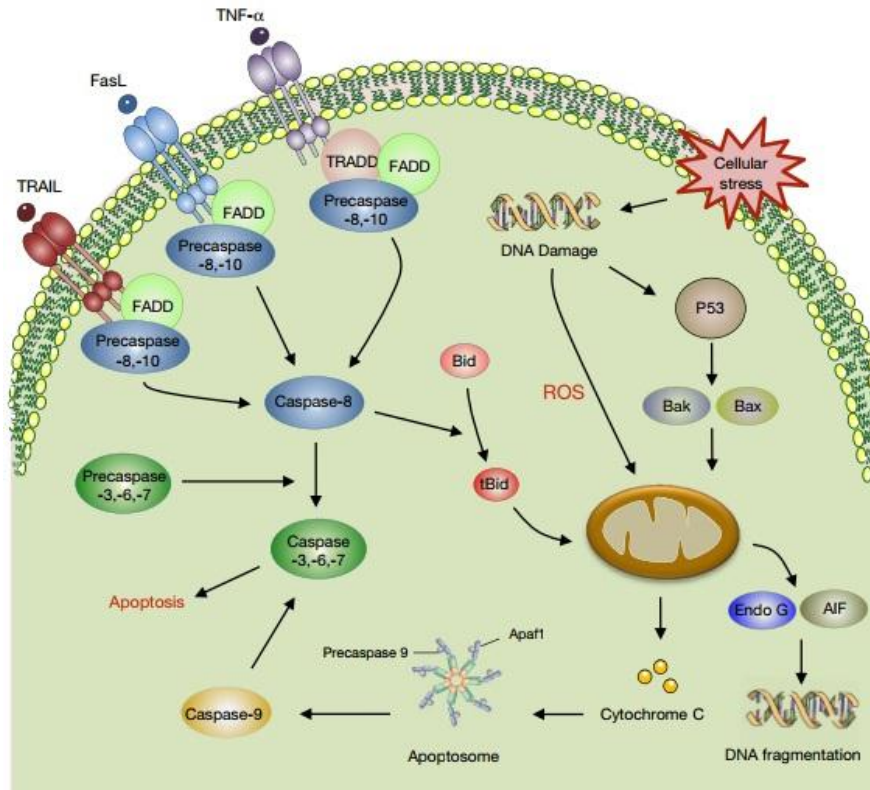
Figure 2. The overview of apoptotic signaling pathway. There two pathways for induction of apoptosis. Extrinsic or death receptor dependent apoptosis pathway can be initiated through the ligation of death receptors (Fas, DR4, DR5, TNF-R1) by their specific ligands e.g., FasL, APO-2L, TRAIL, and TNF. Once death ligand binds to its correspondence receptor, FADD is recruited and apoptotic signals can trigger the activation caspase-8. Active caspase-8 can activate caspases -3,-6, and -7 to induce apoptosis. In addition activated caspase 8 affects mitochondria via truncated BID and causes mitochondrial initiator caspase (caspase-9) activation, leading the activation of effector caspases and later induction of apoptosis. Instrinsic signaling pathway initiate by different strees signals mostly inside of cells. Stress could activate pro-apoptotic Bcl-2 family (Bax/Bak) and eventually leading to caspase dependent or independent apoptosis. Anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xl) counteract pro-apoptotic ones and could delay or inhibit apoptosis [184].

1.7.1. The induction of apoptosis by temozolomide in different sarcomas

As shown in previous studies, temozolomide's mechanism of action in sarcomas known to be closely linked to the activity of DNA repair protein O6 -alkylguanine DNA alkyltransferase, which is encoded by the DNA repair gene, O6-methylguanineDNA methyltransferase (MGMT). This DNA repair protein removes alkyl groups from the O6 position

of guanine [185-189].

An ideal response to TMZ in sarcomas is dependent on low levels of MGMT or the silencing of MGMT [136]. The silencing of MGMT occurs, albeit infrequently, via promoter methylation in soft tissue sarcomas [190]. One study that included 62 patients with different soft tissue sarcomas including liposarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor (MPNST), malignant fibrous histiocytoma (MFH), and synovial sarcoma, indicated that 34% had MGMT promoter methylation [191]. However, in another study that observed 65 patients with MFH, MPNST and leiomyosarcoma, only 15% patients had MGMT promoter methylation [192].

Alkylating agents' ability to trigger apoptosis seems to be dependent on the level of MGMT and activation of the different signaling pathways [193, 194]. The pathways involved in apoptosis induction might be different in each type of sarcoma. It has been discovered that TMZ induces dose/time dependent apoptotic cell death in musculoskeletal sarcomas (MSS) though enhancing the activity of caspase-3 and PARP [147]. XTT assay's were used to compare the sensitivity of MSS cells (SKNMC (Ewing's sarcoma), Saos-2 (Osteosarcoma), NOS1 (Osteosarcoma), NMS-2 (Malignant peripheral nerve sheath tumor), NEPS (Epithelioid sarcoma), FU-EPS-1 (Epithelioid sarcoma), SFT-8606 (Epithelioid sarcoma), HS-SY-II (Synovial sarcoma), SYO-1 (Synovial sarcoma), HT-1080 (Fibrosarcoma), 402-92 (Liposarcoma), ASPS-KY (Alveolar soft part sarcoma)) with U-87 MG cells (human glioblastoma) with U-87 MG cells (human glioblastoma) [147]. The result showed that the half maximal inhibitory concentration (IC50) values for MSS cells after 120 hours treatment, was in the range between 72 to 1167μM and that of for U-87 MG, was 348μM. The IC50 value of TMZ in SKNMC, NOS1, HS-SY-II, SYO-1 and 402-92 cells was lower than the IC50 for U-87 [147].

This leads to the suggestion that TMZ is more potent in Ewing's sarcoma, osteosarcoma, synovial sarcoma, and liposarcoma in compare to U87-MG cells. HT1080, NMS-2, Saos-2, ASPS-KY, NEPS, FU-EPS-1 and SFT-8606 cell lines have been observed to be highly resistant to TMZ compared to other srcoma and glioblastoma cells [147].

While temozolomide's general mechanism of action is well understood, there is little information about how this alkylating agent specifically affects sarcomas at the cellular level. The obtained findings regarding cell viability in different types of sarcomal cell lines indicates considerable variability in TMZ's ability to induce cell death [147]. These results further indicate that it the mechanism of action of TMZ differs in various sarcomas.

It is known that TMZ induces apoptosis through activation caspase-3 and PARP in MSS cells. Additionaly, PI3K/Akt and ERK1/2 MAPK signaling pathways were also found to be involved in regulation of apoptosis in MSS cells. In SYO-1, HT-1080, NMS-2 and ASPS-KY cell liness, the level of p-Akt was observed to be decreased for 24 hrs after treating with TMZ. However, the expression of phosho-Akt was shown to be enhanced at 72 h after treatment with TMZ [147]. The basal activation ERK1/2 was also reported MSS cells. In the presence of 250 μM TMZ, the levels of p-ERK1/2 were marginally decreased in ASPS-KY and NEPS cells following 48 h treatment. Activation of ERK1/2 generally promotes cell survival by regulation of BCL-2 family proteins [195]. However, under certain conditions, ERK1/2 can have pro-apoptotic functions [196]. It has been shown that ERK1/2 activation can suppress apoptotic pathway by regulation of cell death receptors (i.e. Fas, TNF, and TRAIL) [197].

1.8. Autophagy

Autophagy is a conserved physiological process of cellular self-eating, which plays an

essential role in cellular housekeeping activity by targeting cytoplasm, damaged and dysfunctional organelles; misfolded and toxic aggregate-prone mutant proteins, and intracellular pathogens [198-200]. It has been shown that autophagy is implicated in several fundamental biological processes, including stress adaptation, aging, development, immunity and protection against neurodegeneration [201, 202]. Autophagy can be activated by a variety of cell stresses including changing nutrient conditions, starvation or pathogen infection [203, 204]. Depending on the route of cytoplasmic material that is delivered to the lysosomes, there are at least three distinct forms of autophagy: chaperone-mediated autophagy, microautophagy and macroautophagy [198, 205]. Microautophagy, a non-selective mechanism, is the sequestration of small portions of cytoplasm that directly traps small proteins and organelles at the lysosome or vacuole, resulting in degradation of the materials [206]. Chaperone-mediated autophagy (CMA) is a selective mechanism for the degradation of specific cytosolic proteins through the CMA substrate chaperone [206]. Macroautophagy (from here on abbreviated to autophagy), plays a major physiological role and is better characterized than other forms of autophagy. This process is initiated by the formation of double-membrane vesicles, known as autophagosomes, which form around cargo and are then able to fuse with the vacuole in yeast or the lysosome in mammalian cells [201]. This fusion between the lysosome with autophagosome forms a structure known as autophagolysosome where lysosomal enzymes are then able to hydrolyze and degrade the cargo [207, 208]. As a result of degradation, amino acids, fatty acids, and nucleotides are produced and are exported back to the cytosol to be reused for energy metabolism, and in both macromolecular production and biosynthesis [209, 210].

Autophagy is divided into several different phases including, induction, nucleation,

expansion, fusion and degradation [211]. Autophagy is controlled by autophagy-related genes (ATGs), which encode proteins involved in autophagy [212, 213]. The proteins have been further divided in five groups and all have different functions [213]. The proteins include: (i) a protein serine/threonine kinase complex, which acts as an initiator protein, responding to upstream signals including target of rapamycin (TOR) kinase (Atg1/ULK1, Atg13 and Atg17); (ii) a lipid kinase group, which is implicated in nucleation complex i.e. Atg6/Beclin1, Atg14, Vps34/ PI3KC3 and Vps15; (iii) two ubiquitin-like conjugation cascades that allow expansion of the vesicle (the Atg8 and Atg12 conjugation systems); (iv) a recycling pathway that is involved in the disassembly of Atg proteins (Atg2, Atg9, Atg18); and (v) vacuolar permeases that permit the efflux of amino acids from the degradative compartment (Atg22) [214-216].

Autophagy is initiated in response to signals from stimulators, such as starvation. Under stress conditions, inhibited mammalian TOR (mTOR) kinase phosphorylates Ulk1-Atg13-FIP200-Atg101 complex (an autophagy machinery core), triggering VPS34-Beclin 1-class III PI3-kinase complex [217, 218]. Next, Beclin 1 binds to Vps34 and contributes in autophagosome nucleation [219, 220]. At this point, Atg (autophagic related proteins) are involved in initiation of formation of phagophore and then autophagosome [221] followed by conversation of the LC3β-I (free form), a microtubule-associated protein 1 light chain 3 (LC3), to LC3β-II (phosphatidylethanolamine-conjugated form) [222, 223]. This process will further proceed until completion of the autophagosome. Cargo is engulfed in autophagosome by a process dependent on P62 [224]. After that, lysosome is fused with autophagosome to form autophagolysosome, where the cargo is digested [225] (Figure 3).
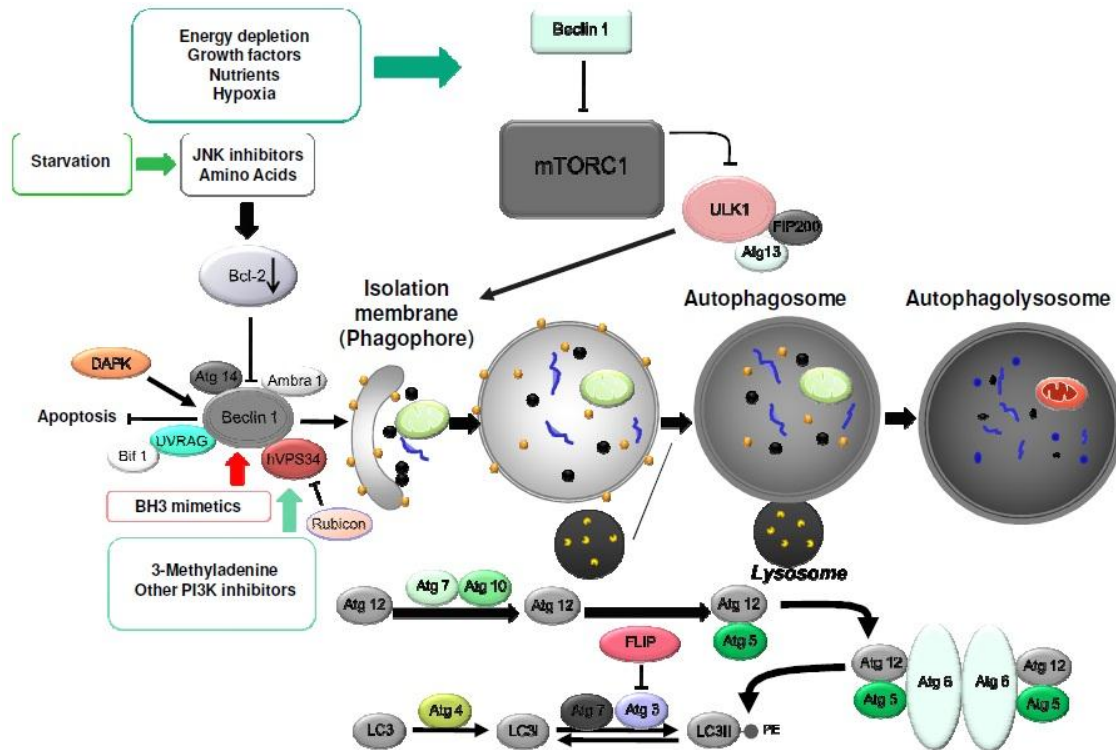
Figure 3. The overview of autophagy signaling pathway. Autophagy is a homeostatic process for the degradation and recycling of unnecessary cellular compartments, which that is controlled by many cellular pathways and 32 evolutionary conserved autophagy-related genes (ATG). This pathway has different regulated steps such as induction, nucleation, expansion and completion, fusion and degradation. The mTOR is known as the key regulator of autophagy induction and can be inhibited through ULK1, causing the induction of VPS34-Beclin 1-class III PI3-kinase complex which is involved in autophagy induction. Several different membrane pools contribute to the formation of the phagophore. The Atg proteins (Atg2, Atg9, Atg18) are essential for phagophore formation. The Atg5-Atg12-Atg16 and LC3 conjugation system also participate in autophagosome membrane formation and elongation. Later the autophagolysosome is formed by the fusion of the autophagosome and lysosome to digest and the cargo [216].

1.8.1. The involvement of autophagy in rhabdomyosarcoma

Autophagy is also known to be a crucial processes in the maintenance of cellular viability and proliferation in rhabdomyosarcoma [226]. In 2014, Zhou and colleagues demonstrated that loss of autophagy leads to a reduction in growth and proliferation of rhabdomyosarcoma cells [227]. Using chloroquine, it was also shown that the inhibition of autophagy induced by anticancer drugs can suppress cellular viability in human rhabdomyosarcoma RD and RH30 cells [227, 228]. Consistently, inhibition of autophagy by knocking down Atg7 or through Baf-A1

treatment, has been observed to result in the depression of cell growth and viability in rhabdomyosarcoma cells [228].

SIRT1 and SIRT2 are deacetylase enzymes that belong to the mammalian Sirtuin (SIRT) family, which are known to be involved in tumorigenesis [229]. Overexpression of SIRT1 and SIRT2 has been observed in both human rhabdomyosarcoma biopsies as well as human rhabdomyosarcoma cell lines [229]. Importantly, the loss of SIRT1 and SIRT2 can result in inhibition of stress-induced autophagy through AMPK pathway dependent manner, sensitizing tumor cells to death [230, 231]. A recent study has reported that pre-treatment with neferine, a major alkaloid derived from the embryos of Nelumbo nucifera Gaert, can impair hypoxia induced autophagy by blocking the expression of Beclin1 and PI3KCII in RD cells. This finding suggests that neferine is capable of protecting RD cells from autophagic cell death via activation of the PI3K/AKT/mTOR pathway[232]. In another investigation, accumulation of the amyloid precursor protein (APP) and β-amyloid were reported when human rhabdomyosarcoma CCL136 cells were incubated with TNF-α or rapamycin. Interestingly, the reduction of APP as well as β-amyloid was found in another study under the condition that there was a loss of macroautophagy [233].

1.9. Connection between autophagy and apoptosis

Autophagy and apoptosis are two independent processes, while under certain conditions they can make mutual relationship to regulate the turnover of cells within organisms and of organelles and proteins within cells [234, 235]. However, the inter-connection of apoptosis and autophagy and how they are mutually connected are highly context-dependent [236]. It has been explained that in the majority of cases autophagy and apoptosis have suppressive effect on each other [237, 238]. Generally, autophagy is rapidly induced after exposing with low dosage

of stress, whereas apoptosis is initiated followed by long-term exposing with high doses of stress, reflecting that the autophagy–apoptosis crosstalk supports the instinct of the cell to adapt to stress and decrease the sensitivity of cells to death stimuli [236]. However, in special conditions, autophagy may also contribute in induction of cell death by either the activation of programmed cell death type II (autophagic cell death) or through linking with cell death signaling pathways (apoptosis or necrosis) [239, 240]. In autophagic cell death, autophagy blockage can prevent cell death and the final cell death is determined by the level of autophagy flux and not by other cell death pathways (i.e. apoptosis or necrosis) [201, 241-243]. Autophagy also can act as a facilitator of apoptosis by providing ATP during stress conditions to promote ATP- dependent apoptotic mechanisms [244]. ATP is essential for 'eat me' signal phosphatidylserine. It has been shown that suppression of autophagy in embryoid bodies led to reduce the level of the ''come-get-me'' signal (lower level in secretion of lysophosphatidylcholine) and consequently can lead to failure of dead cell clearance during apoptosis [244]. Apoptosis may also be facilitated by autophagy activation through maintaining ATP level necessary to maintain active membrane blebbing in apoptosis which is a ATP-dependent process [245].

1.9.1. Apoptosis-regulating proteins in the modulation of autophagy

There are key molecular regulators of the crosstalk between autophagy and apoptosis. BCL-2 family proteins can play a dual role to regulate autophagy and apoptosis. The interaction between BCL-2 family proteins leads to the regulation of apoptosis followed by stimulation and/or neutralization of proteins with pro-apoptotic and anti-apoptotic functions [246]. BCL-2 family proteins including Bad, Bid, Bnip3, Nix, Noxa, and Puma have also been

27

shown to activate autophagy through competitively disrupting the inhibitory interactions between Beclin-1 and Bcl-2/Bcl-xl or Mcl-1 [247-249]. The use of pharmacological BH3 mimetics in different studies also has shown the same effect [248-250]. The sequestration of anti-apoptotic proteins (Bcl-2 and Bcl-xl) and Beclin-1 can result in induction of the PI3K activity of the autophagy protein vacuolar protein sorting 34 (VPS34), thereby suppression of autophagy [251]. During stress conditions, Bcl-2 should be displaced from Beclin-1 and Bax to activate autophagy and apoptosis, respectively (Figure 4) [235]. It has been reported that Bcl-2 localized in the endoplasmic reticulum (ER) interacts with the nutrient-deprivation autophagy factor-1 (NAF-1) and stabilizes its interaction with Beclin-1, preventing Beclin-1-mediated starvation-induced autophagy [252-254]. Moreover, mitochondrial Bcl-2 was known to play inhibitory role on AMBRA1-induced autophagy activation, by releasing AMBRA1 (Beclin-1-interacting protein) from Beclin-1 [255]. In the same study it was revealed that the interaction of mitochondrial Bcl-2 and AMBRA1 is decreased during apoptosis [255]. The stimulatory role of Nix (BNIP3L), a BCL-2 family protein, on autophagy has been reported. The interaction of Nix and GABA receptor-associated protein (GABARAP), a functional homologue of the autophagy protein light chain 3 (LC3), has been explained as potential crosslink between apoptosis and autophagy [256]. Bim, another pro-apoptotic BH3 only protein, was found to have inhibitory effect on Beclin-1 dependent autophagy by interacting directly to Beclin-1 and mislocalizing it to dynein light chain 1 (DLC1) [257].

The tumor suppressor protein P53 can induce extrinsic apoptotic signaling pathway when it is in nucleus by enhancing the expression of the Fas receptor and TRAIL receptor. P53 in the cytoplasm can activate intrinsic apoptotic signaling pathway by either triggering pro-apoptotic proteins (PUMA, Bax, Bid, and Noxa) or Apaf-1 of the apoptosome [238, 258].

In addition, the important role of P53 in autophagic signaling pathway has been identified [259, 260]. Interestingly, in the nucleus, activated P53 triggers damage-regulated autophagy modulator (DRAM), a stimulator of autolysosome formation, thereby leading to the activation of autophagy [261]. In the cytoplasm, however, activated P53 inactivates AMP kinase and suppresses autophagy by stimulating of mTOR signaling [262]. P53 is also considered to be responsible for activation mitochondria-specific autophagy through activating of PUMA, leading to mitochondria degradation [259]. For this function, PUMA requires Bax/Bak activation but not Bcl-2/Bcl-xl and Beclin-1 interaction. PUMA/Bax-mediated autophagy is required to recruit Atg5, Atg10, and Atg7 which can be involved in apoptosis activation [235]. In addition, PUMA's initiation of autophagy can trigger cytochrome c release from mitochondria, resulting in apoptosis [263].
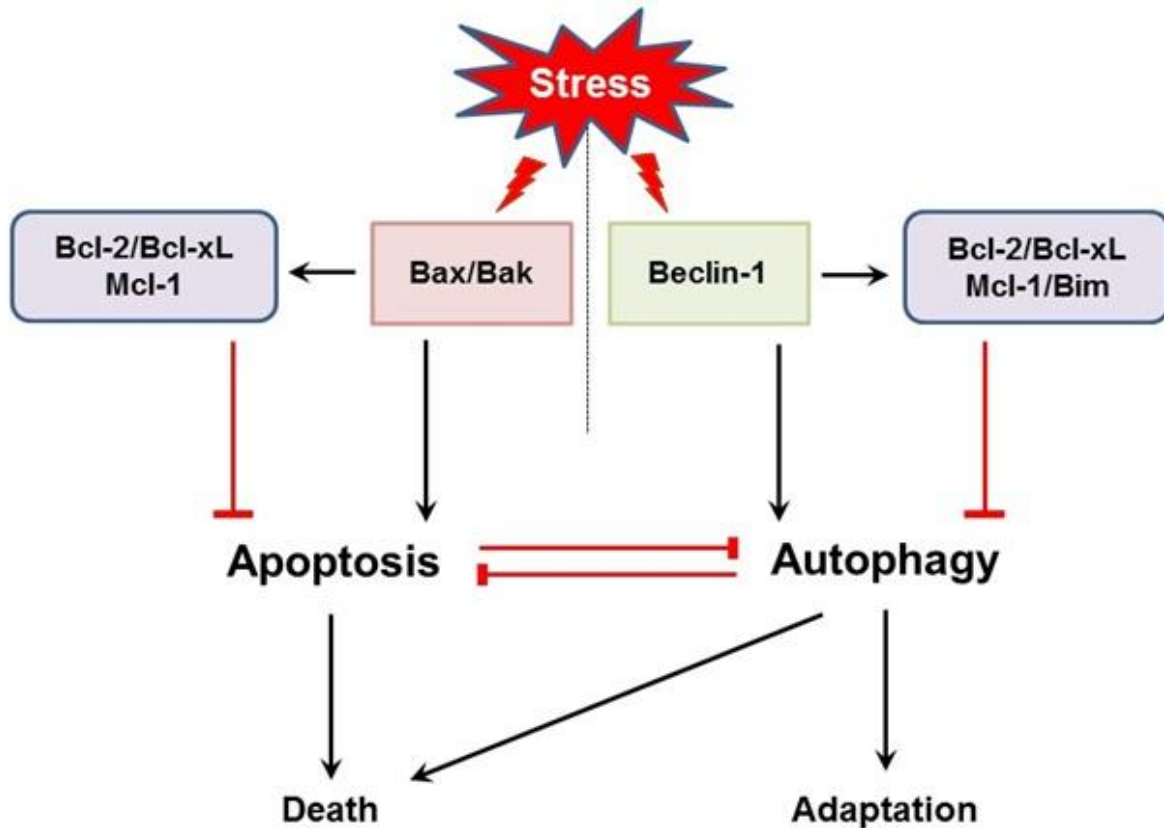
Figure 4. Crosslink of autophagy and apoptosis via Bcl1 family protein interactions. When cells are in normal condition, antiapoptotic proteins (such as Bcl-2, Mcl-1, Bcl-xl) bind to pro-apoptotic proteins including Bax/Bak and Beclin-1, thereby, preventing induction of apoptosis and autophagy, respectively. However, when cells are under stress, anti-apoptotic proteins tends to dissociate from pro-opoptotic proteins and Beclin-1 which then can result in activation of apoptosis and autophagy [264].

Autophagy and apoptosis can link together through other proteins like autophagic related proteins i.e. Atg12, Atg5, and Atg3. Atg12 binds to Mcl-1/Bcl-2 complex and inactivates the complex, which results in mitochondrial apoptosis activation. In addition, knock-down of Atg12 can suppress the pro-apoptotic protein (Bax) and inhibit cytochrome c release from mitochondria; whereas, ectopic expression of Atg12 represses Mcl-1 activity [265].

1.10. Rationale of the Study

Rhabdomyosarcoma is a lethal disease in children, and novel ideas can help to develop therapeutic strategies in this field. Since in many studies TMZ is found to be a potent active agent in cancer therapy, different clinical trials are testing TMZ in combination therapy with other drugs. However, the underlying mechanisms of action of TMZ remains unclear. These exciting and innovative studies will lay the molecular foundation to advance our understanding of this therapeutic strategy to promote programmed cell death of RMS cells. We will use the results of the current project, investigating how TMZ induces apoptosis in RMS cells, as a platform to understand how RMS cells can become resistant to TMZ therapy. This knowledge will enable us to design combinatorial approaches that will expand the clinical usefulness of TMZ as a chemotherapy agent.

1.11. Hypothesis and objectives

1.11.1. Hypothesis:

Autophagy is involved in regulation of TMZ-induced apoptosis in RMS cells (Figure 5).
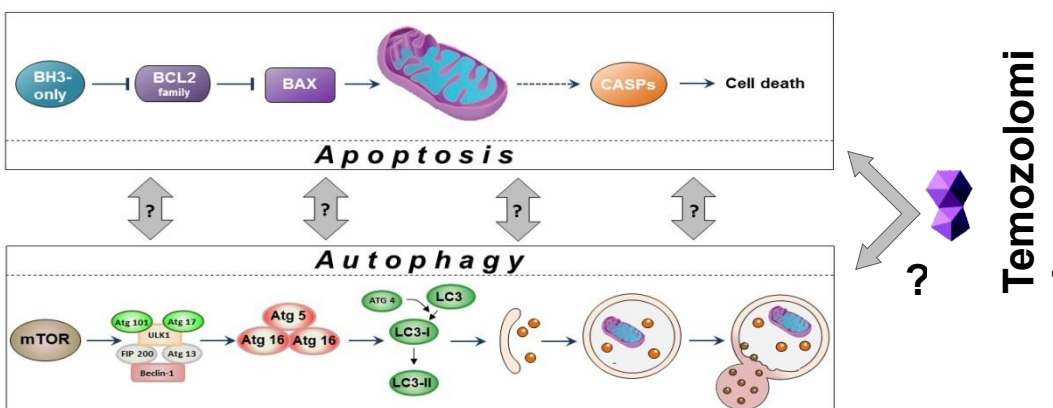
Figure 5. A concise overview of the hypothesis of the study focusing on the modulatory role of autophagy on apoptosis induced by TMZ.

1.11.2. Objectives:

A. Investigate if TMZ induces autophagy.

B. Investigate if TMZ induces autophagy using Baf-A1to inhibit autophagy flux.

C. To determine if autophagy inhibition (pharmacological inhibitors) modulates TMZ-induced apoptosis in RMS cell lines.

D. Investigate caspase activation and luminescence caspase assay to further confirm autophagy inhibition on TMZ induced apoptosis.

2. CHAPTER 2: Materials & Methods

2.1. Materials and Antibodies

Cell culture plastic ware, penicillin, and streptomycin were purchased from VWR (Toronto, ON, Canada). Cells were cultured in Roswell Park Memorial Institute (RPMI-1640) with L-Glutamine & 25mM Hepes (BioWhittaker; Cat #: 12-115Q) and Dulbecco's Modified Eagle's Medium (DMEM) (CORNING; Cat #: 50-003-PB) with 10% Fetal Bovine Serum (FBS) (Gibco™; Cat #: 16000044). Autophagy inhibitor Bafilomycin-A1 (Baf-A1), rabbit anti-human/mouse/rat LC3 (L8918, 1:3,000), anti-mouse IgG (A8924, 1:3,000), and anti-rabbit IgG (A6154, 1:5,000), propidium iodide (PI), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT) were purchased from SIGMA-Aldrich Canada Co, Oakville, CA. All western blotting primary and secondary antibodies are listed in Table 1 & 2. Casapase-Glo®-3/7, Caspase-Glo®-8 and Caspase-Glo®-9 assay were purchased from Promega (Toronto, ON, Canada). Enhanced chemiluminescence (ECL) and Enhanced chemiluminescence (ECL) prime regents (Western Blotting Detection Reagent) were purchased from Amersham-Pharmacia Biotech. Hyperfilm™ ECL was purchased from fisher scientific. All other biochemicals were American Chemical Society grade and were purchased from either Fisher Scientific (Winnipeg, MB, Canada) or SIGMA-Aldrich Canada Co.

Table 1. Primary antibodies used in western blotting

Primary Antibody	Dilution	Source Cat No.	Molecular size (KDa)
LC3B-I	1:2500	SIGMA; # L7543	18

LC3B-II			16 kDa
P62	1:1000	Cell signaling; #8025	62 kDa
Bax	1:1000	Cell signaling; #2774	20 kDa
Bcl-2	1:1000	Cell signaling; #2870	26 kDa
Bcl-xl	1:1000	Cell signaling; #2762	30 kDa
Mcl-1	1:1000	Cell signaling; #4572	40 kDa
Bak	1:1000	Cell signaling; #12105	25 kDa
Nix	1:1000	Cell signaling; #12396	38,76 kDa
Beclin-1	1:1000	Cell signaling; #3495	60 kDa
Bid	1:1000	Cell signaling; #2002	15, 22 kDa
PARP	1:1000	Cell signaling; #9532	116, 89 kDa
Cleaved Caspase-3	1:1000	Cell signaling; #9661	17,19 kDa
Cleaved Caspase-7	1:1000	Cell signaling; #8438	19 kDa
Cleaved Caspase-9	1:1000	Cell signaling; #9505	35 kDa
Phospho-Histon H2A.X (Ser139)	1:1000	Cell signaling; #2577	15 kDa
GAPDH	1:1500	Santa Cruz; sc-47724	37 kDa
Actin	1:400	Santa Cruz; sc-1616 (1-19)	40 kDa

Table 2. Primary antibodies used in western blotting

Secondary Antibody	Dilution	Source Cat No.
Anti-Rabbit IgG (whole molecule)–Peroxidase antibody produced in goat	1:5000	SIGMA; # A-6154
Anti-Mouse IgG (Fab specific)–Peroxidase antibody produced in goat	1:3000	SIGMA; # A-8924

2.2. Cell culture

The human rhabdomyosarcoma cell line (RH30) [RC13, RMS 13, SJRH30] (ATCC® CRL 2061™) (Human muscle cancer cells) and mouse muscle cell line (C2C12) (ATCC® CRL-1772™) were used in this project. RH30 cell lines were cultured in (RPMI-1640) with L-Glutamine & 25mM Hepes media and C2C12 cells were cultured in (DMEM) with high Glucose media. Both media were supplemented with FBS (10%), penicillin (1%), and streptomycin (1%). Cells were grown to 35-40% confluency on a 100 mm cell culture plate, 6-well plates, and 96 well plates. Cells were maintained in a humidified incubator with 95% air and 5% CO2 at 37˚C and were passaged once every 2 to 3 days.

2.3. Cell viability assay

Cell viability in RH30 and C2C12 cells was measured under different treatment conditions, using MTT assay [266-268]. The RH30 cells (30,000 cells/ml) and C2C12 (20,000 cells/ml) were seeded in 96 well plates and treated with different concentrations of TMZ in different time points (0–1000 μM, 0–96 h). Cells were also treated with various concentrations (0.1, 1, 2.5, 5 or 10 nM) of Bafilomycin A1, an autophagy inhibitor, and cell viability was assessed after 48 and 72 hrs. In each time point, 20 μl of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5mg/ml), is the aqueous solubility of the reduced formazan product, was added into each well and incubated for 3 hours. Then, media gently were removed from each well using pipette and 200ul of solvent control (DMSO) was added to each well and mixed very well by pipetting to solubilize the MTT formazan. The plates were analyzed at a test wave length of 570 nm on a plate reader, following a 20 minute incubation at room temperature (RT). Readings were then compared to a control well, that did not contain any MTT. Results are expressed as percentage of corresponding time point control that had been treated with dimethyl sulfoxide (DMSO) vehicle only and represent the mean ± SD of 15 replicates in three independent experiments (****, $P<0.001$). The assay is based on Tetrazolium dye reduction, which is dependent on cytosolic NAD(P)H-dependent oxidoreductase enzymes in the cell. NAD(P)H-dependent cellular oxidoreductase enzymes are active only in living cells with metabolic activity. Viable cells with active metabolism can convert MTT into a purple colored formazan product. When cells die, they lose the ability to change MTT into formazan, thus color formation can be used as a marker of only in live cells [269, 270].

2.4. Measurement of apoptosis by flow cytometry

Apoptotic cells were assessed by flow cytometry with propidium iodide (PI), using the

Nicoletti method [271, 272]. RH30 and C2C12 cells were treated with TMZ (100 μM, 72 hrs) in cells cultured in 12 well plates. In every time point cells were detached by EDTA buffer and centrifuged at 1500 g for 5min at 4 °C. Then, cells were washed by PBS once. The cells were permeabilized and treated with a fluorescent dye that stains DNA quantitatively, using hypotonic PI lysis buffer (0.1% Triton X-100, 1% sodium citrate, 0.5 mg/ml RNase A, 40μg/ml propidium iodide). Prior to flow cytometry analysis, cells were incubated for at least 1 h, at 4ºC, and in the dark to prevent photo-bleaching. Measurement was in red fluorescence (460 nm) for 10,000 cells. To analyze the data, the flow cytometer was properly calibrated in order to accurately gate out debris. Finally, after elimination of residual debris the percentage of normal and apoptotic nuclei were estimated by analysis of the DNA histogram [273]. The nuclei of apoptotic cells were located on the left side of the G1 peak. Apoptotic nuclei have less DNA compared to nuclei of healthy G0/G1cells, causing an increase in sub-G1 section in the fluorescence histogram which can be applied for distinguish apoptotic cells in samples. In each sample, sub-G1 peak was measured and statistically compared with other samples.

2.5. Luminometric caspase assay

A luminometric assay was done in order to detect the proteolytic activity of caspase-8 (IETD-ase), -3/-7 (DEVD-ase), -9 (LEHDase), using the Caspase-Glo®-3/-7, -8, and -9 kits (Promega). The procedure was followed by the guidance of manufacturer's instructions and our previous reports [201, 272, 274]. In this experiment, cells were seeded in 96-well plate (15,000 cells/well) and treated with TMZ (100 μM, 72h). Fresh reagent prepared as follow: first, the Caspase-Glo® buffer and lyophilized Caspase-Glo® substrate were equilibrated in RT. Second, the Caspase-Glo® buffer was transferred and into the   amber bottle containing Caspase-Glo® Substrate and mixed well. Mixing was continued to make sure the substrate is thoroughly

dissolved and the Caspase-Glo® reagent was formed. The caspase reagents contain z-DEVD-Luciferin or z-LEHD-Luciferin, z-LETD-Luciferin, and whole protein cell lysate extract buffer. Using 96-well plates we added the 1:1 ratio of Caspase-Glo® reagent volume to sample volume. 25µl of Caspase-Glo® reagent was added to each well of a white-walled 96-well plate containing 25µl of treated cells and/or 25µl blank as negative control, in culture medium. Then, plates were put in a plate shaker and mixed at 300–500 rpm for 30 sec, followed by a 90 min incubation at RT. Luminescence of samples were then measured in a plate-reading luminometer. The results obtained for samples were compared to negative control [201, 272, 274].

2.6.   Western blotting

Western blot analysis was used to assess markers of DNA damage and cell cycle, apoptosis, and autophagy in RH30 and C2C12 cells. We examined hallmarks of intrinsic or extrinsic apoptotic pathway and autophagy signaling pathways, while GAPDH and Actin were used to normalize the results. After treatment, cells were collected and protein extracts were made using NP-40 lysis buffer (20 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 0.5 mM PMSF, 100µM β-glycerol 3-phosphate and 0.5% protease inhibitor cocktail). The extracts were kept in -20˚C until all extracts from different time points were collected. Samples were then sonicated in 5 times/5 cycle using ultrasound sonicator, followed by centrifugation at 13000g for 10 min to collect the supernatant protein. Protein content was then determined via a Lowry protein assay and protein samples were made. Prepared samples, of a volume between 15-20 µl, were heated at 90˚C for 5 min before loading into 10- 15% polyacrylamide gels (depends on the molecular weight of the proteins). Additionally, 10µl of a standard molecular weight marker (Thermo Fischer Scientific, ON, Canada) was loaded on each gel, as an approximate indicator of protein molecular weights. Proteins were immediately transferred under reducing conditions in transfer

buffer (500nM glycine, 50mM Tris-HCl, and 20% methanol) to Immun-Blot PVDF Membranes (Bio-Rad; #1620177), at RT and 100 volts for 2-2.5 hrs. After the transferring was complete, membranes were carefully transferred into 5% non-fat dried milk in 1X Tris-buffered saline containing Tween (TBS/0.025% tween 20; TBST) and placed on the shaker in the cold room for overnight or RT for 2 h. Following this blocking step, membranes were incubated with proper dilution of primary antibodies in 1% milk made in 1X TBST and kept in cold room (4ºC) overnight. Membranes were washed three times with 1X TBST (0.025% Tween) for 20 min and membranes were incubated with secondary antibodies (HRP) for 2 hrs on the shaker at RT. Membranes were washed again three times for 20 min and incubated with enhanced chemiluminescence (ECL) regents (Amersham-Pharmacia Biotech) for 2-3 min. The signals were visualized by autoradiography. Obtained protein bands were evaluated for changes in the autophagy and apoptosis signaling pathways. In order to assess even loading, membranes were incubated in milk 1% with primary antibodies against GAPDH or Actin overnight, washed three times and probed with a secondary antibody to visualize the signals. In the instances of re-probing of other proteins on the same membrane, blots were incubated with stripping solution containing 200 nM glycine, pH 2.5, 0.005 Tween20 for 15 min in RT and followed the same instruction as after blocking for these blots.

2.7. TMRM Staining for Mitochondrial Membrane Potential Measurement

Healthy mitochondrial membranes hold electrical potential difference between the exterior and interior of the cell, called to as a membrane potential. It is an important process which is linked to a host of mitochondrial functions. Tetramethylrhodamine methyl ester (TMRM), a cell-permeant dye, can accumulate inside the healthy and active mitochondria with intact membrane potential and then becomes fluorescent. TMRM fluorescent signal can be disappeared when

there is loss of the mitochondrial membrane potential. TMRM fluorescence can be detected with fluorescence microscopy and allows quantification of mitochondrial membrane potential. RH30 and C2C12 cells were cultured in 6 well plates (30000 cells/ml) and treated with Temozolomide (100µM, 60h). To increase sensitivity, cellular growth media was removed prior staining and C2C12s were washed with Phosphate Buffered Saline (1x, Hyclone), while RH30 were not, as RMS cells can be easily detached. TMRM (Tetramethylrhodamine Methyl Ester Perchlorate), a mitochondrial membrane dye, and Hoechst, a nuclear dye, at concentrations of 100 nM and 10 µM respectively, were diluted in media and added to cells for 30 minutes at 37 °C. Cells were imaged using an Olympus epifluorescence microscope. Fluorescence intensity was measure by ImageJ software (NIH, Bethesda, MD, USA), minimum 20 cells per condition. In each condition, fluorescence intensity for 20 cells (one by one) randomly was measured and then intensity was averaged out for 20 cells and quantified with the technical assistance of Simone D S Rosa. Data are mean ± SEM and were analyzed by Student's t test or ANOVA, followed by post-hoc analysis. If $p<0.05$, results were considered statistically significant.

2.8. Live cell imaging: LC3-GFP

GFP-LC3 is a specific marker for occurrence of autophagosomes formation [275, 276]. GFP-LC3 is fusion of the green fluorescent protein (GFP) and LC3 and can behave in a similar manner as endogenous LC3 [277, 278]. The GFP-LC3 is localized on the autophagosome membrane and green punctate signals are observed [278]. To confirm TMZ induced-autophagy and autophagy flux inhibition through Bafilomycin A1 (100nM), cells were transfected with a green fluorescent protein plasmid called LC3-GFP (Addgene, #24920), a vector to visualize autophagosome formation in real time. With the technical assistance of Simone D S Rosa, C2C12s were transfected using JetPrime Polyplus reagent, while RH30 cell line was transfected

using Qiagen's Effectene reagent, as per manufacturer's instructions. After 48h of transfection, cells were treated with TMZ (60h) and Bafilomycin (3h before imaging). Lysotracker Red staining (Molecular Probes™; LysoTracker® Red DND-99; L7528) was used to detect lysosomal activity and MitoTracker Red CMXRos at a concentration of 50 nM to detect active mitochondrial membrane potential. Cells were stained for 30 min in 37 °C incubator. Using this approach, instances where LC3-GFP puncta co-localized with lysotracker were considered with be autophagic, while LC3-GFP co-localization with mito-tracker was interpreted as mitophagy.

2.9. Immunocytochemistry

For detection of autophagy flux we used immunocytochemistry (ICC) in RH30 cells [279]. Briefly, RMS cells were cultured on cover slips in 6 well plates in RPMI media with 10% FBS. The cells were then treated with TMZ (100 µM) or vehicle control and for 72hrs (based on the concentration obtained by MTT, and FACS assay and the results of immunoblot experiments). 4hrs before time point Bafilomycin-A1 (100 nM) was added which already has been treated with TMZ. At the indicated time point, ICC will be performed using the protocol, which has been described previously (Lysosomes will be stained with Lysotracker red (Molecular Probes; 100 nM, 10 minutes) prior to fixation and permeabilization) [266, 280]. GFP-LC3 punctuate localized with activated lysotracker red will be identified as autophagic cells [280].

2.10. Transmission electron microscopy (TEM)

TEM was used to evaluate autophagy activation in both cell lines following treatment with TMZ and/or BAF-A1. TEM imaging was performed by Dr. James. A. Thliveris according to a protocol described previously [281, 282]. (TEM is available at the Platform for Histomorphology and Ultrastructural Imaging at Department of Human Anatomy and Cell Science). Briefly, RH30

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and C2C12 cells were seeded in 100 mm plates (300,000 cells/dish) in RPMI and DMEM (high glucose) media, respectively, supplemented with 10% FBS. Cells were treated with TMZ and Baf-A1 and were then collected using EDTA for detachment of cells. Cells centrifuged three times (1500 g) and then fixed (2.5% glutaraldehyde in PBS, pH 7.4) for 1 hrs at 4 ºC. Additionally, cells were treated with a post-fixation step using 1% Osimium tetraoxide, followed by an alcohol dehydration series, prior to embedding in Epon. TEM was performed with a Philips CM10, at 80 kV, on ultra-thin sections (100 nm on 200 mesh grids) 72 hours after treatment. Cells were stained with uranyl acetate and counterstained with lead citrate for 3 min sequentially. Finally, grids were washed with water for 1 min and dried completely to be ready for imaging. Autophagy induction and inhibition were evaluated based on the formation and accumulation of autophagosome and autophagolysosome in ultra-structure of the studied cells. TEM was done to confirm the autophagy induction by TMZ and autophagy suppression by Baf-A1 in the cells. For autophagy flux examination, RMS cells was prepared and studied at time points over 72hrs. The cells were treated with TMZ and vehicle control and then Bafilomycin-A1 (100 nM) was added to the TMZ and corresponding time match controls. At the indicated time point, TEM was performed using an established protocol [266, 283, 284]. Autophagy flux induction was evaluated based on autophagosome, and autophagolysosomes formation in ultra-structure of the studied cells.

2.11. Statistical analysis

All results were presented as mean ±SD and the differences between the groups were tested by one-way ANOVA or two-way ANOVA analysis (non-parametric, Brown–Forsythe test), using GraphPad Prism 7.0. The confidence interval in each analysis was 95% and $P < 0.05$ was considered statistically significant.

3. CHAPTER 3: RESULTS

3.1. Cell viability

3.1.1. TMZ induces cell death in normal mouse myoblast cell lines (C2C12)

Our results showed that TMZ induces cell death in C2C12 cell line. We treated C2C12 with TMZ (0, 50, 100, 250, 500 and 1000 µM in 48, 72, 96 hrs) and cell viability was examined by MTT assay. TMZ induced cell death in a dose and time dependent manner in C2C12 (Figure 6) [(48 and 72 h, P< 0.01 for concentrations ≥50µM), (72h & 96 h, P< 0.001 for concentrations ≥100 µM)].
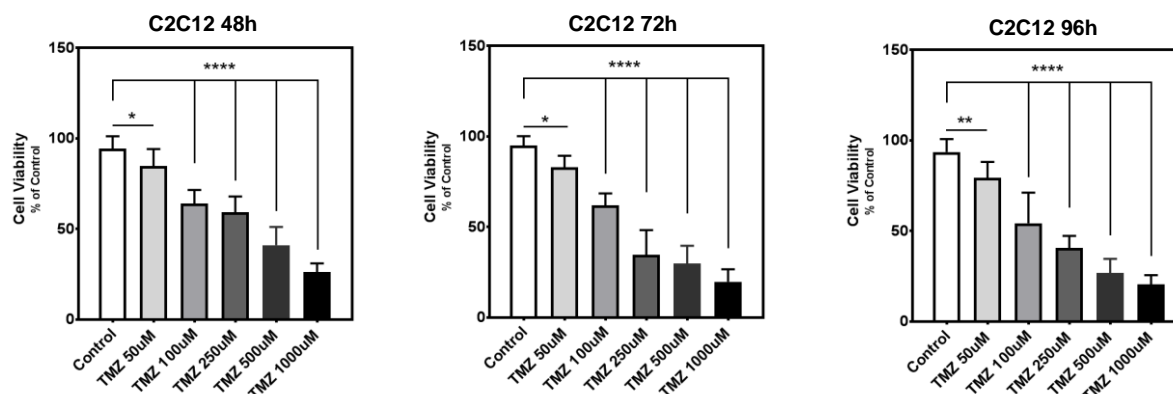
Figure 6. Cell viability assay (MTT assay). TMZ induces cell death in myoblast cell lines (C2C12). C2C12 cells were treated with TMZ in different concentrations ranging from 0 to 1000 µM and cell viability was evaluated in various time points (48, 72 and 96 hrs) by MTT assay. Results are expressed as percentage of corresponding time point control and represent the means ± SD of 15 replicates in three independent experiments (****, P<0.001).

3.1.2. TMZ induces cell death in rhabdomyosarcoma cell lines (RH30)

As a comparison, we also evaluated the effect of TMZ (0, 50, 100, 250, 500 and1000

μM) on the induction of cell death in the rhabdomyosarcoma cell line (RH30) at different time points (48, 72 and 96 hrs). Cell viability was assessed by MTT assay, using the same protocol for RH30 cells as for C2C12 cells. The results revealed that TMZ induced cell death in a dose- and time-dependent manner; however, the rhabdomyosarcoma cell lines (RH30 cells) was more sensitive to TMZ compared to C2C12 cells (Figure 7) [(72h & 96 h, P< 0.001 for concentrations ≥50 μM)].
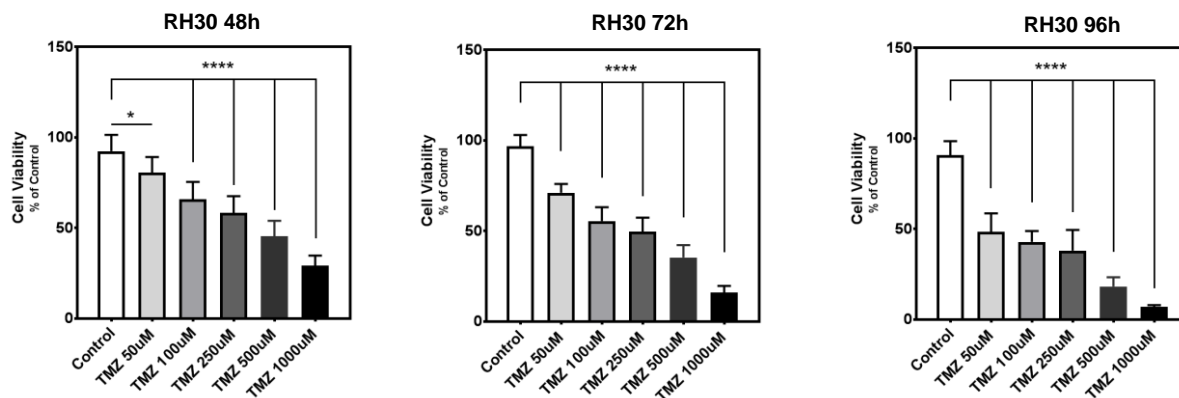
Figure 7. Cell viability assay (MTT assay). TMZ induces cell death in rhabdomyosarcoma cell lines (RH30). RH30 cells were treated with TMZ in different dosage ranging from 0 to 1000 μM and cell viability was assessed in different time points i.e. 48, 72 and 96 hrs by MTT assay. Results are expressed as percentage of corresponding time point control and represent the means ± SD of 15 replicates in three independent experiments (****, P<0.001).

3.2. TMZ induces different apoptotic response in C2C12 and RH30 cells

3.2.1. Apoptosis induction by TMZ in C2C12

We confirmed that TMZ (100μM, 72h) increases apoptotic cell death in C2C12 cells using a propidium iodide (PI)- dependent method (Nicoletti assay) for flow cytometry. As shown

in Figure 8, apoptotic cell population was detected as sub-G1 population in flow cytometry assessment (as described in Methods and Materials). The percentage of apoptosis activation in control group of C2C12 was identified 10.95%, while in C2C12 cells treated with TMZ the percentage of apoptosis was increased to 29.64%. These findings indicated that TMZ induced accumulation of sub-G1 cell population approximately three times more than control C2C12 cells.
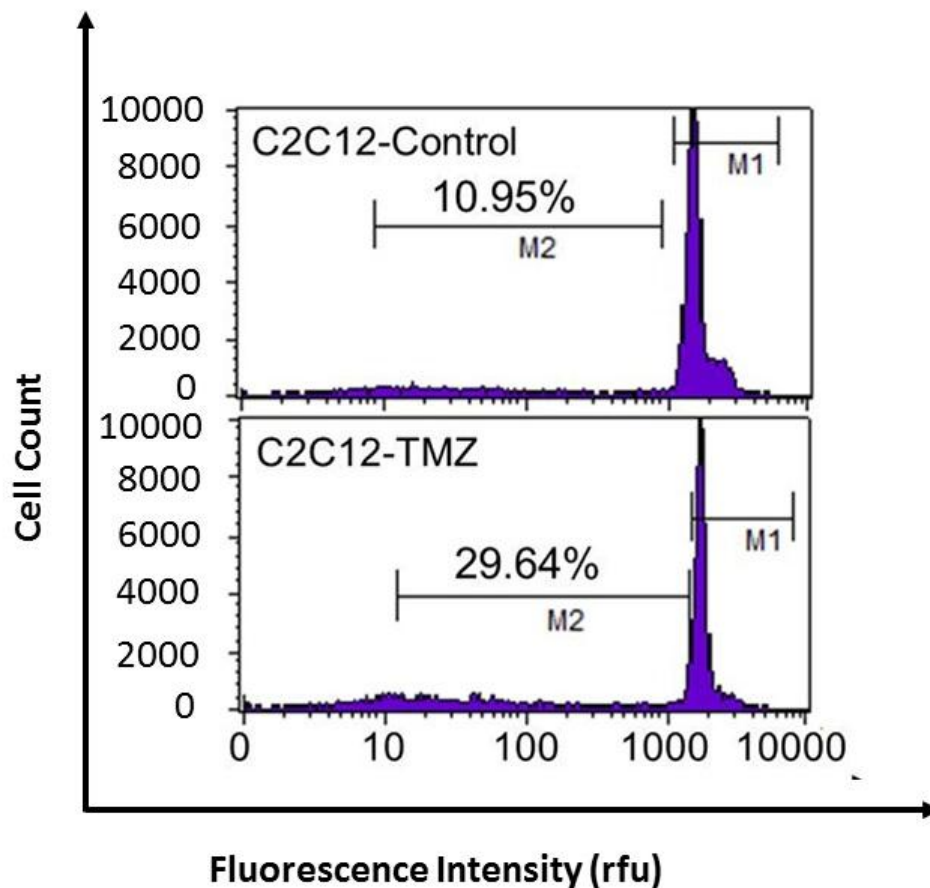
Figure 8. Apoptotic cells were assessed by flow cytometry (Nicoletti method) with propidium iodide (PI)-method in C2C12 cell lines treated with 100uM in 72hrs. Each histogram represents 10.000 cells. The finding revealed that there is no significant difference in apoptosis cell death between C2C12 treated cell (29.64%) and its control (10.92 %). The result is representative of 4 independent replicate. On way ANOVA (Tukey's multiple comparisons test) with the correlation of 95%.

3.2.2. Apoptosis induction by TMZ in RH30 cells

Our finding using flow cytometry with propidium iodide (PI)-method revealed that TMZ (100μM, 72h) induced accumulation of sub-G1 cell population in RH30 cell lines, representing apoptotic cells. The apoptotic percentage in control group of RH30 was found 2.11%, while in RH30 cells treated with TMZ the percentage of apoptosis activation was displayed 29.64%. Interestingly, TMZ induced apoptosis by 17.5 times compared to control RH30 cells (Figure 9).
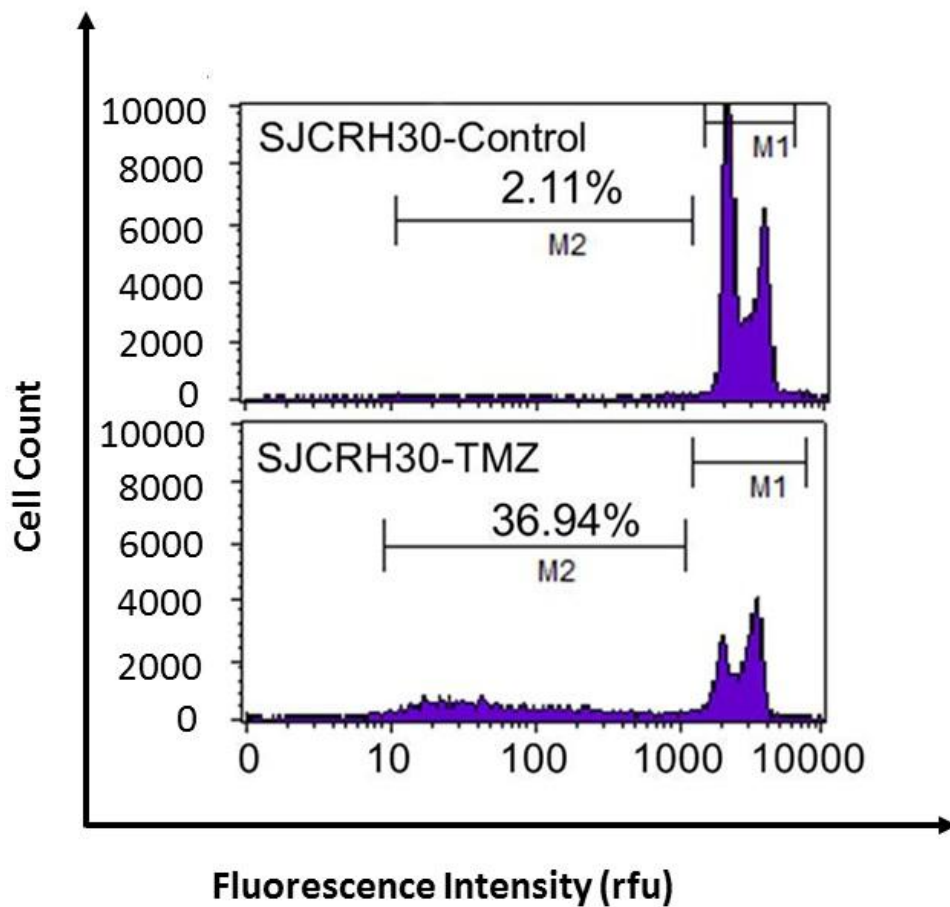
Figure 9. The results showed that there is a significant accumulation of sub-G1 population in RH30 cells after treating with TMZ (100μM, 72h). The number of cells in sub-G1 was found 2.11%, whereas that for RH30 cells treated with TMZ was 36.94%. The result is representative of 4 independent replicate. On way ANOVA (Tukey's multiple comparisons test) with the correlation of 95%.

3.2.3. TMZ activated higher level of apoptosis in RH30 cells than C2C12

Propidium iodide (PI)-method results for C2C12 and RH30 cells were compared. We observed that apoptotic activation was significantly higher in RH30 cells than C2C12 cells (Figure 10).
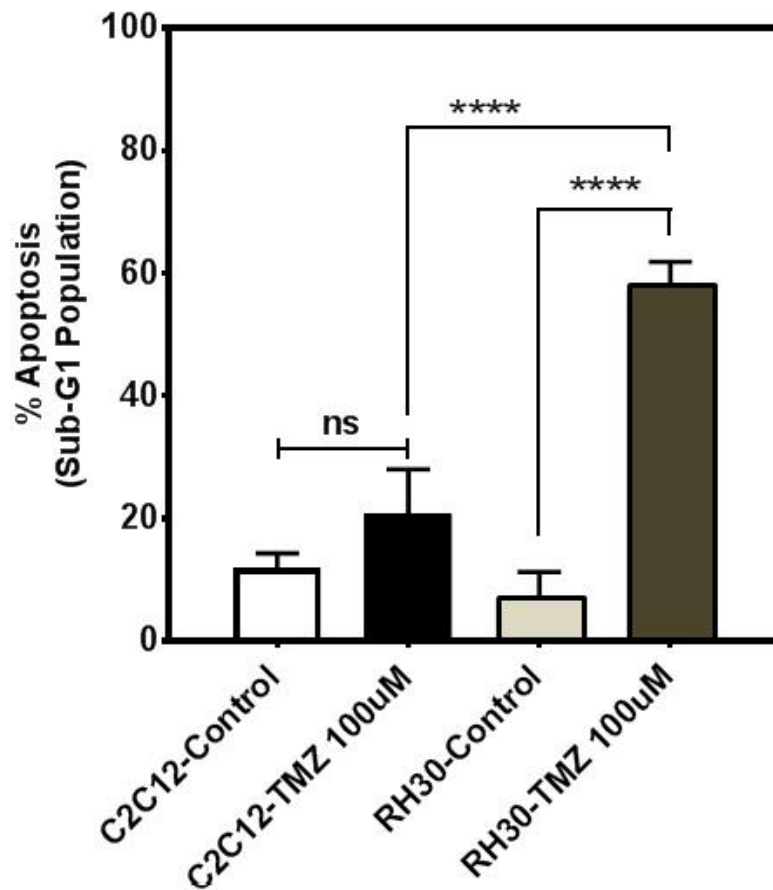
Figure 10. Comparison of induction of apoptosis caused by TMZ in RH30 and C2C12 cells. Higher induction of apoptosis was found in RH30 cells treated with TMZ than C2C12 cells treated with TMZ. Apoptotic induction were quantified for both cells compared to their correspondent controls graphs were prepared by GraphPad Prism using two way ANOVA (non-parametric, Brown–Forsythe test). P-value ≤ 0.05 and lower were considered as statistically significant.

3.3. Apoptosis and TMZ

3.3.1. TMZ and DNA damage in RH30 cells

In order to elucidate how TMZ can induce apoptosis in rhabdomyosarcoma cells, we determined the effect of TMZ treatment on DNA double strand breaks by examination of the appearance of phosphorylated H2A histone variant (γH2AX) in RH30 cells. γH2AX is a DNA damage marker and indicator of early apoptosis [285]. Western blot analysis was done and results revealed that TMZ caused significantly higher expression of γH2AX in RH30 cells compared to time matched controls (Figure 11).
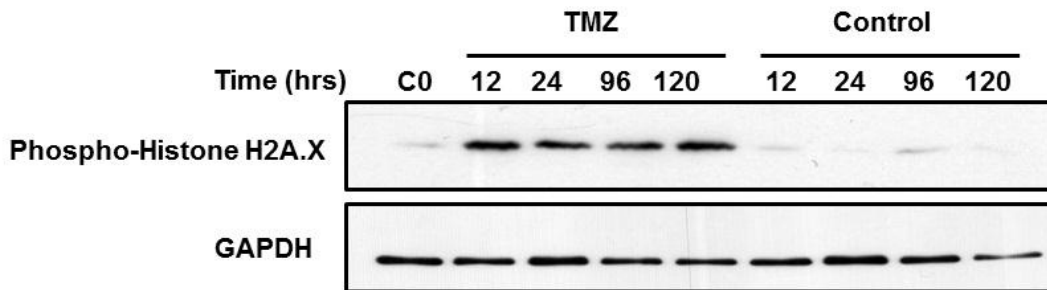
Figure 11. TMZ increases DNA double strand breaks (γH2AX) in RH30 cells. Rhabdomayosarcoma cells (RH30) cells were treated for 72 hrs with TMZ (100 μM) and Phospho-Histone H2A.X protein level were analyzed. Results showed that TMZ can stimulate the expression of γH2AX in RH30 cells.

3.3.2. TMZ affects intrinsic apoptotic pathway in C2C12 and RH30 Cells

We asked the question how TMZ can induce apoptosis signaling pathways in normal muscle cells (C2C12) and rhabdomyosarcoma cells (RH30). To address this, we have tested the expression of t-Bid in the cells using western blot. The mechanism of activation of Bid is a caspase-8-dependent cleavage resulting in the appearance of a truncated protein, called t-Bid,

which is a cytochrome c releasing factor and is the linkage between extrinsic and intrinsic apoptosis pathways. The appearance of t-Bid is interpreted that there are activation of extrinsic apoptotic signaling pathways [286]. Our results demonstrated that Bid is not truncated after treatment with TMZ (Figure 12), suggesting that the extrinsic apoptotic pathway may not be involved in TMZ-induced apoptosis.
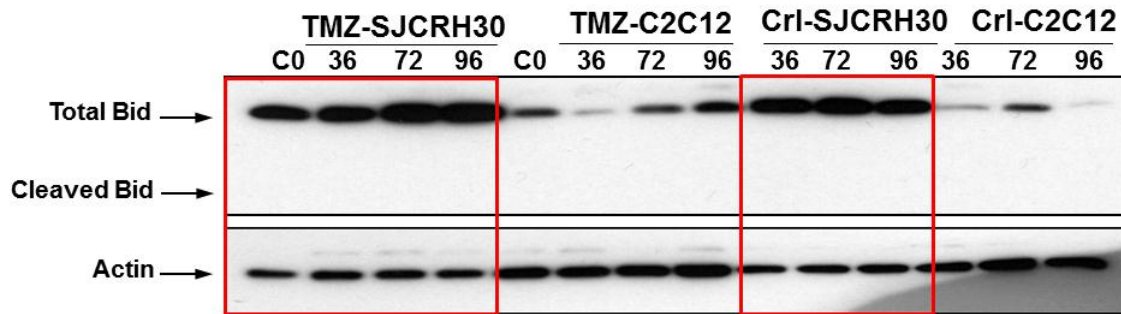
A
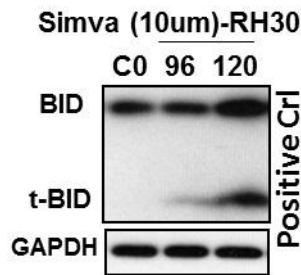
B

Figure 12. Total Bid are not truncated (t-Bid) in treated RH30 and C212 cells. (A) Bid is a crosslink between extrinsic and intrinsic apoptotic pathway, also known as a caspase-8-dependent cytochrome c releasing factor, resulting the induction of apoptosis. Western blot analysis revealed no expression of t-Bid in C2C12 cells and RH30 cells after treatment with TMZ. To have a positive control in order to validate the truncated-BID antibody we treated RH30 cells with simvastatin (10 uM) for 96 hrs and 120 hrs (B) and the result revealed that after treatment there is expression of truncated-BID after 96 hrs and 120 hrs.

3.3.3. TMZ induces caspase-9 cleavage

To further investigate the role of TMZ in the induction of the intrinsic or extrinsic apoptotic pathways, RH30 cells we assessed for expression of caspases -3, -7, and -9 activation using western blotting. Since caspase-3 and -7 are effector caspases, they can be activated if there is stimulation of their up-stream regulators (caspase-9 or caspase-8). Caspase-9 is activated by the intrinsic apoptotic pathway and caspase-8 is activated by the extrinsic apoptotic pathway. Analysis of caspase-9 and -8 activation was performed. As shown in Figure 13, western blotting results showed that TMZ treatment could increase the expression of cleaved caspase-9 in RH30 cells, implicating the involvement of intrinsic apoptotic pathway in RH30.
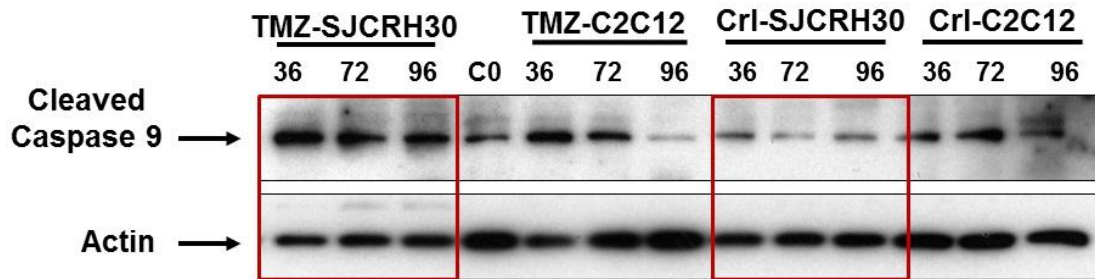
Figure 13. TMZ induced cleavage of caspase-9 in RMS cells. The cleavage of caspase-9 has also been tested by Western blot in cell lysates treated or not with TMZ (100 μM) for different period of time (0-96 hrs).

3.3.4. TMZ differentially alters the expression of BCL-2 proteins, but does not significantly affect mitochondrial membrane potential in RH30 and C2C12 cells.

Analysis of mitochondrial membrane potential by Tetramethyl rhodamine methyl ester (TMRM) analysis revealed a trend towards higher membrane potential in TMZ treated RH30 and C2C12 cells compared to control cells. However, these increases were not statistically significant (P>0.05) (Figure 14). In addition, to see which mitochondrial proteins are regulated by TMZ we

evaluated the expression of BCL-2 family proteins in both cell lines.
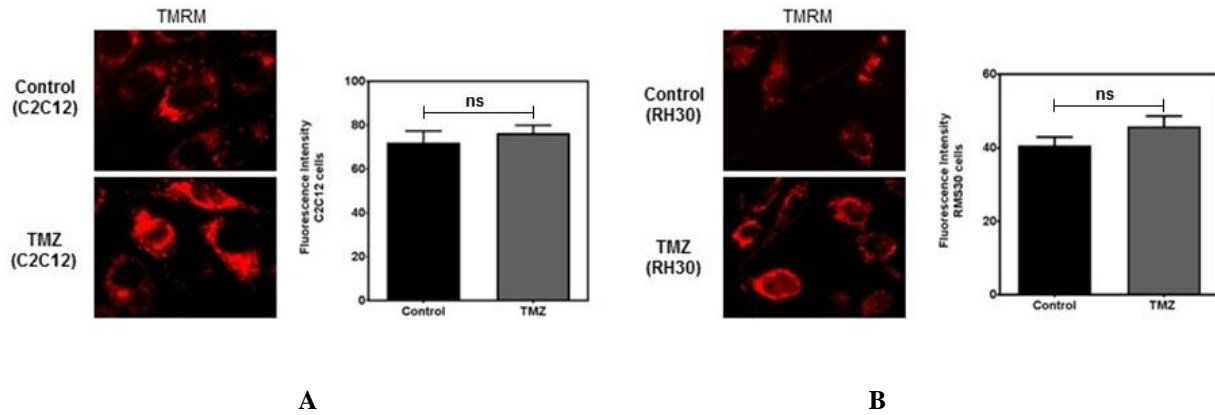
Figure 14. Mitochondrial membrane potential measured by TMRM. (A–B) representative images collected from control, TMZ-treated cells; (D) graph showing the comparison of depolarization of mitochondrial membrane potential in TMZ-treated cells and non-treated cells. Data were collected from 3 independent experiments and presented as means ± SD. Red color denotes TMRM staining.

To evaluate whether TMZ (100 µM) treatment could affect the apoptosis by regulating members of the Bcl-2 family proteins in C2C12 and RH30 cell lines, we studied the expression of both anti-apoptotic (Bcl-2, Bcl-xl, and Mcl-1) and pro-apoptotic (Bax and Bak) members using western blot analysis. As shown in Figure 15, a different amount of Bcl-2 family protein is expressed in the C2C12 cells compared to the RH30 cell line. The obtained data indicated that MCL-1 was expressed at a higher level in control RH30 cells, and was down-regulated by TMZ treatment. On the contrary, Mcl-1 expression was increased in time dependent manner in C2C12 following treatment with TMZ. BCL-2 was showed to be up-regulated in RH30 cell, but in C2C12, Bcl-2 expression was decreased after treatment with TMZ. The expression of BAX was not changed significantly by TMZ in RH30 cells; however, the level of Bax expression was slightly increased in C2C12 cells. Bcl-xl expression was not altered by TMZ treatment in either cell line (C2C12 and RH30).
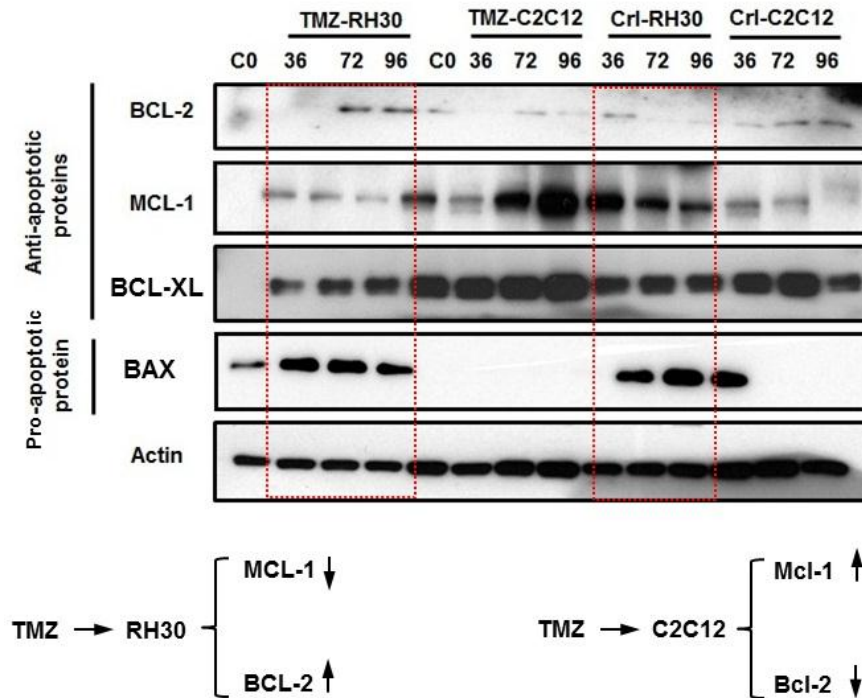
Figure 15. TMZ affects Bcl-2 family proteins in RH30 and C212 cells. TMZ differentially changes the expression of Bcl-2 family proteins in normal and RMS cells. BCL-2 expression was detected to be up-regulated in TMZ treated cells (RH30), however, Bcl-2 was decreased in C2C12 cells treated with TMZ compared to its control. TMZ had no effect on the Bcl-xl and BAX in RH30, while TMZ had no effect on the Bcl-xl but increases Bax expression in C2C12. The expression of MCL-1 decreased in RMS cells after treatment with TMZ, while in C2C12 cell the expression of Mcl-1 up-regulated. Based on these results, TMZ (100 μM) applies its affect by regulation of ani-apoptotic proteins including Mcl-1 and Bcl-xl in both cell lines. The result is representative of 3 independent replicate.

3.4. Autophagy and TMZ

3.4.1. TMZ induces autophagy hallmarks in RH30 cells

Our results demonstrated that TMZ induces autophagy in the RH30 cell line. We treated RH30 cells with TMZ (100 μM) for 36, 72 and 96 hrs. We showed up-regulation or down-regulation of important hallmarks involved in the activation of autophagy. Western blot results demonstrate lipidation of LC3B and degradation of P62 in RH30 cells, indicating autophagy

activation by TMZ (Figure 16).
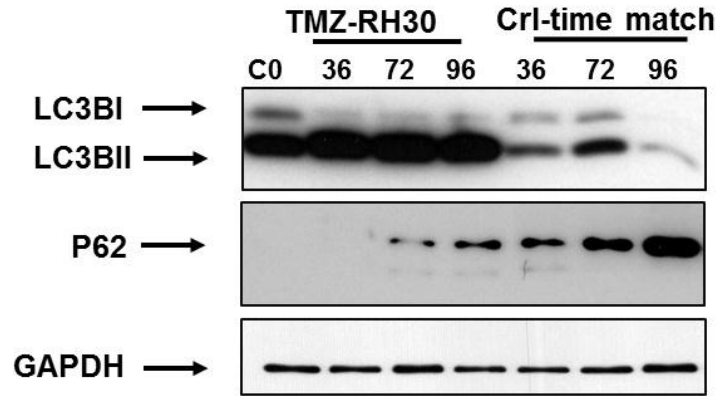
Figure 16. Effects of TMZ on autophagy induction in RH30. TMZ regulates cells were treated with TMZ (100 μM) and autophagy hallmarks were assessed by western blotting in different time points. TMZ treatment induces autophagy hallmarks including LC3B lipidation, P62 degradation in RMS cell (RH30). The result is representative of 3 independent replicate.

3.4.2. TMZ accumulates autophagosome in RH30 cells

Transmission electron microscopy (TEM) was performed by Dr. James. A. Thliveris (TEM core) as described in the Methods and Materials chapter, in order to confirm the autophagy activation in cells treated with TMZ. We observed the formation of autophagosome-like structures in cells treated with TMZ (Figure 17). These results demonstrating accumulation of autophagosomes validate that TMZ activates autophagy of RH30 cell.

C2C12 (Control)   **C2C12 (TMZ)**

SJCRH30 (Control)   **SJCRH30 (TMZ)**

Autophagosome

A   **B**
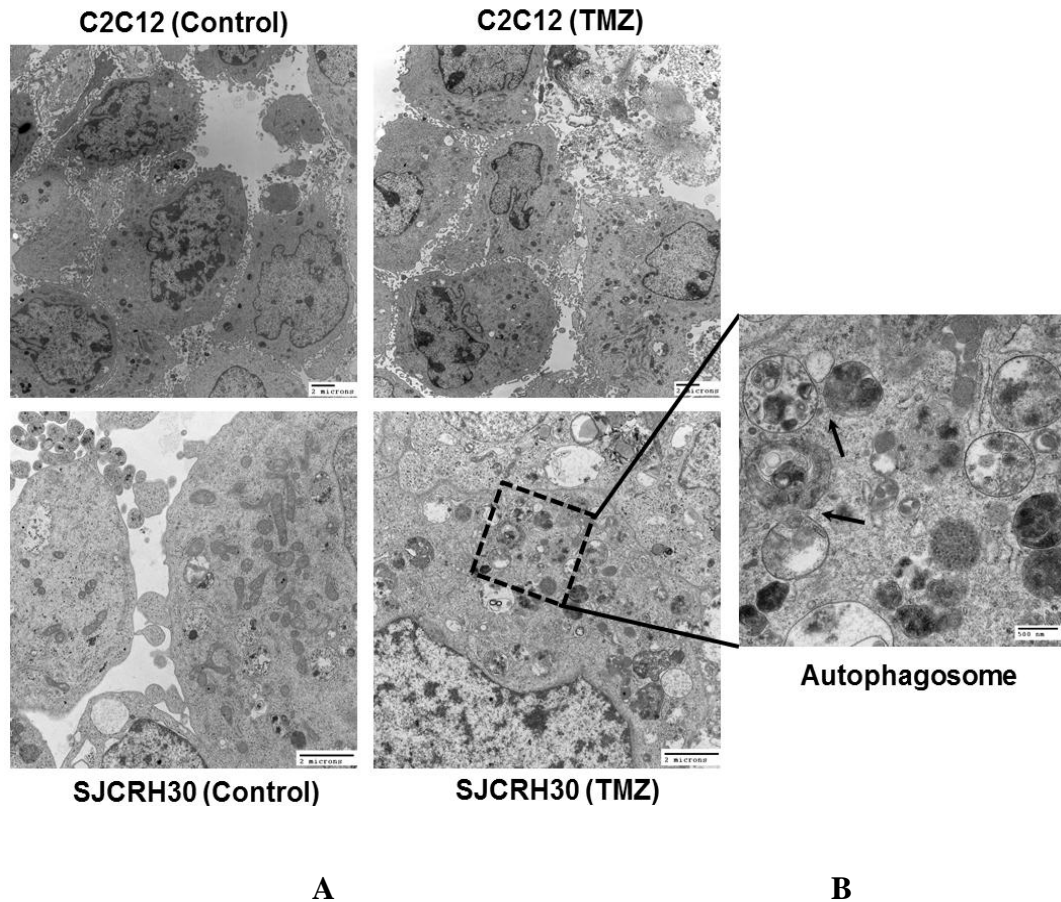
Figure 17. TMZ induces autophagy in RH30 cells following TMZ treatment.

Transmission electron microcopy was shown that in treated RH30 cells there are accumulated autophagosome like structures compared to control and normal cells after 72 hours treatment. Arrows show the forming autophagolysosomes containing the cargo (A) (magnification 11600x), (B) (magnification 20500x).

3.4.3. TMZ increases LC3-GFP signal and lysosome activity in RH30 cells

Lysosomes were stained with Lysotracker red (Molecular Probes; 100 nM, 10 minutes) prior to fixation and permeabilization, as described previously [266, 280]. LC3 punctuate localized with activated lysotracker red was identified as autophagic cells [280]. In addition, I transfected cells with LC3-GFP, and observed GFP-puncta and lysosomal activation after

treatment with TMZ for 60 hrs in both C2C12 and RH30 cells, highlighting that autophagy is activated in both cell lines (Figure 18). These results obtained by fluorescent microscopy analysis are also were in agreement with results obtained by western blotting and TEM.
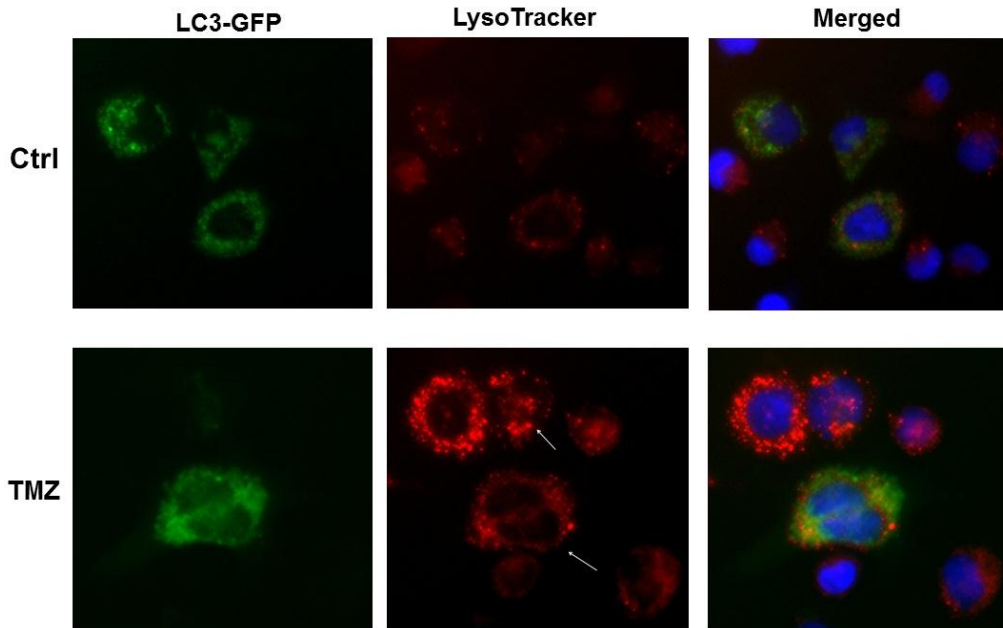
Figure 18. TMZ induces activation of lysosomes in RMS cells in presence of LC3 punctuate. RMS were treated with TMZ (100 μM, 60 hrs) and media (control) and lysosomal activation and LC3 punctuate were detected using immune fluorescence microscopy.

3.5. Chemical inhibition of autophagy by Baf-A1 enhances TMZ-induced apoptosis RH30 cell lines.

We performed a cell viability assay (MTT) to establish an appropriate concentration of Baf-A1 which can suppress autophagy signaling with the least toxic effect. RH30 cells were treated with different concentration of Baf-A1 (0.1-10 nM) for 48 & 72 hrs. Our results revealed

that Baf-A1 (4-6 nM) had low cytotoxicity at 72hrs in both cell lines (Figure 19A). In addition, cells were treated with Baf-A1 (4 & 6 nM) for 48 hrs & 72 hrs and autophagy markers were studied in both cell lines by western blotting. The results show that Baf-A1 (4 & 6 nM) can suppress autophagy by accumulating lipidated LC3B and reduced degradation of P62 (Figure 19B).
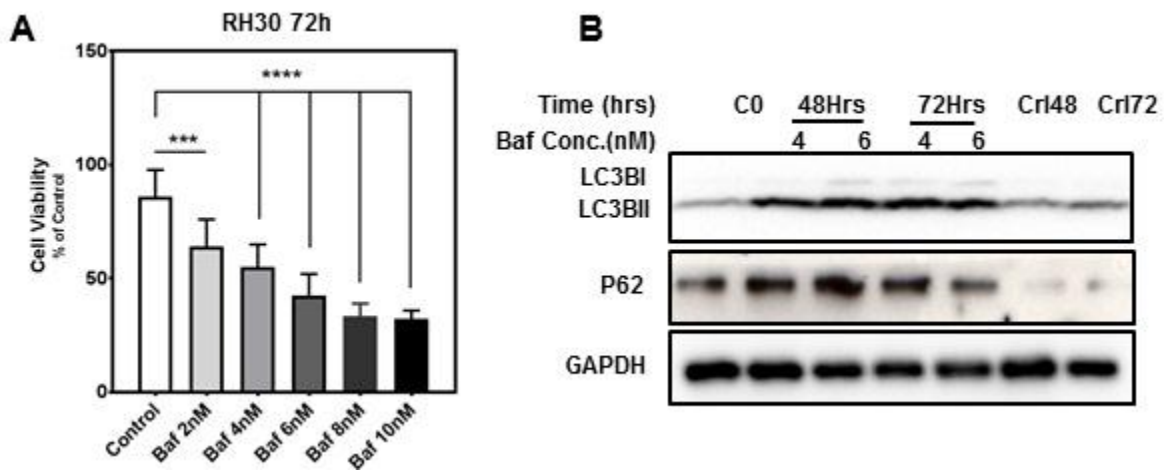
Figure 19. Treatment of cells with Bafilomycin A1 (0.1-10nM) can suppress autophagy in RH30 cells.

(A) Cytotoxicity assay for Baf-A1in RH30 cells. RH30 cell lines were treated with a wide range of Baf-A1 (0.1, 2, 4, 6, 8 and 10 nM) and cytotoxicity was evaluated in 72hrs using MTT assay. Results are expressed as percentage of corresponding time point control and represent the means ± SD of 15 replicates in three independent experiments (****, P<0.001). Results were showed that Baf-A1 can induce cell death mechanism in time and dose dependent manner. (B) Treatment of cells with Baf (4 nM & 6 nM) showed there are LC3BII lipidation and up regulation of P62, confirming suppression in autophagy pathway in C2C12 cells by Baf-A1. GAPDH was also measured as control for loading. It has been done in two independent experiments.

Next, we treated RH30 cells with combination of temozolomide (100 uM/ml) and Baf-A1 (4 & 6 nM) and compared with their controls. The results demonstrate that autophagy is suppressed by Baf-A1 which was exhibited by higher LC3B-II lipidation and P62 expression (Figure 20).
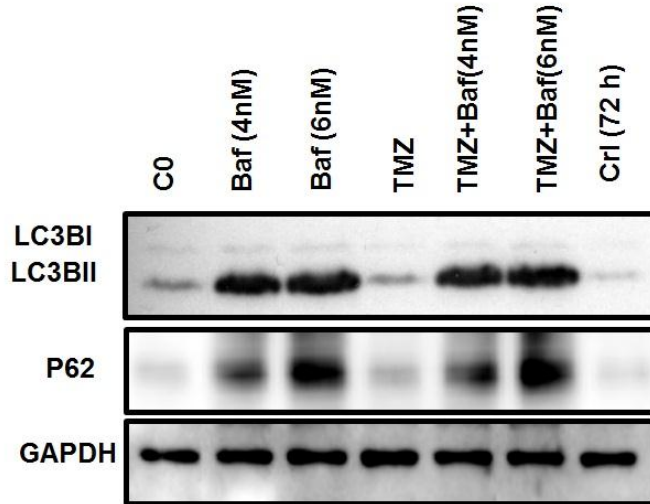
Figure 20. RH30 cells were treated with TMZ (100 uM) and co-treated with Bafilomycin A1 (1nM) 72 hrs. Two control groups were assigned to compare with treated groups. C0 (control zero) receive no treatment and cells were collected at the beginning of the experiments at time point 0 (before treatment). A time-matched Control group (Crl) is also assigned and received no treatment, but cells are collected 72 hrs after treatment. The time-matched Control group provides a comparison with the treatment group at each time point. Cell lysates were collected and using western blotting the abundance level of autophagy markers were analyzed for both cell lines. GAPDH was also measured as control for loading. The blots for LC3 and P62 were tested for both cells and compared to their correspondent controls. It has been done in 2 dependent.

3.6. Autophagy suppression have additive effect on apoptosis in RH30 cells

Flow cytometry with propidium iodide (PI) staining confirmed that co-treatment of RH30 cell with TMZ (100μM) and Baf-A1 (4nM) has an additive effect on apoptosis. As shown in Figure 21, both TMZ and Baf-A1 increased apoptotic cell population compared to control. The percentage of apoptosis activation in TMZ group was identified 32.80%, while in group treated with Baf-A1 the percentage of apoptosis activation was increased to 29.62%. Interestingly, the percentage of apoptosis in combination treatment of TMZ and Baf-A1 was found 61.92%. These findings indicated that suppression of autophagy can accumulate sub-G1 cell population compared to the control groups.
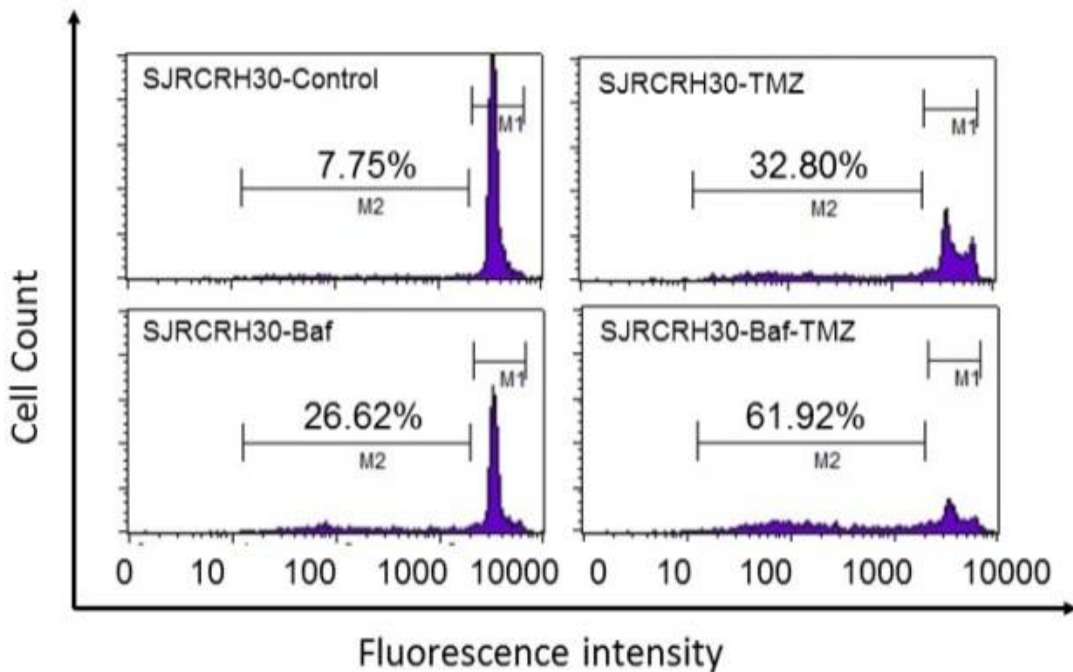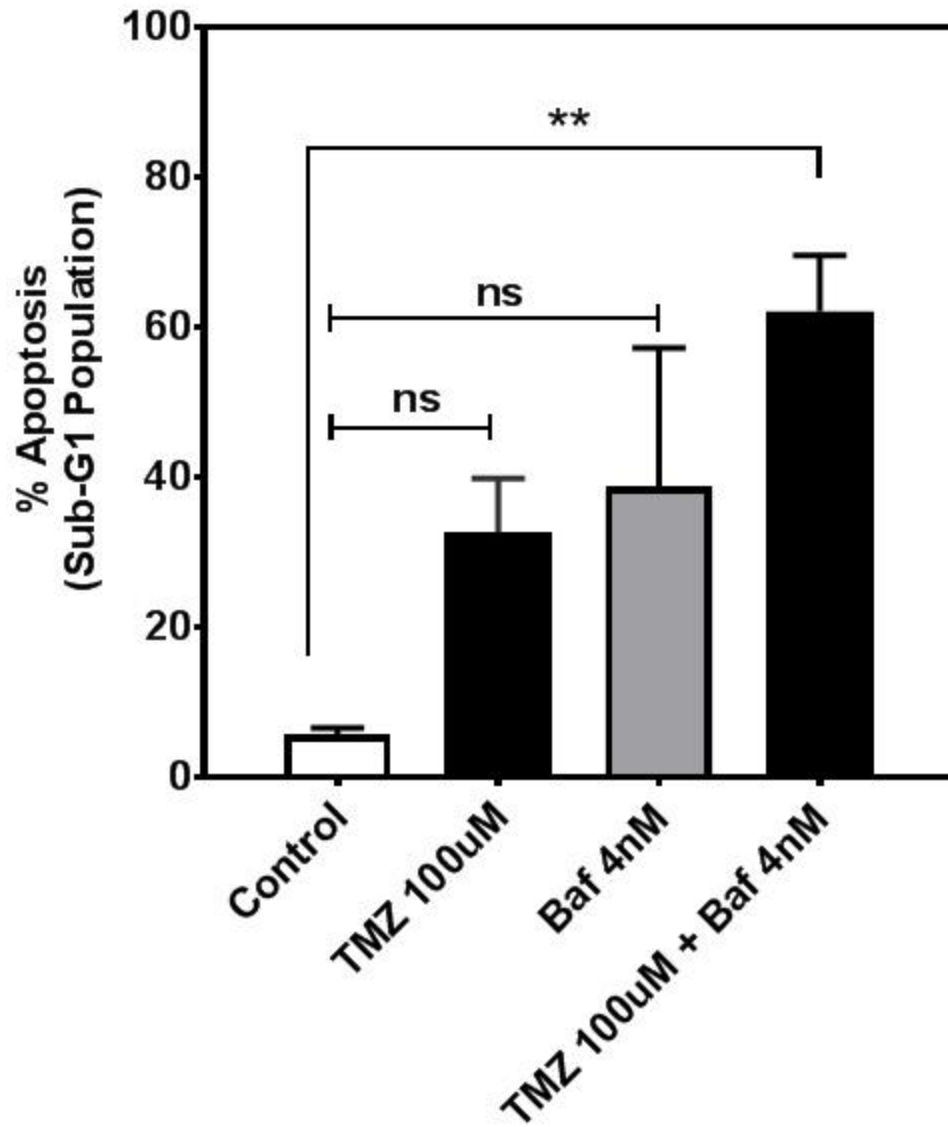
Figure 21. Apoptotic cells were assessed by flow cytometry with propidium iodide (PI)- method in autophagy inhibitor (Baf-A1). Each histogram represents 10,000 cells. Flow cytometry was shown that using TMZ+Baf-A1 can increase apoptosis rate in treated RH30 cells compared to groups using TMZ and Baf-A1 alone. It has been done in 4 dependent experiments.

3.7. Chemical inhibition of autophagy (Baf-A1) changes Bcl-2 family proteins in RH30 cell lines treated with TMZ

To study if suppression of autophagy using Baf-A1 in cells treated with TMZ (100 µM) could affect the apoptosis by effecting to the members of the BCL-2 family proteins in RH30 cells, we evaluated the expression of anti-apoptotic (BCL-2, BCL-XL, and MCL-1) and pro-apoptotic (BAX and BAK) proteins by Western blot analysis. As shown in Figure 22, Baf-A1 treatment induces expression of the pro-apoptotic protein BAX. In addition, when Baf-A1 is applied in combined with TMZ, BAX expression is increased further. BCL-2 expression was increased in cells treated with TMZ, while no change was observed in the expression of BCL-XL. TMZ + Baf-A1 treatment also showed a slight increase in expression of MCL-1 in RH30 cells. Moreover, results showed a modest increase in BID truncation after co-treatment with TMZ and Baf-A1, which suggests the possibly that extrinsic apoptosis signaling may also can be involved in regulation of cell death after suppression of autophagy by Baf-A1 (Figure 22).
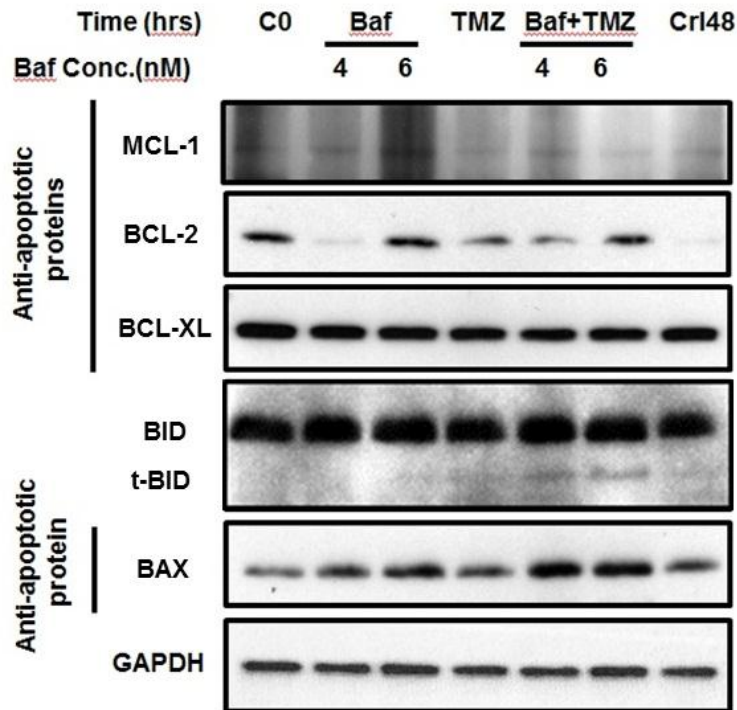
Figure 22. Combination therapy TMZ and Baf-A1 affects BCL-2 family proteins in RH30.

TMZ differentially changes the expression of BCL-2 family proteins in normal and RMS cells. BCL-2 expression was detected to be up-regulated in TMZ treated cells (RH30) in compared to control time match. BCL-2 expression was increased in RH30 cells treated with TMZ + Baf-A1 compared to its control. TMZ had no effect on the BCL-XL. The expression of MCL-1decreased in RMS cells after treatment with TMZ, while Baf-A1 caused an elevation in expression of MCL-1. BAX as pro-apoptotic protein detected to be up-regulated caused by Baf-A1. Furthermore, BID was found to be truncated after using Baf-A1 in cells treated with TMZ. It has been done in 2 dependent experiments.

3.7.1. TMZ up-regulates luminescence caspase-9 and caspase-3/7 activities after suppression of autophagy

To determine if suppression of autophagy using Baf-A1 (4nM) in cells treated with TMZ (100 μM) could affect apoptosis, we performed caspase Glo assays to assess cleavage of caspases -3/7 and -9. The Caspase-Glo Assay is the most sensitive caspase assay available [287]. These results demonstrate that TMZ (100μM, 72h) did activate caspase-9 as well as caspase-3/-7

in RH30 cell lines (Figure 23). However, the combination of TMZ (100 uM) and Baf (4nM) treatment showed significantly higher cleavage of caspase-9 and caspases -3/7 compared to the groups treated with Baf-A or TMZ alone.
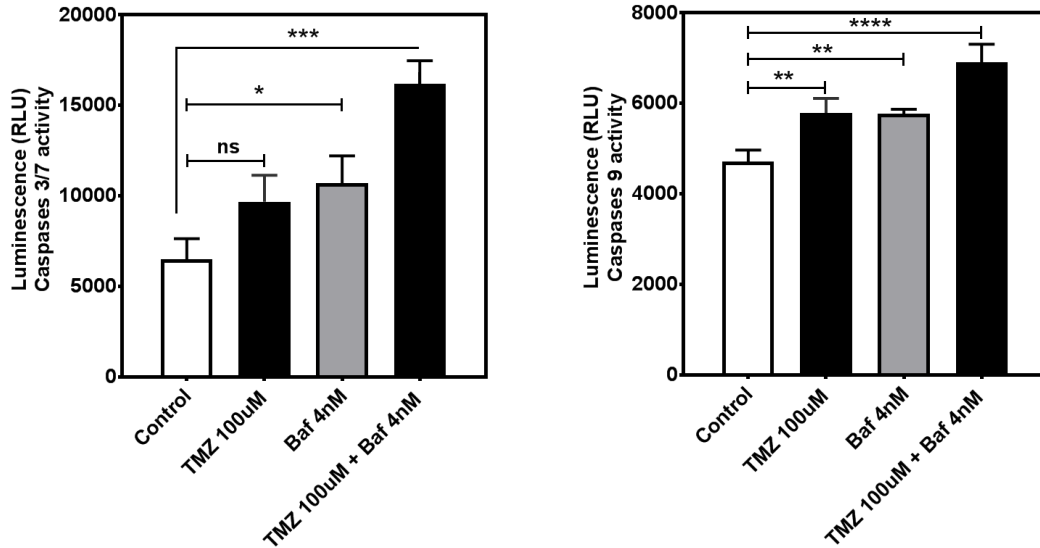
Figure 23. TMZ induced luminescence (RLU) caspase-9 as well as luminescence (RLU) caspase-3 and -9 in RMS cells (48 hrs). The result is representative of 3 independent replicates.

3.8. TMZ treatment Induces Autophagy Flux in RMS cells

We applied higher concentration of Baf-A1 (100 nM) to study if the accumulation of LC3B-II is because of increasing autophagic flux or preventing autophagic proteolysis. A high concentration of Baf-A1 with short exposure times decreases lysosome numbers, causes fusion block, suppresses autolysosome destruction, by interfering with late-stage autophagosome-lysosome fusion [288]. Treatment with Baf-A1 (100 nM) 2, and 4 h prior to harvesting in cells without TMZ treatment markedly increased the accumulation of levels of LC3-II and P62 in

RH30 cells, determined by western blotting. Cells treated with a combination of TMZ (100 uM, 72 hrs) and Baf-A1 (100 nM) at 1, 2, 3, and 4 h prior to harvesting showed a time-dependent accumulation of LC3B-II compared to treated cells with Baf-A1 (100 nM) alone (Figure 24 A). These results indicated that TMZ induced autophagy flux in RH30 cells. The same results were achieved in the GFP-LC3 (or endogenous LC3) puncta formation assay. RH30 cells treated with a combination of TMZ and Baf-A1 (100 nM) increased GFP-LC3 fluorescent signals during the 3 hrs incubation period, compared to cells treated with Baf-A1 (100 nM) alone (Figure 24 B). Lysosomal activity was found to be lower in cells after exposing with Baf-A1 (100 nM) alone as well as combined TMZ (100 uM, 72 hrs) and Baf-A1 (100 nM, 3 hours) compared to control and TMZ group, confirming lysosomal acidification inhibition and blockage of the autophagy flux (Figure 24 B). TEM analysis showed that in RH30 cells treated with 100 nM Baf-A1 possessed considerably fewer dark lysosomes. However, Baf-A1 at high concentration caused accumulation of double-membrane autophagic structures which appeared to be contained un-degraded cargo (Figure 24 C "B,D"). All three sets of results of autophagy flux examination were in line together and showed that higher concentration of Baf-A1 (100 nM) can suppress autophagy flux, suggesting that downstream steps of autophagy in both cells are active after treatment with TMZ.

A

B
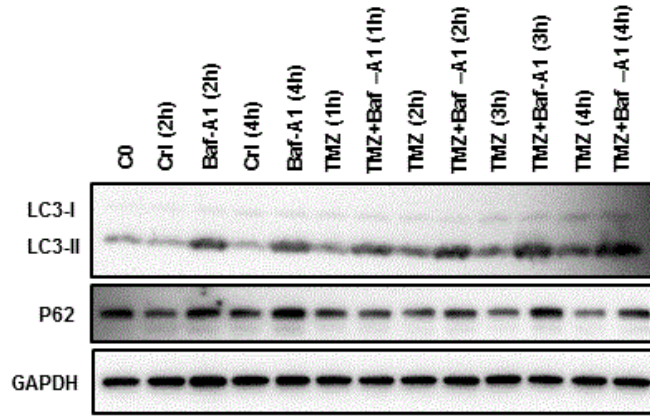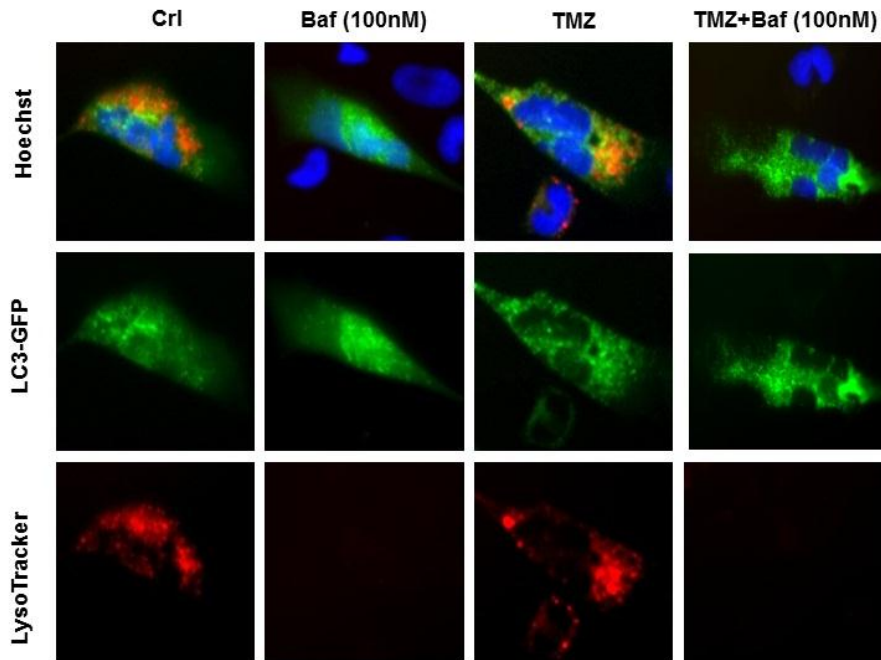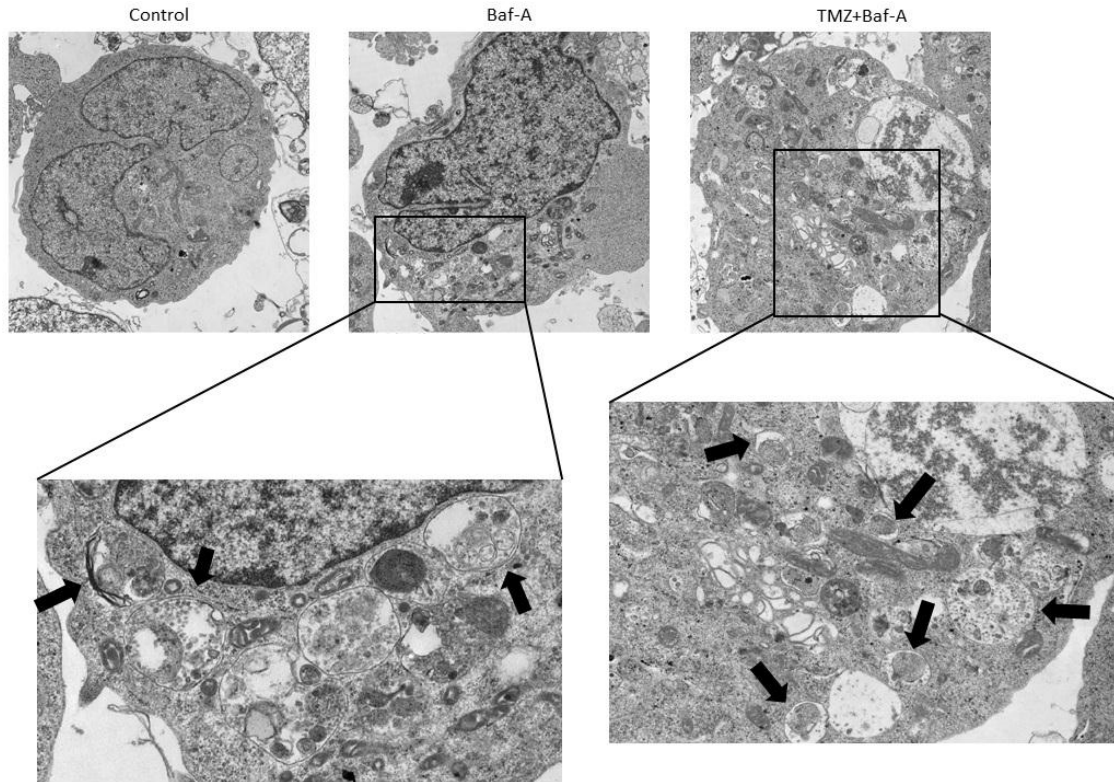
Figure 24. Baf-A1 at high concentration suppresses autophagosome flux and lysosome acidification in RH30 cells.

Two control groups were assigned to compare with treated groups. C0 (control zero) receive no treatment and cells were collected at the beginning of the experiments at time point 0 (before treatment). Control zero can show the state of cells at the time of the treatment. Control time match group (crl) is also assigned and received no treatment, but cells collected 72 hrs after treatment of the groups. Control time match group is to have comparison with the treatment group after time point (72 hrs after treatment). In RH30 cells treated with 100 nM Baf-A1 resulted in accumulation of LC3-II and P62; whoever, combination of TMZ (100uM) + Baf-A1 (100 nM) could accumulate higher protein levels of LC3-II in RH30 cells (A). The blot shown is typical of 3 independent experiments. As a loading control GAPDH abundance was determined for each sample. GFP-LC3 and LysoTracker fluorescence were determined to study the recruitment of labeled GFP-LC3-II into the autophagosomes and lysosome activity, respectively. Values are expressed as means±SD compared with control group in 2 independent experiments. Cell nuclei were stained with Hoechst (blue). In agreement with the obtained results of western blotting, the (TMZ+Baf-A1)-treated cells revealed higher punctate pattern of GFP-LC3 fluorescence compared to group treated with Baf-A alone and group treated with TMZ alone, representing the higher recruitment of LC3-II into the autophagosomes. However, Lysosomal acidification or the number of lysosomes was found to be suppressed by Baf-A1 at higher concentration (B). In addition, TEM was perfprmed by Dr. James. A. Theliveris (TEM core) and results (C) showed that Baf-A1 at high concentration with TMZ can increases double-membrane vesicles in RH30 compared to other groups. Group (Ca) is control group revieved no treatment; group (Cb) received TMZ (100 uM); group (Cd) received Baf-A (100nM) and group (Cd) received TMZ (100uM)+Baf-A(100nM) (2 microns).

4. CHAPTER 4: DISCUSSION

Alveolar rhabdomyosarcoma is described as a rare, metastatic, and aggressive soft-tissue malignant tumor occurring mostly in children and adolescents. Children with metastatic alveolar RMS have a very poor prognosis, albeit survival in these patients has not improved appreciably in the past years. At present, there are few therapeutic options available for patients with metastatic RMS. Standard therapy for this type of cancer, in addition to surgery and radiotherapy, is treatment with vincristine, dactinomycin, and cyclophosphamide (VAC) [76]. In order to improve therapeutic effect of chemotherapy in RMS, an alkylating agent temozolomide (TMZ) has recently received considerable interest. TMZ is a well-tolerated oral alkylating agent with a broad spectrum of antitumor activity and relatively low toxicity [185, 186]. Several Phase I trials suggest that using TMZ alone or in combination is well tolerated in RMS when used in relapse settings [149]. The combination of irinotecan and TMZ showed a synergistic antitumor activity against RMS [149, 163, 289, 290]. In addition, TMZ was found to be effective in other diseases such as gliomas [289, 291-294]. The introduction of TMZ appears to be advantageous and hold great impact on the outcome for patients with gliomas [295, 296]. We have shown in our study that TMZ could induce cell death mechanisms including apoptosis and autophagy in RH30 cells. Autophagy is known to be important in preventing cells from cancer initiation and promotion [297]. However, several studies have pointed out that autophagy acts as a pro-survival pathway which allow cells to survive in the existing stress in the tumor microenvironment [298]. It is also known that autophagy can be an important protector of cells against treatment with anti-cancer agents [211]. Autophagy starts its function by forming two layers membrane vacuole called autophagosome and then engulfing cargo and damaged cytoplasmic organelles into

autophagosome [299]. Autophagy preserves cells integrity by degrading and recycling damaged and dysfunctional organelles and providing energy to the cells by digesting those organelles to very small particles i.e. amino acids and fatty acids [300]. These products generated by autophagy are used for ATP production, providing alternative energy to cells to survive [301]. This function of autophagy appears to be ideal for cancer cell survival in the face of unfavorable starvation conditions [302]. It also is evident that under certain circumstance autophagy can be a mechanism of cell death (ACD) [303, 304]. Autophagy might kill cells through selectively degradation of important cellular components such as mitochondria, or by non-selective degradation of cellular components like active degradation of apoptotic proteins [305, 306]. Thus, autophagy and apoptosis have multiple direct and indirect interactions with each other [307]. It was reported that autophagy sometimes can change the way that cells die. Thorburn J *et al.,* in 2009 indicated that glioma cells dying by targeted toxin is not caspases-dependent, while inhibiting autophagy caused a slight increase in cell death via caspases activation [308].

Our results determined that TMZ regulates expression of autophagy associated proteins, such as LC3-II, P62, and Atg 5-12 in both cell lines (RH30 and C2C12). Autophagy function is dependent on lysosomal activity [242]. Using lysotracker we identified that TMZ increases a fluorescent intensity of acidic vesicle reproducibly in RH30 cells. We observed for the first time that exposure to TMZ (100 μM) for 3 days is able to increase the formation of two-layer membrane autophagosomes in RH30 cells using TEM. We identified that the autophagy suppressor Baf-1 increases the anti-tumor effect of TMZ against RMS cell by induction of apoptosis. Our findings are in line with the results obtained by other research groups investigating autophagy activation in other cancer type (U373-MG cells) after treatment with TMZ. They found an increase in formation of autophagosomes and secondary lysosomes

formation in U373-MG cells treated with TMZ compared to untreated U373-MG cells [309]. This group also tested the involvement of LC3 in TMZ-treated U373-MG cells using a stable clone transfected with LC3 fused to green fluorescent protein (GFP-LC3). They also observed that treatment of TMZ in GFP-LC3 transfected U373-MG cells enhanced fluorescence intensity as well as punctate pattern in number [309]. In U87MG [310], U251 [310, 311], U-118 [312], Rat C6 cells [313], LN229 [310], GBM8901 [314] cell lines the activation of autophagy by TMZ has been published [315-317]. There are also other reports showing that TMZ can induce autophagy in patients with malignant gliomas [318]. Our data presented here resonate with other recent work showing that TMZ can activate autophagy in RMS cells, suggesting there is accumulation of autophagosome formation in RMS cells after exposure to TMZ.

We determined that TMZ can activate apoptosis in a time and dose dependent manner in RH30. The enhancement of apoptosis was found to be associated with higher expression of gamma H2X phosphorylation. Histone H2A-X phosphorylated at Ser139 is a marker of DNA double strand breaks, a type of DNA damage that can direct cells to death if unrepaired [319, 320]. The mechanism of TMZ induced apoptosis was found in previous studies in other cancer cell lines to be consistent with our results showing that TMZ induce DNA double strand damage [124, 321-323]. Our results demonstrated that intrinsic or mitochondrial pathway is involved in induction of apoptosis after exposing with TMZ. In RH30 cell, anti-apoptotic proteins, such as MCL-1, was downregulated and Bcl-2 slightly up-regulated. However, no changes were found in expression of t-Bid and pro-apoptotic protein (Bax) in both cell lines. Mitochondrial Bcl-2 family proteins are known to be essential for the apoptotic response in chemotherapy, emphasizing that these proteins are potential therapeutic

targets [184, 324, 325]. Bcl-2 family proteins are considered to be principal apoptosis regulators [326]. A study showed TMZ causes higher expression of Bcl-xL and lower expression of Mcl-1 in glioma cells, sensitizing cells for better drug responsiveness of glioma [327]. Using Bax targeting BH3 mimetic compound (BTC-8), this report showed an additive effect on apoptosis induced by TMZ in U87MG glioma cells [328]. Therefore, it can be concluded that mitochondrial Bcl-2 family proteins are involved in regulation of TMZ-mediated cytotoxicity in RMS cells, where ani-apoptotic proteins MCL-1and BCL-2 appear to be key players. Our results highlighted the importance of the involvement of anti-apoptotic proteins after TMZ treatment in this specific cancer (RMS). The effect of TMZ in our study, altered expression of Bcl-2 family proteins increased the activation of cleaved caspase-9, indicating that TMZ-induced apoptosis in RMS cells is mitochondria-dependent. These results are in agreement with other studies in other cancer cells in regards to TMZ-induced mitochondrial apoptotic cell death [329, 330]. Our investigations extend our mechanistic understanding of how TMZ induces cell death in RMS cell line and reinforces the concept that TMZ induced -autophagy and -apoptosis are controlled by overlapping pathways. These pathways are to the cross-talk between apoptosis and autophagy.

It has long been known that Baf-A1 is specific suppressor of autophagy [288, 331], targeting lysosomal protein pump (vacuolar H^+-ATPase) [331]. We found that BafA1 alone inhibits the baseline autophagy in RH30 cells, indicating there is a basal autophagic activity in RMS cells (low level). A study on rhabdomyosarcoma showed that the maintenance of cellular viability and proliferation in rhabdomyosarcoma is dependent on the baseline of autophagy [226]. The loss of autophagy negatively regulated cell growth and proliferation of rhabdomyosarcoma cells [227]. Knocking-down ATG7 or using of Baf-A1 led to the suppression

of autophagy, resulting in reduction of cell growth and viability in rhabdomyosarcoma cells [228]. We found that co-treatment of Baf-A1 with TMZ leads the suppression of TMZ-induced autophagy (higher level). It has been recently reported that TMZ induces autophagy in many cell types including Glioblastoma multiforme (GBM) cells [332], malignant glioma cells [333-335] and lung cancer [336] [21]. Our study on the effect of autophagy specific inhibitor on apoptosis showed that the combination treatment of Baf-A1 and TMZ has additive effect on apoptosis compared to treatment of cells with TMZ alone and Baf-A1 alone. This suggests that autophagy plays a survival role in TMZ treated RH30 cells. The autophagy inhibitor enhances the anti-tumor effect of TMZ against RMS cell by activation of apoptosis. This result was consistent with findings by Kanzawa et al., 2004 identified that Baf-A1 after LC3 localization could increase the effectiveness of TMZ against malignant glioma cells by activation of apoptosis [309]. In glioblastoma C6 cells, autophagy inhibition decreases sensitization of the cells to TMZ induced cell death [337]. In U87 glioblastoma cells, TMZ induces autophagy and apoptosis and its apoptotic effect is synergistically increased after inhibiting autophagy by Chloroquine [313]. Our group has recently shown that autophagy inhibition increases the sensitization effect of glioblastoma cells (U87 and U251) treated with simvastatin and TMZ (unpublished).

Analysis of BCL-2-family proteins after co-treatment with Baf-A1 and TMZ exhibited up-regulation of pro-apoptotic protein (Bax) and truncated Bid. These results implied that the extrinsic apoptotic signaling pathway may also be involved in regulation of apoptosis after blocking of autophagy. These results indicated that higher apoptotic activity after suppression of autophagy might be because of involvement of extrinsic apoptosis signaling pathway. The truncation through activation of extrinsic apoptotic pathway and its effect on pro-apoptotic proteins (BAX or BAK) cause to release cytochrome c from mitochondria, inducing of

70

apoptosis [338]. The activation of cascade truncted Bid/Bax was found to be associated with caspases activation and thereby apoptosis [338]. However, how Baf-A1 triggers apoptosis in combination therapy is not fully understood and needs to be more investigation.

In our study inhibition of autophagy flux by chemical inhibition using Baf-A1 (100 nM) was performed in RH30 cells. The obtained results were showed that Baf-A1 (100nM) for short time (1,2,3, and 4 hours) inhibits autophagy flux, validating the hypothesis that TMZ can also activate autophagy flux in RH30 cells. Autophagy flux is the complete process of autophagy, the dynamic process of autophagosome synthesis, delivery of autophagic substrates to the lysosome, and degradation of autophagic substrates inside the lysosome and is a more reliable indicator of autophagic activity than measurements of autophagosome numbers.[339, 340]. Autophagy has been defined as a defensive mechanism against unfavorable stresses in the tumor microenvironment. It can increase the survival of the cancer cells and keep them in a healthy condition through eliminating the damaged organelles and aggregated or misfolded proteins harmful to the cancer cells [341].

Our findings mentioned here show the essential role that autophagy plays in the TMZ-induced apoptosis in RH30 cells. Targeting autophagy can be considered as a potential target for developing new treatment options against rhabdomyosarcoma.

5.   Significance and Future Direction:

Rhabdomyosarcoma is a lethal disease in children, and novel ideas can help to develop therapeutic strategies in this field. Since in many studies TMZ is found to be a potent active agent in cancer therapy, different clinical trials are trying to test it as combination therapy with other drugs. However, its underlying mechanisms are remains unclear and under debate. These

exciting and innovative studies will lay the molecular foundation to advance our understanding of this therapeutic strategy to promote programmed cell death of RMS cells. We will use the results of the current project, investigating how TMZ kills RMS cells, as a platform to understand how RMS cells can become resistant to TMZ therapy. This knowledge will enable us to design combinatorial approaches that will expand the clinical usefulness of TMZ as a chemotherapy agent.

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