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THE HISTONE DEACETYLASE INHIBITOR, MS-275, SENSITIZES METASTATIC OSTEOSARCOMA TO FASL-INDUCED CELL DEATH: A ROLE FOR C-FLIP

Krithi R. Bindal

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**THE HISTONE DEACETYLASE INHIBITOR, MS-275, SENSITIZES METASTATIC
OSTEOSARCOMA TO FASL-INDUCED CELL DEATH: A ROLE FOR C-FLIP**

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OSTEOSARCOMA TO FASL-INDUCED CELL DEATH: A ROLE FOR C-FLIP**

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston

and

The University of Texas
M.D. Anderson Cancer Center
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in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR of PHILOSOPHY

by

Krithi Rao-Bindal, MS

Houston, Texas
December, 2011



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DEDICATION

I am truly blessed to have been surrounded by extreme unconditional love. To my devoted
parents and to my entirely selfless husband...

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I am extremely grateful to my PhD advisor, Dr. Eugenie Kleinerman. She has truly been a role model to me as a scientist, a medical leader and as a woman. I thank her for taking me on initially as a graduate student and molding me into the scientist I am today. I am sincerely appreciative of her guidance, encouragement and support throughout my training. I have also been fortunate to have Drs. Joya Chandra and Felipe Samaniego as committee members over the years. They have wholeheartedly contributed to the success of my project as well as my PhD education. I am especially grateful to Dr. Chandra for her helpful advice and suggestions. My recent committee members, Drs. Gary Gallick and Bradley McIntyre, have given me great advice over the past couple of years and have really helped in refining my project. Past and present lab members including Dr. Nancy Gordon, Dr. Nadya Koshkina, Dr. Mario Holloman, Dr. Zhichao Zhou, Dr. Thomas Yang, Dr. Kazumasa Nishimoto, Dr. Ling Yu, Dr. Gangxiong Huang, Dr. Keri Schadler, Dr. Randala Hamdan, Sergei Guma and Janice Santiago have truly been like family to me. The Pediatrics department has been an extremely amiable environment to work in.

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To my husband, Dr. Ankur Bindal – you have been my right hand throughout this entire process. Having to live in different cities while we follow our dreams hasn't been easy, but truly demonstrates your loving support. I am extremely excited to continue to pursue our dreams on this amazing journey together.

MS-275 SENSITIZES METASTATIC OSTEOSARCOMA TO FASL-INDUCED
CELL DEATH: A ROLE FOR C-FLIP

Publication No. _____

Krithi Rao-Bindal, MS

Supervisory Professor: Eugenie S. Kleinerman, MD

The purpose of this study was to determine the effects of the histone deacetylase inhibitor, MS-275, on the Fas signaling pathway and susceptibility of osteosarcoma (OS) to Fas ligand (FasL)-induced cell death. OS metastasizes almost exclusively to the lungs. We have shown that Fas expression in OS cells is inversely correlated with their metastatic potential. Fas⁺ cells are rapidly eliminated when they enter the lungs via interaction with FasL, which is constitutively expressed in the lungs. Fas⁻ OS cells escape this FasL-induced apoptosis and survive in the lung microenvironment. Moreover, upregulation of Fas in established OS lung metastases results in tumor regression. Therefore, agents that upregulate Fas expression or activate the Fas signaling pathway may have therapeutic potential.

Treatment of Fas⁻ metastatic OS cell lines with 2 μ M MS-275 sensitized cells to FasL-induced cell death *in vitro*. We found that MS-275 did not alter the expression of Fas on the cell surface; rather it resulted in increased levels of Fas within the membrane lipid rafts, as demonstrated by an increase in Fas expression in detergent insoluble lipid raft fractions. We further demonstrated that following MS-275 treatment, Fas colocalized with GM1⁺ lipid rafts and that there was a decrease in c-FLIP (cellular FLICE-inhibitory protein) mRNA and protein. Downregulation of c-FLIP correlated with caspase activation and apoptosis induction. Transfection of cells with shRNA to c-FLIP also resulted in the localization of Fas to lipid

rafts. These studies indicate that MS-275 sensitizes OS cells to FasL by upregulating the expression of Fas in membrane lipid rafts, which correlated with the downregulation of c-FLIP.

Treatment of nu/nu-mice with established OS lung metastases with oral MS-275 resulted in increased apoptosis, a significant inhibition of c-FLIP expression in tumors and tumor regression. Histopathological examination of mice showed no significant organ toxicity. Overall, these results suggest that the mechanism by which MS-275 sensitizes OS cells and lung metastases to FasL-induced cell death may be by a reduction in the expression of c-FLIP.

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INTRODUCTION

Chapter 1

Introduction: background, rationale and research plan

Osteosarcoma

Osteosarcoma (OS) is the most common primary malignant tumor of the bone in pediatric patients. It arises primarily in the long bone and very rarely in the soft tissues and is generally characterized by the formation of immature bone or osteoid by tumor cells (1). Diagnosis is determined by radiographs, computed tomography (CT), magnetic resonance imaging (MRI). Elevated serum alkaline phosphatases, which occurs in greater than 40% of patients with OS, can be used to assist with diagnosis (2). Treatment includes surgery combined with neoadjuvant chemotherapy. The standard of care includes a combination of high-dose methotrexate, cisplatin, adriamycin and ifosfamide. Mifurtamide (liposomal muramyl tripeptide phosphatidyl ethanolamine; Mepact[®]) has recently been approved in the European Union for the treatment of high-grade, resectable non-metastatic osteosarcoma (3). The cure rate for patients with nonmetastatic OS is 60-70% (4, 5).

Since OS metastasizes through the hematogenous route, the most common sites of metastasis are the lungs followed by bone, which occurs at a lower frequency. About 80% of patients present with microscopic pulmonary metastases and 10-20% with macroscopic disease at diagnosis. Although survival of patients with nonmetastatic disease has improved dramatically, patients who present with metastasis, primarily to the lung, have poor prognosis with 5-year survival rates of less than 20% (6, 7). Once metastases occur, aggressive treatment is necessary, which includes surgical resection and intensive chemotherapy. The most effective combination has been found to include cisplatin, doxorubicin and high-dose methotrexate plus leucovorin. However, the likelihood of response with these combinations and others in clinical trials has been found to be less than 10% in patients with refractory or recurrent disease after chemotherapy (8). Therefore, the need for novel therapeutic strategies for metastatic OS is ongoing.

Since metastatic disease is clearly an indicator of poor outcome in patients with solid tumors such as OS, there is an increasing interest in understanding the molecular events associated with metastatic potential. Ezrin, a membrane-cytoskeleton linker protein, was shown to be expressed at high levels in primary tumors from OS patients. This high expression correlated with a shorter disease-free survival and a greater risk for metastatic disease (9). Matrix metalloproteinases (MMPs) are known to play a role in the migration and invasion of tumor cells and are associated with poor prognosis. MMP-2, -8, -13, and -26 have been found to be overexpressed in metastatic tumor tissue in OS patients, which implicates their role in the metastatic process (10). Overexpression of vascular endothelial growth factor (VEGF) has also been linked to pulmonary metastasis and poor prognosis of OS patients (11-13). The focus of our laboratory is on understanding the mechanisms of metastasis and the role of the lung microenvironment in the potentiation of metastatic tumor formation and growth.

The role of the Fas/FasL pathway in OS metastasis

Our laboratory has demonstrated that the Fas/Fas ligand (FasL) pathway plays an important role in the metastatic potential of OS. This is significant since the lung is one of the few organs to constitutively express FasL (14-17). Using human SAOS-2 OS cells, we created a series of highly metastatic sublines by recycling the cells through the lungs of nude mice. With each passage, a subline was created with more metastatic potential. The LM7 subline had a 100% incidence of forming pulmonary metastases in nude mice (18). We observed lower Fas expression in LM7 than the SAOS-2 parental cell line, supporting the hypothesis of an inverse correlation between Fas expression and metastatic potential (19). We demonstrated in OS *in vivo* models that OS primary tumor in the bone contained a population of both Fas⁺ and Fas⁻ cells, pulmonary metastases were Fas⁻, confirming our hypothesis that Fas expression was inversely correlated with metastatic potential (14). In addition, pulmonary metastases from

patients were found to be Fas-negative (15). We have demonstrated that Fas⁺ OS cells are cleared in the lung by activation of Fas signaling and apoptosis, while Fas⁻ cells have the ability to evade this and survive to form metastatic lesions (14-16). In particular, we showed a correlation between Fas expression and the clearance of OS cells from the lung (14). Fas⁺ OS cells were cleared within 24 hours while Fas⁻ cells remained (Figure 1).

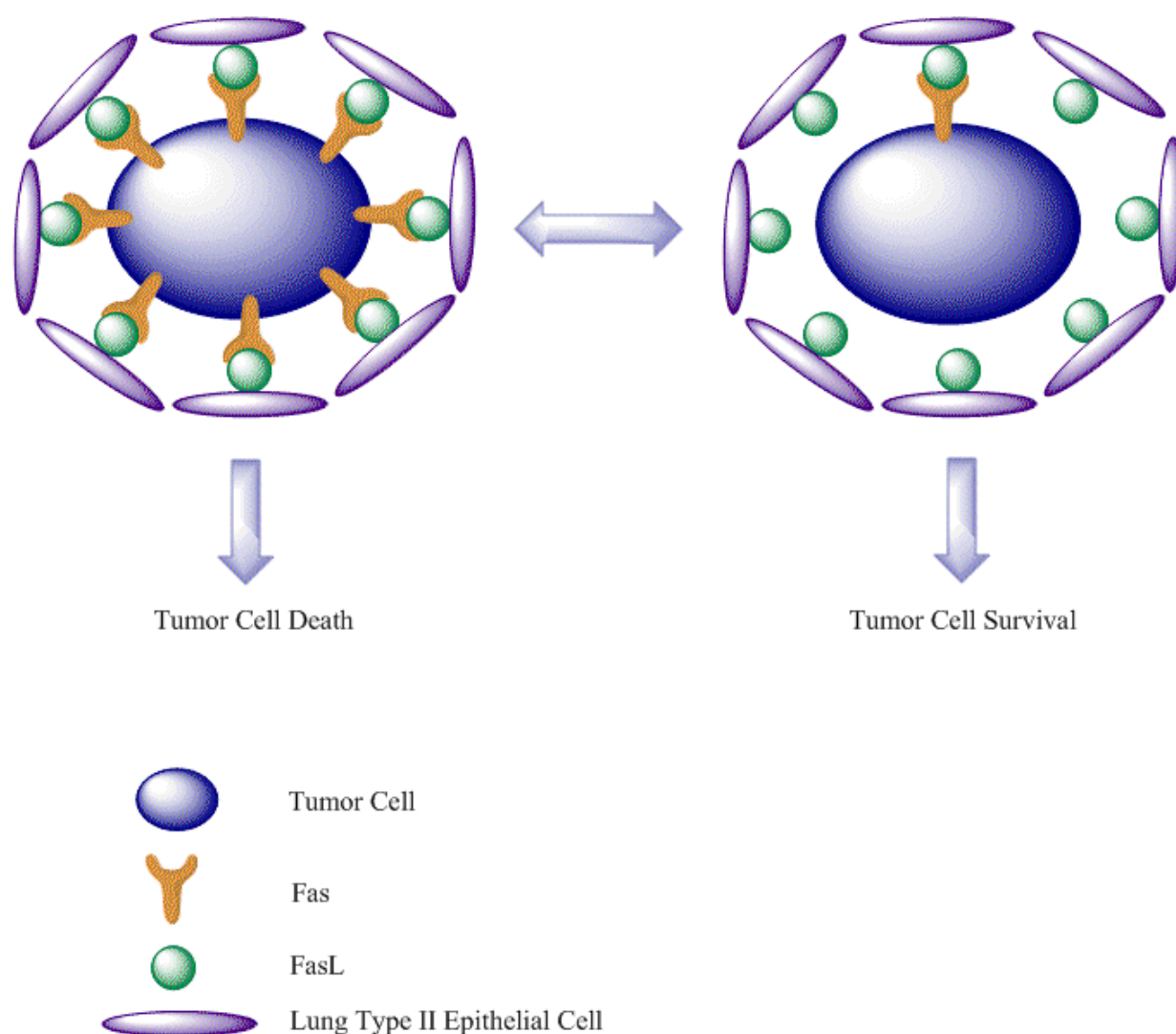


Figure 1. Model diagram of selection of Fas⁻ OS cells in the lung microenvironment. FasL in the lung interacts with Fas⁺ OS cells to induce cell death while Fas⁻ OS cells can survive to form metastases.

To confirm our hypothesis further, studies in our laboratory involved the injection of mouse OS cells intravenously into FasL-deficient (*gld*) mice. The lung metastases in the mice contained a mixed population of Fas⁺ and Fas⁻ cells, further validating our findings that FasL is necessary for the selective formation of Fas⁻ metastases (20). Finally, disruption of the Fas pathway by transfecting cells with FADD-dominant negative (FDN), resulted in the formation of Fas⁺ lung metastases (14). Overall, the data confirms the importance of Fas/FasL signaling pathway and the tumor microenvironment in the formation of OS lung metastases.

The Fas signaling pathway

The Fas (CD95)/FasL signaling pathway has been implicated in the pathogenesis of several tumor types and malignancies (Figure 2). The Fas receptor, a member of the tumor necrosis factor (TNF) receptor superfamily, is known to induce apoptosis by binding to FasL. Receptor-ligand interaction induces the recruitment of Fas-associated death domain (FADD) and procaspase-8 to form the death-inducing signaling complex (DISC). Interaction of procaspase-8 at the DISC leads to its autocatalytic cleavage and activation, which results in caspase cleavage either via the mitochondrial pathway or by direct activation of the effector caspases. Inhibition of Fas-mediated apoptosis is regulated by FLICE-inhibitory protein (FLIP), the structural homologue of procaspase-8 (21). Cellular FLIP (c-FLIP) competes with procaspase-8 for recruitment to FADD at the DISC (22, 23). c-FLIP has been found to be overexpressed in numerous cancer cell lines and primary cells and tissues from patients (6, 24-31). Since overexpression of c-FLIP is associated with increased resistance to death receptor pathways, several investigators have found that downregulation of c-FLIP results in the sensitization of tumor cell lines to Fas-mediated apoptosis.

Targeting the Fas/FasL pathway in OS

Several studies by our group confirmed that OS lung metastases express little to no Fas in a microenvironment constitutively expressing FasL. Therefore, we hypothesized that treatment of metastases with therapeutic agents that can upregulate Fas may result in the sensitization of cells to FasL. Our laboratory first demonstrated that interleukin (IL)-12 treatment of human metastatic OS cells resulted in the upregulation of Fas expression and sensitized cells to cross-linked anti-Fas antibody (32). Intranasal or aerosol therapy is a method used to deliver agents directly to the lungs to increase exposure and minimize toxicity. Use of intranasal IL-12 gene therapy in a nonviral polyethylenimine vector in mice with lung metastases was successful in upregulating Fas expression and eradicating metastases (33). Similarly, both intranasal and aerosol gemcitabine treatment of mice with lung metastases upregulated Fas expression and resulted in the regression of metastatic tumor nodules (34, 35). This result was later confirmed to be dependent upon a functional Fas signaling pathway since corruption of the pathway using FADD-dominant negative (FDN), reduced the effect of aerosol gemcitabine (20). Since our data suggest that upregulating Fas expression with therapeutic agents results in the regression of OS lung metastases, we proposed the use of epigenetic modifiers to re-express Fas expression. However, treatment of metastatic cells with the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) did not upregulate Fas or sensitize cells to FasL (36). This suggested the possibility of histone modifying agents such as histone deacetylase inhibitors, which have increasingly gained popularity as anti-cancer agents.

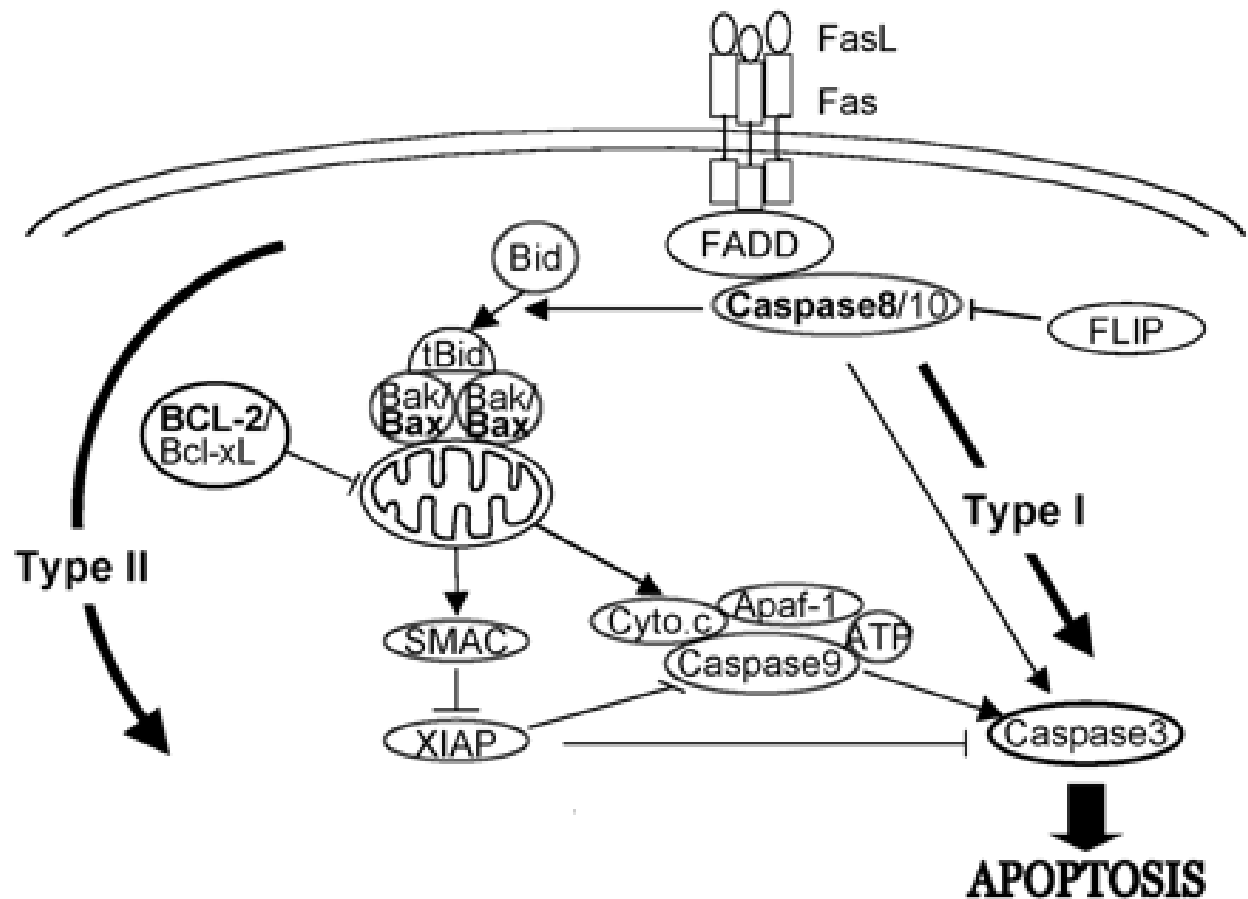


Figure 2. The Fas signaling pathway. Upon binding with FasL, Fas undergoes trimerization and associates at the death domain (DD) with Fas-associated death domain (FADD). FADD then interacts with caspase-8 at the death effector domain (DED) which results in its cleavage and activation. Fas, FADD and caspase-8 together form the death-inducing signaling complex (DISC). Caspase-8 activation results in activation of the effector caspases and apoptosis either directly or via the mitochondrial pathway. The inhibitor of apoptosis protein, FLICE-inhibitory protein (c-FLIP) prevents caspase-8 activation by competitively binding at the DISC. *Reprinted from F. Hua The Journal of Immunology 2005; 175(2)985-995 with permission from The Journal of Immunology. Copyright 2005. The American Association of Immunologists, Inc.*

Epigenetic Regulation

Since epigenetic-targeted therapy has gained popularity as anti-cancer agents; the field of epigenetics has been one of the most recently studied areas in cancer research. The classical definition of epigenetics is a heritable change in gene expression or phenotype that results from changes other than alterations in the underlying DNA sequence. Some of the most commonly studied epigenetic mechanisms include DNA methylation, histone modifications and other chromatin remodeling factors (37). Histone post-translational modifications may include acetylation, methylation, phosphorylation, ubiquitination and sumoylation (38). Importantly, epigenetic mechanisms are often dysregulated in human cancers, which have increased the interest in studying these underlying mechanisms.

In particular, histone modifications by acetylation and deacetylation have been shown to play a key role in tumorigenesis. Two enzymes are responsible for these histone modifications and resulting remodeling of chromatin: histone deacetylases (HDACs) and histone acetyltransferases (HATs). HDACs are responsible for the removal of acetyl-groups from histone tails that results in a closed chromatin conformation and transcriptional repression. While, HATs acetylate histone tails leading to a relaxed chromatin state and transcriptional activation (38-40) (Figure 3). There exist four classes of HDACs. Class I includes HDAC-1, -2, -3 and 8, while class II contains HDAC-4, -5, -7, and -9. Class IIa differs from class II by the number of catalytic sites and includes HDAC 6 and 10. Class III contains sirtuins and class IV includes HDAC-11 (41). Knockout analyses suggest HDAC-specific functions and even show variation in their localization in the cell (42-47). Aberrant HDAC function and overexpression has been associated with several human cancers and has been correlated with poor patient prognosis (48-52). Thus, the rationale to target HDACs is highly validated.

Histone Deacetylase (HDAC) Inhibitors

HDAC inhibitors are classified by both structure and function. The structural classes include hydroxamic acids, cyclic peptides, aliphatic acids and benzamides. HDAC inhibitors are also functionally classified as pan-HDAC inhibitors, class I and class II-specific inhibitors, and sirtuin inhibitors (42, 53). Vorinostat (SAHA) is a pan-HDAC inhibitor that is classified under the hydroxamic acids and is the first HDAC inhibitor to be approved by the Food and Drug Administration (42, 54). Several other HDAC inhibitors are currently in clinical trials for both hematological malignancies and solid tumors (55).

The more well understood mechanism of action of HDAC inhibitors involves the inhibition of HDACs that results in the accumulation of acetylated histones. The presence of histone acetylation leads to an open chromatin conformation and results in transcriptional activation. The resulting change in gene expression has been shown to affect such processes as cell proliferation, cell death and cell migration. Interestingly, tumor cells are more sensitive to cell death induced by HDAC inhibition than are normal cells. While there are many theories explaining this finding, several groups have demonstrated that HDAC inhibitors target cell cycle checkpoints, which are often defective in tumor cells (56, 57). Others have found that treatment of cells with HDAC inhibitors result in the accumulation of reactive oxygen species (ROS) selectively in tumor cells but not in normal cells. Their findings indicated that the HDAC inhibitors induced the expression of thioredoxin, an antioxidant, primarily in normal cells which inhibits ROS-induced cell death (58). In general, the mechanism of HDAC inhibitors seem to vary by which HDACs are inhibited, dose, duration of treatment and what molecular changes exist in the tumor cell (42).

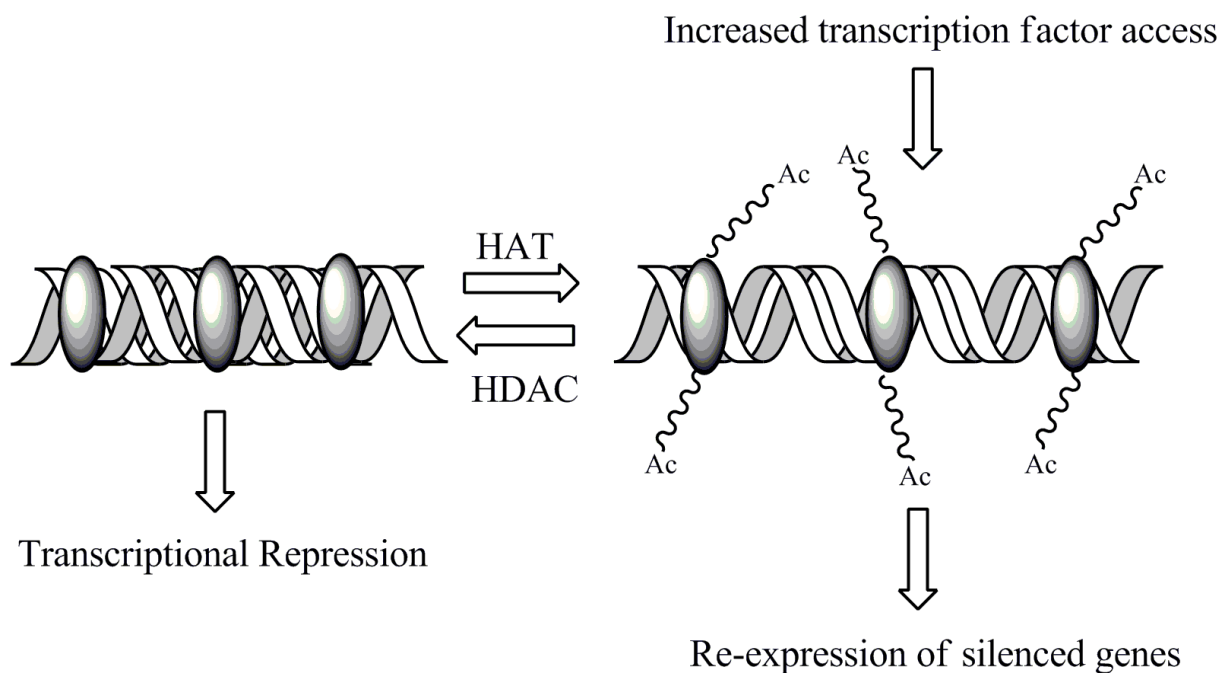


Figure 3. Role of histone modifications in the activation or repression of transcription. Histone acetyltransferases (HAT) are enzymes that acetylate amino acids on histone tails, which result in an open chromatin conformation and transcriptional activation. While, histone deacetylases (HDAC) are a class of enzymes that remove acetyl groups which results in tightly packed chromatin and transcriptional repression.

Recent studies have provided evidence that in addition to histone proteins, non-histone proteins can be targets of HDAC inhibitors (59). These findings have demonstrated that many of the effects that often result from HDAC inhibition, such as cell cycle control, DNA damage response, nuclear transport and RNA splicing have correlated with changes in non-histone protein acetylation (60, 61). Even miRNA expression has been shown to be affected by HDAC inhibitors (62). For this reason, HDAC inhibitors are more appropriately referred to as deacetylase (DAC) inhibitors (59). It is important to note that protein acetylation regulates protein degradation, which gives an explanation as to why gene expression both increases and decreases following HDAC inhibition (63, 64).

MS-275 (SNDX-275, Entinostat; Syndax Pharmaceuticals Inc.) is a histone deacetylase inhibitor in the benzamide structural class (Figure 4). In tumor cells, MS-275 has been shown

to inhibit class I HDACs with more specificity for HDAC 1 and 3 and almost no activity against HDAC 8 (65). Both histone acetylation and non-histone protein acetylation have been demonstrated following treatment that resulted in both downregulation and upregulation of gene expression (66). In particular, MS-275 treatment has been shown to affect various molecular processes such as death receptor and ROS-dependent apoptosis, cell cycle arrest and differentiation and response to DNA damage both *in vitro* and *in vivo* (67-70). To date, four phase I studies have been completed for MS-275 and have reported both safety and tolerability in patients with lymphoid malignancies (71-74). Phase II trials in metastatic melanoma were recently completed and several phase I and phase II trials are currently in progress either as a single agent or in combination (75). Overall, MS-275 has demonstrated efficacy and safety in animal models and clinical trials and therefore shows promise for evaluation in other cancers.

Histone deacetylase inhibitors and the Fas pathway

HDAC inhibitors have been identified to induce cell cycle arrest and apoptosis *in vitro* and *in vivo*. Specifically, many HDAC inhibitors have been shown to sensitize cells to Fas-mediated apoptosis. However, the specific mechanism of how this may occur has been shown to vary by tumor type and drug. For example, upregulation of Fas or FasL expression has been demonstrated in neuroblastoma, promyelocytic leukemia and uveal melanoma cells following treatment with the HDAC inhibitors CBHA, apicidin and depsipeptide, respectively (76-79). VPA increased the sensitivity of OS cells to Fas-mediated cell death by reducing the secretion of sFas (80). Induction of cytotoxicity of acute leukemia cells by the HDAC inhibitor, PCI-24781, has been shown to be dependent on caspase-8 and FADD (78, 79). Additionally, downregulation of c-FLIP expression by the HDAC inhibitor depsipeptide has been observed in both chronic lymphocytic leukemia (CLL) cells and OS cells (78, 79). The HDAC inhibitor, MS-275, has also been shown to inhibit c-FLIP expression in CLL cells, which was followed

by the induction of caspase-dependent apoptosis (79). However, few studies have examined the use of HDAC inhibitors to treat pediatric solid tumors, including OS (31, 79, 81).

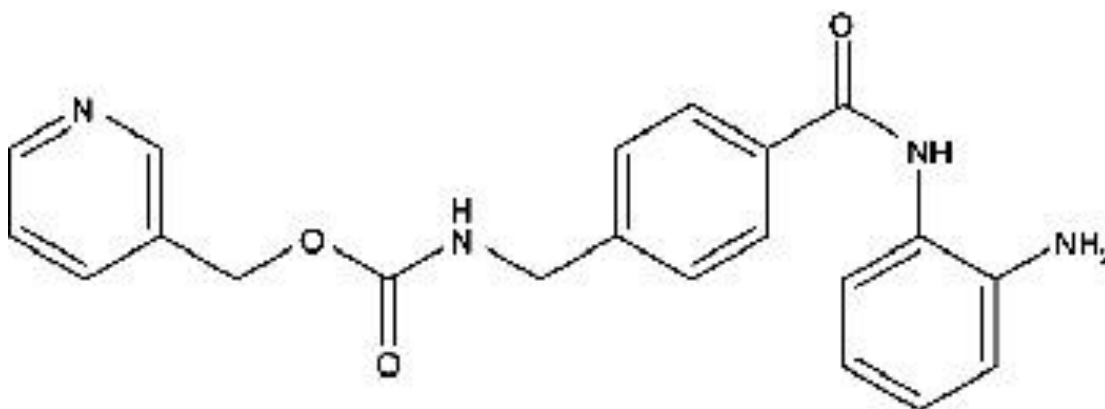


Figure 4. Molecular structure of MS-275. MS-275 (SNDX-275, Entinostat; Syndax Pharmaceuticals, Inc.) is structurally categorized in the benzamide class and is a class I-specific HDAC inhibitor. Adapted from *J. Knipstein Expert Opinion on Investigational Drugs 2011; 20(10)*.

Lipid raft microdomains and Fas

Recent findings have demonstrated that FasL sensitivity of cancer cells is not only determined by levels of Fas expression, but also by the localization of the receptor in membrane compartments known as lipid rafts (82, 83). Lipid rafts are portions of the cellular membrane that contain higher levels of cholesterol and sphingolipids than the plasma membrane (84). The high levels of cholesterol cause the membrane in lipid rafts to be rigid and tightly packed. Due to this rigid nature, lipid rafts often serve as signaling platforms for receptors, which cause receptors to be localized in a close proximity to each other (85). In fact, receptor localization in either rafts or non-rafts may be a mechanism of regulating signaling pathways (84). It has recently been shown that compartmentalization of receptors in lipid rafts

prevent interaction with membrane phosphatases, which degrade proteins and are often found in the plasma membrane. This further confirms the role of rafts in promoting receptor signaling (82, 83). In addition, lipid raft localization has been linked to intracellular receptor internalization and trafficking (83).

Recent studies have shown that upon Fas stimulation, the Fas receptor forms an aggregate and is then redistributed to lipid rafts (86-88). It is within these lipid rafts that FADD and caspase-8 are recruited to form the death-inducing signaling complex (DISC) to initiate apoptosis (88). It has been reported that Fas signals through ceramide-rich lipid rafts and that pre-treating these cells with cholesterol-blocking reagents, which disrupt membrane lipid rafts, inhibited Fas signaling leading to apoptosis (89). In addition, it has been found that treatment of cells with some chemotherapeutic drugs can also induce the lipid raft translocation of Fas (90-93). Following lipid raft translocation, the receptor is then internalized in a clathrin-dependent manner, a process that has been shown to be required for the induction of Fas-mediated apoptosis (94). These studies all suggest that Fas redistribution to lipid rafts occurs upon stimulation and is required for Fas-mediated apoptosis. In identifying the mechanism of Fas translocation to lipid rafts, some groups have investigated the role of post-translational modifications of Fas. It was demonstrated that Fas was palmitoylated or modified by addition of a 16-carbon fatty acid to cysteine residues, which directed Fas to lipid rafts microdomains (Figure 5). Site-directed mutagenesis confirmed that the palmitoyl group is attached to C(cysteine)199. Further, cells expressing Fas that was palmitoylation-deficient decreased the sensitivity of cells to Fas-mediated cell death (87, 95). These data are consistent with the findings that proteins that are post-translationally modified by myristoylation or palmitoylation are directed to lipid rafts (96).

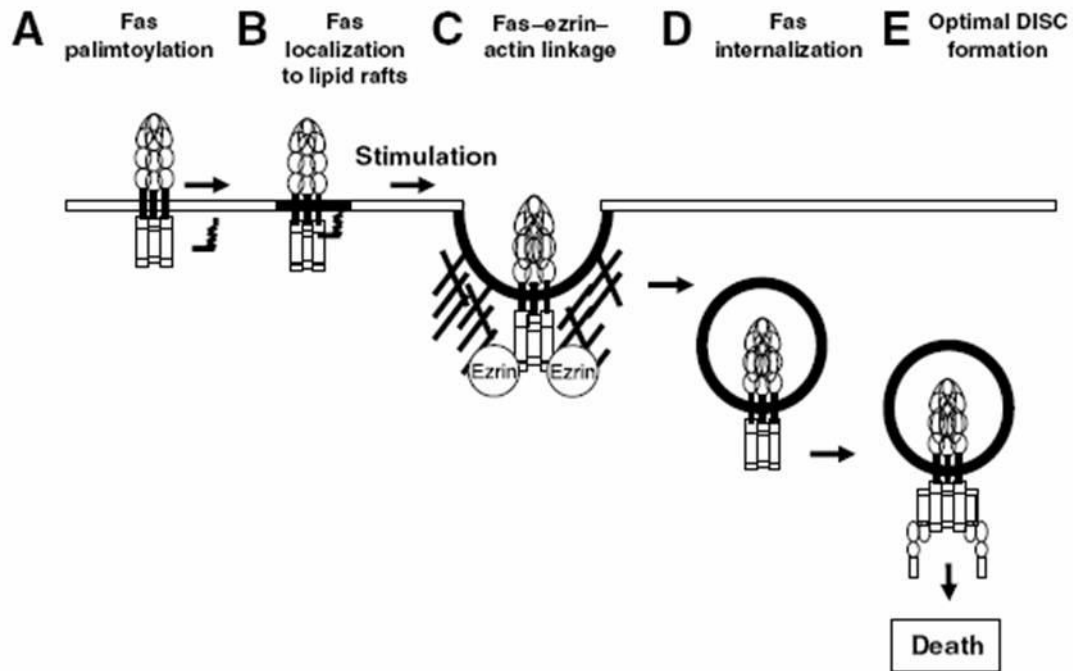


Figure 5. Lipid raft localization of Fas is required for induction of Fas-mediated apoptosis. The Fas receptor is palmitoylated and directed to membrane lipid rafts. Following stimulation with FasL, Fas is internalized in a clathrin-dependent manner which leads to caspase activation and induction of apoptosis. *Reprinted from K. Chakrabandhu The EMBO Journal 2007; 26(209 – 220) with permission from The EMBO Journal.*

Modulating the localization of death receptors in lipid rafts

Some of the initial studies investigating drug-induced apoptosis and the link to lipid rafts involved the mechanistic analysis of early chemotherapeutic agents. For example, cytarabine and daunorubicin induced sphingomyelinase activity specifically in lipid raft microdomains. This increase in activity correlated with the induction of apoptosis in myeloid leukemia cells. This is the first study to describe the importance of lipid rafts in the apoptosis-inducing effect of chemotherapeutic agents (97, 98). Around the same time, the treatment of human leukemic cells with the alkyl-lysophospholipid analogue, edelfosine, was the first compound observed to induce apoptosis through the translocation of Fas to membrane lipid rafts (99). This observation opened a whole new area of interest studying the drug-induced modulation of death receptor localization in rafts independent of ligand. Many of these chemotherapeutic agents have been shown to increase lipid raft localization by altering raft composition and lipid composition (100, 101). However, other mechanisms of action have been reported such as the chemotherapeutic-induced downregulation of c-FLIP, which correlated with death receptor redistribution from non-rafts to lipid rafts (102).

Only one reported study has investigated the use of an HDAC inhibitor in modulating the localization of death receptors in lipid rafts. Treatment of prostate cancer cells with depsipeptide did not change the cell surface levels of the Tumor Necrosis Factor-Related Apoptosis Inducing Ligand Receptors, TRAIL-R1 and TRAIL-R2. Instead, depsipeptide treatment increased the expression of the receptors specifically in membrane lipid rafts and resulted in increased sensitivity to the ligand TRAIL. However, considering the new emergence of this field, the molecular mechanism of how HDAC inhibitors can modulate death receptor localization is not well understood (103).

Aim of the study

Previously published studies and our preliminary data (discussed in chapter 2), suggest the effectiveness of the HDAC inhibitor, MS-275, in osteosarcoma cells. Our central hypothesis for this study is that treatment of Fas-negative OS cells with MS-275 will increase the sensitivity to FasL-induced cell death. Further, we speculate that the mechanism of action will involve the upregulation of Fas expression and/or activation of the Fas signaling pathway. Considering the necessity of novel therapeutic agents for the treatment of OS lung metastases, we investigate the efficacy of oral administration of MS-275 in a lung metastases mouse model. Understanding the effectiveness of MS-275 and elucidating its molecular mechanism is critical for moving this therapeutic concept forward into a clinical trial.

RESULTS

Chapter 2

The histone deacetylase inhibitor, MS-275, sensitizes osteosarcoma cells to FasL-induced cell death in a caspase-dependent manner

RATIONALE

Our group has previously demonstrated that upregulation of Fas using therapeutic agents was successful in sensitizing OS cells to FasL-induced cell death *in vitro* and inducing the regression of OS lung metastases *in vivo* (17, 33, 35, 104). Moreover, corruption of the Fas pathway by transfecting cells with a FADD-dominant negative (FDN) plasmid increased their metastatic potential and abrogated the effect of these therapeutic agents (20). These data support our hypothesis that therapeutic agents that can either upregulate Fas or activate the Fas signaling pathway may be beneficial in the treatment of OS pulmonary metastases. Because of the finding that HDAC inhibitors can activate the Fas pathway in some solid tumors, we sought to determine whether the HDAC inhibitors could sensitize Fas-negative OS cells to FasL. The effect of the HDAC inhibitors, SAHA and MS-275, were tested in OS cell lines (Figure A3). Our preliminary experiments demonstrated that in comparison to SAHA, MS-275 sensitized cells to sFasL-induced cytotoxicity even at subtoxic doses. Therefore, the subsequent studies focus on the use of the half-maximal inhibitory concentration (IC₅₀; 2 μ M) of MS-275 to sensitize OS cells to FasL.

In chapter 2, clonogenic assays and apoptotic assays were performed to determine whether pre-treating with MS-275 increased the sensitivity of OS cells to sFasL. To determine whether the sensitization of cells to sFasL following treatment with MS-275 was caspase-dependent, caspase activity assays were performed for caspase-8 and the effector caspase, caspase-3. In addition, the pan-caspase inhibitor, Z-VAD-fmk, was utilized to determine caspase-dependency.

RESULTS

MS-275 sensitizes OS cells to FasL-induced cell death

The effects of MS-275 on clonogenic growth were examined in the human OS cell lines, LM7 and CCH-OS-D. Both cell lines induce lung metastases following injection in nude mice and had relatively low levels of Fas expression (Figure A1). As expected, both cell lines were minimally sensitive to the effects of sFasL (Figure 6). While MS-275 alone partially inhibited cell survival, the combination of MS-275 and sFasL resulted in a greater inhibition of clonogenic survival (Figure 6).

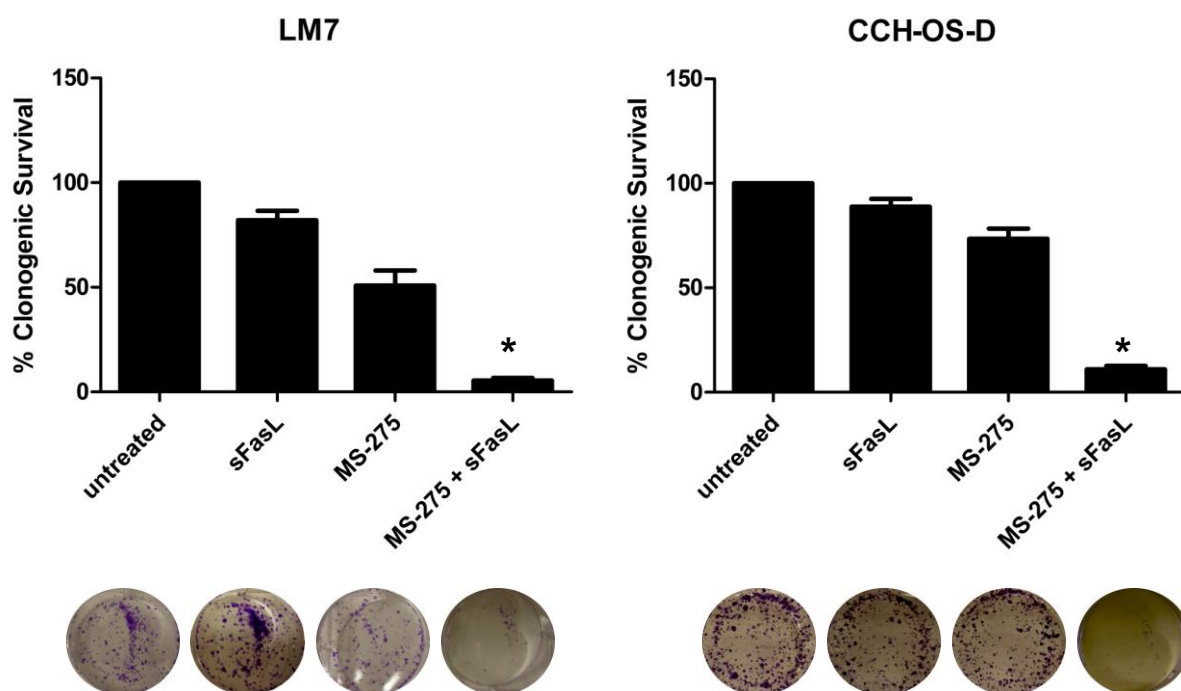


Figure 6. MS-275 sensitizes OS cells to FasL-induced cell death. LM7 and CCH-OSD cells were treated with 2 μ M MS-275 for 48 hours with or without sFasL for 24 hours. Drug was removed at the end of the treatment period and replaced with fresh medium. Cytotoxicity was quantified 15 days later by counting colonies following crystal violet staining. Colonies were counted as 50 cells per colony. *P < 0.05 compared to control and either agent alone. Data represents average and standard deviation of three independent experiments, while pictures are representative of one such experiment.

In addition, MS-275 sensitized cells to FasL-induced apoptosis, as detected by the ethidium bromide/acridine orange (EB/AO) apoptosis assay (Figure 7). This result suggested that pretreatment of OS cells with MS-275 sensitized Fas⁺ cells to sFasL-mediated apoptosis.

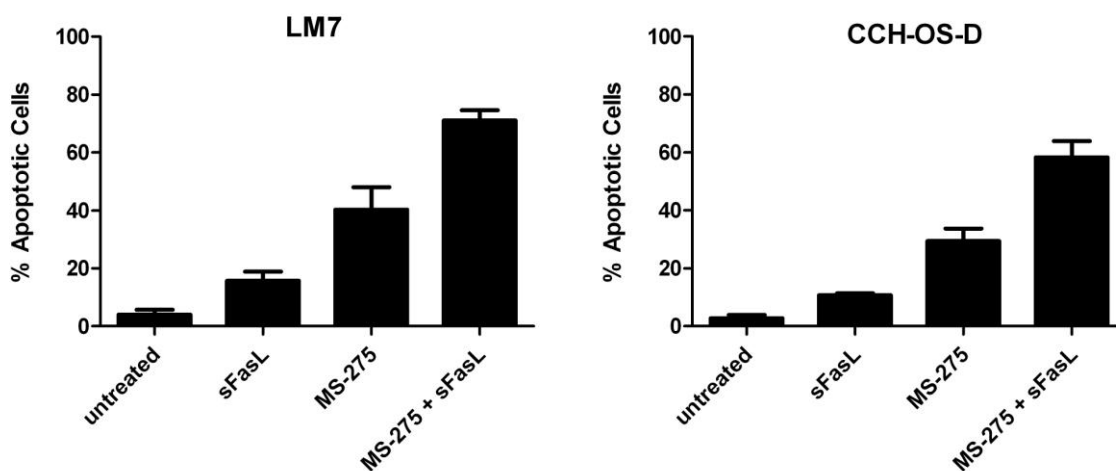
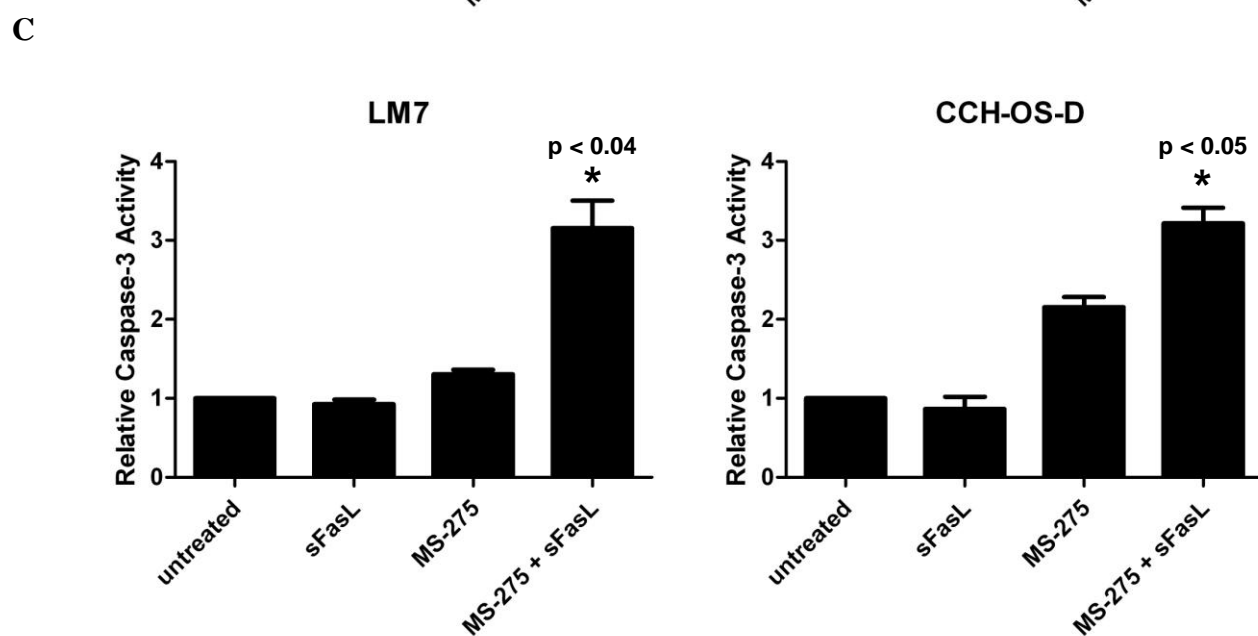
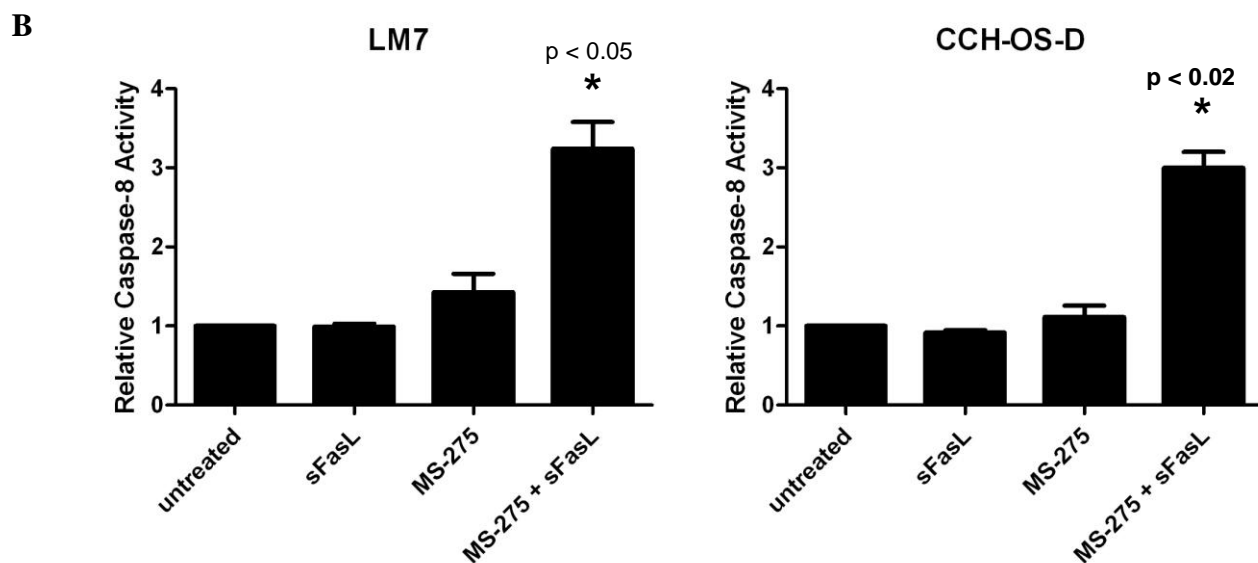
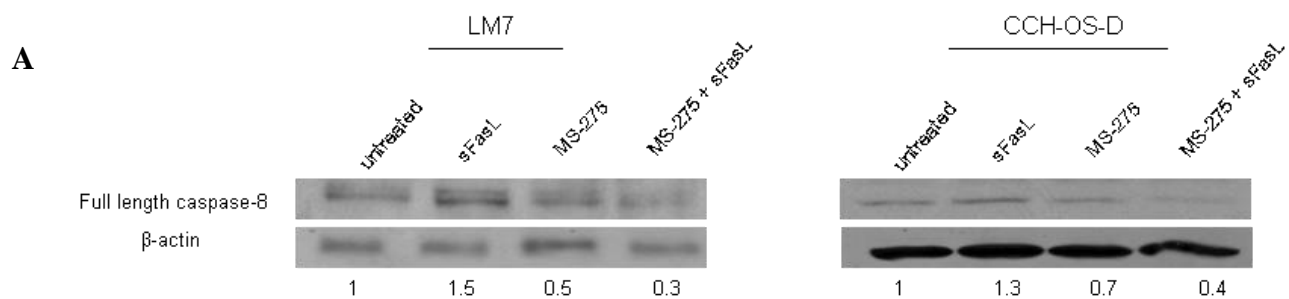


Figure 7. MS-275 sensitizes OS cells to FasL-induced apoptosis. LM7 and CCH-OS-D cells were treated with sFasL for 24 hours, 2 μ M MS-275 for 48 hours or a combination of both. The percentage of apoptotic cells was determined using the ethidium bromide and acridine orange (EB/AO) staining assay. Stained cells were visualized using a fluorescent microscope and apoptosis was determined as percentage of apoptotic cells out of 100 cells counted. Average and standard deviation of three independent experiments is shown.

MS-275-mediated sensitization of OS cells to FasL is caspase-dependent

To determine the ability of MS-275 and sFasL to induce caspase-dependent apoptosis, we examined the caspase activity in cells after treatment. Since caspase-8 interacts with Fas and FADD at the DISC, we investigated the effects of treatment on caspase-8 activity. As expected, no decrease in full length caspase-8 was observed in cells incubated with sFasL alone. We observed lower levels of full-length caspase-8 following treatment with both MS-275 and sFasL than after treatment with either agent alone (Figure 8A), indicating increased caspase cleavage. To validate our data quantitatively, we performed caspase activity assays in

both cell lines. Once again we saw no significant change in caspase activity in cells treated with sFasL alone. However, pretreatment of cells with MS-275 significantly increased sFasL-induced caspase-8 and caspase-3 activity (Figures 8B and 8C). Pretreatment with the pan-caspase inhibitor, Z-VAD-fmk, inhibited cell death following treatment with MS-275 alone and with sFasL, further confirming that MS-275 sensitizes Fas⁻ OS cells to sFasL in a caspase-dependent manner (Figure 8D).



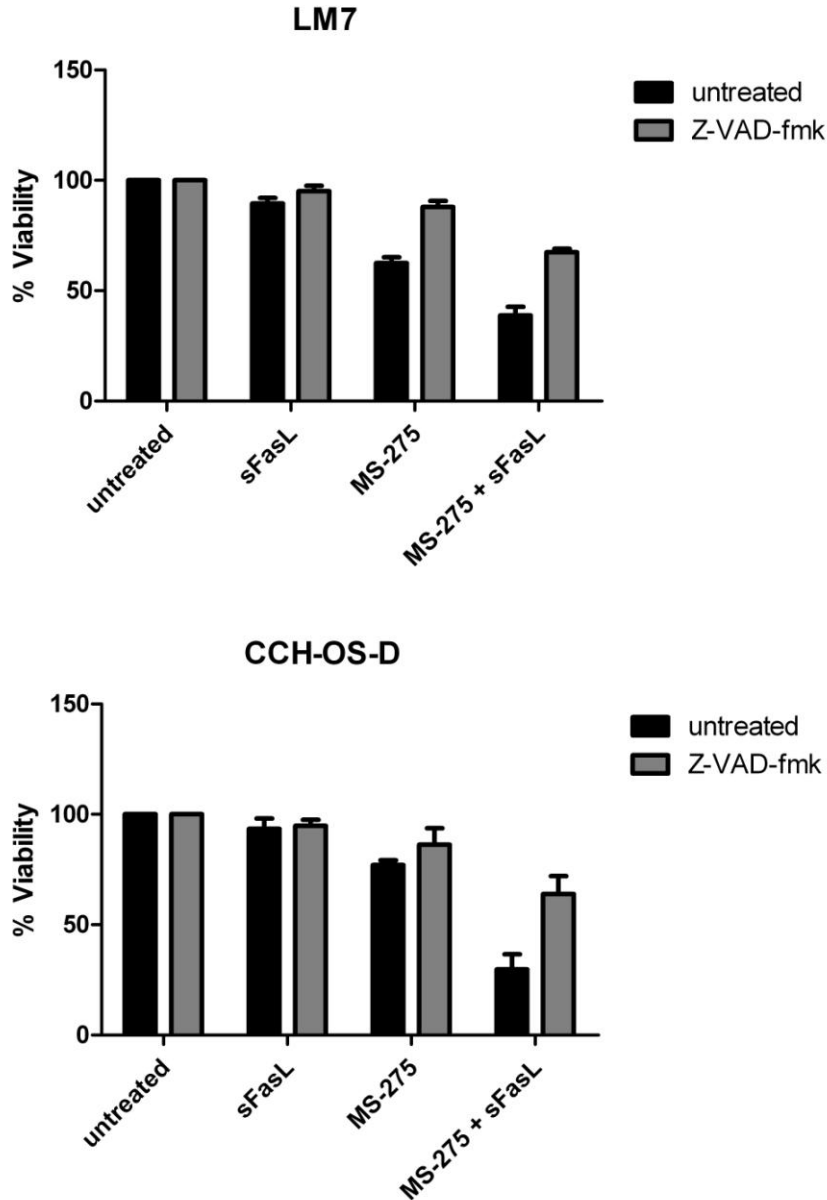
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Figure 8. Effect of MS-275 on caspase activity in OS cells. LM7 and CCH-OS-D cells were treated with 2 μ M MS-275 for 48 hours with or without sFasL for 24 hours. Cell lysates were separated by 10% SDS-PAGE. (A) Immunoblot analysis was done using an antibody against pro-caspase-8. Numbers represent densitometry changes relative to β -actin and compared to the untreated sample. (B) Caspase-8 and (C) caspase-3 activity assays following treatment. *P-values are compared to control and either agent alone. (D) LM7 and CCH-OS-D cells were pretreated with 20 μ M Z-VAD-fmk for 2 hours followed by treatment with 2 μ M MS-275 for 48 hours with or without sFasL for 24 hours. MTT assay was performed to assess cytotoxicity. Comparison of combination group between Z-VAD-fmk treated and untreated cells using student's t-test, yielded a p-value < 0.02. All experiments were repeated three times.

MS-275 induces accumulation of acetylated histone H3

To confirm that MS-275 functions as a HDAC inhibitor, we examined changes in acetylated histone H3 levels by western blot at the dose that inhibited clonogenic survival. MS-275 induced a time-dependent increase in acetylated histone H3 which peaked at 48 hours, the timepoint at which MS-275 was able to both inhibit clonogenic survival and sensitize cells to sFasL. The total levels of histone H3 remained unaltered (Figure 9).

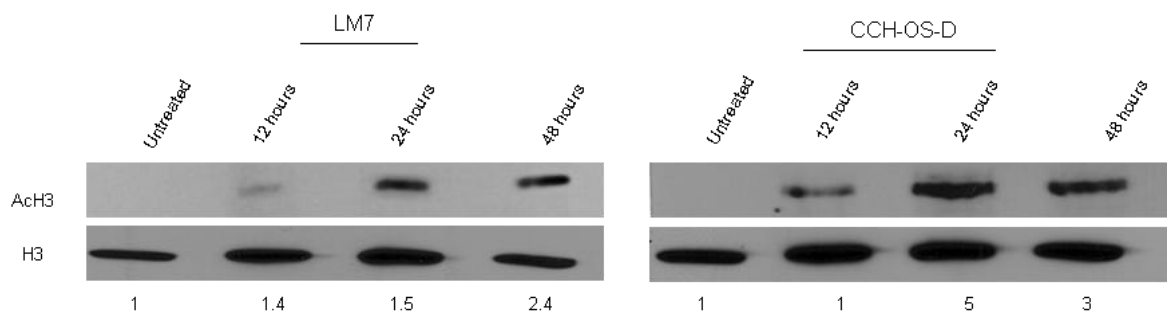


Figure 9. MS-275 induces accumulation of acetylated histone H3. LM7 and CCH-OS-D cells were treated with 2 μ M MS-275 for the indicated times. Histones were then extracted by lysing cells with nuclear extraction buffer followed by centrifugation and sonication of lysates. Lysates were then subjected to separation on 10% SDS-PAGE followed by immunoblot analysis with antibodies against acetylated H3 and total histone H3. Numbers represent densitometry changes relative to histone H3 and compared to the untreated sample.

SUMMARY

These data support our hypothesis that treatment of OS cells with MS-275 sensitizes cells to FasL. I demonstrated that treatment of cells with MS-275 followed by sFasL in combination, reduced the clonogenic growth and increased apoptosis. In addition, I found that MS-275 and sFasL in combination induced caspase-dependent cell death as revealed both by caspase-activity assays and inhibition of caspase activity. To establish that MS-275 was functioning as a histone deacetylase inhibitor in these cells, we confirmed the accumulation of acetylated histone H3 at the dose and timepoints that coincided with sensitization of the cells to sFasL.

RESULTS

Chapter 3

Effect of MS-275 on the Fas signaling pathway

RATIONALE

Our preliminary data revealed the finding that treatment of Fas⁻ OS cells with MS-275 results in its sensitization to FasL-induced cell death. Since histone deacetylase inhibitors have been previously shown to activate Fas signaling in various cancer cell lines (76-79, 105, 106), we sought to determine the importance of the Fas pathway in the mechanism of MS-275-induced sensitization of cells to sFasL. If Fas signaling was essential for MS-275-induced sensitization to FasL, then inhibition of the Fas pathway should abrogate this effect. To test this hypothesis, OS cells were stably transfected with Fas-associated death domain (FADD) dominant-negative (FDN) plasmid. This truncated form of FADD blocks the Fas signaling pathway, which should in turn inhibit the effect of MS-275 if the Fas pathway is involved in the mechanism of action.

We also sought to determine the effect of MS-275 on Fas expression. It has been previously published that selective inhibition of HDAC1/3 upregulates Fas mRNA expression in OS cells. These findings led us to hypothesize that the increased sensitivity of OS cells to FasL following treatment with MS-275 involved the upregulation of Fas expression. Therefore, we performed quantitative real-time PCR, western blot and flow cytometry to determine alterations in Fas expression following MS-275 treatment.

RESULTS

Corruption of the Fas pathway reverses MS-275-induced sensitization of OS cells to FasL

To determine the importance of Fas signaling in the mechanism of MS-275 induced sensitization to sFasL, we stably transfected cells with a Fas-associated death domain (FADD)–dominant negative (FDN) plasmid to block the Fas signaling pathway. LM7 cells were transfected with either empty control vector (LM7/vec) or the FDN construct. Transfection was confirmed by western blotting using an anti-human FADD antibody to detect both the full-length FADD and truncated FADD (Figure 10A). MS-275-induced sensitization to FasL was partially blocked in the LM7/FDN cells as compared to LM7 and LM7/vec controls (Figure 10B). These findings confirm that the Fas signaling pathway is important in MS-275 mechanism of action.

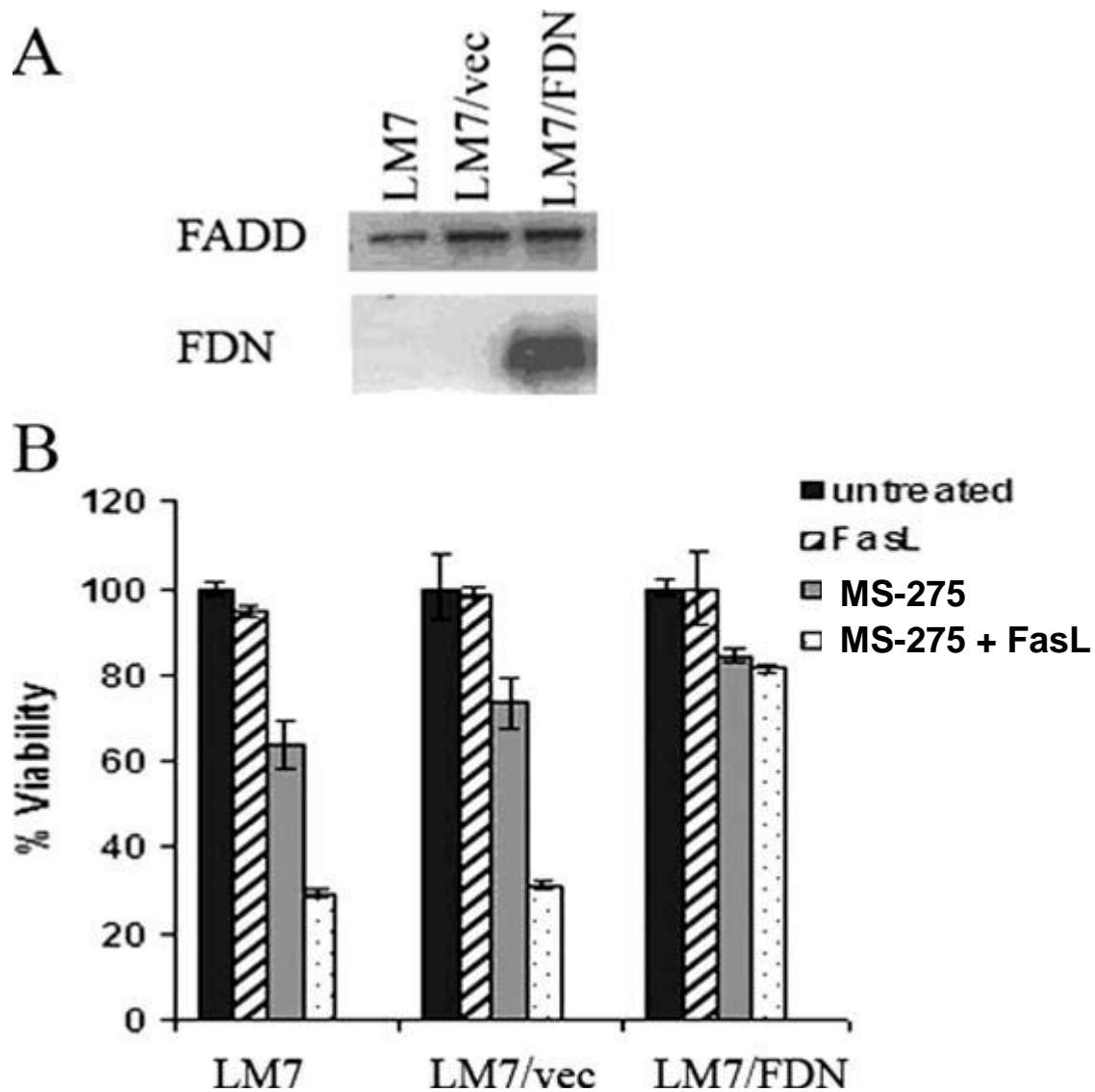


Figure 10. Transfection of LM7 cells with the Fas-associated death domain (FADD) dominant-negative (FDN) plasmid induces expression of the inactive (truncated) form of FADD and reverses MS -275 induced sensitization of LM7 cells to Fas ligand (FasL). (A) LM7 cells were transfected with FDN plasmid (LM7/FDN cells) or with an empty control vector (LM7/vec). Cell lysates were processed using Western blot analysis for expression of the full-length FADD protein and its truncated form, FDN, using an antihuman FADD antibody. (B) LM7, LM7/vec, and LM7/FDN cells were pretreated with 2 μ M MS-275 for 48 hours and then were treated with 10 ng/mL soluble FasL for another 24 hours. Untreated cells and cells that were treated with either agent were used as controls. The cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay. *Figure and figure legend was originally published in Cancer, February 2011 (107). Permission was obtained from Cancer.*

MS-275 increases Fas mRNA and protein expression without a corresponding change in Fas cell surface expression

Histone deacetylase inhibitors act on histone acetylation to regulate gene expression. We therefore sought to determine whether MS-275 induced a change in Fas expression in both LM7 and CCH-OS-D cells. Following treatment of both cell lines with MS-275 there was an increase in Fas mRNA which peaked at 24 hours after addition of MS-275 (Figure 11).

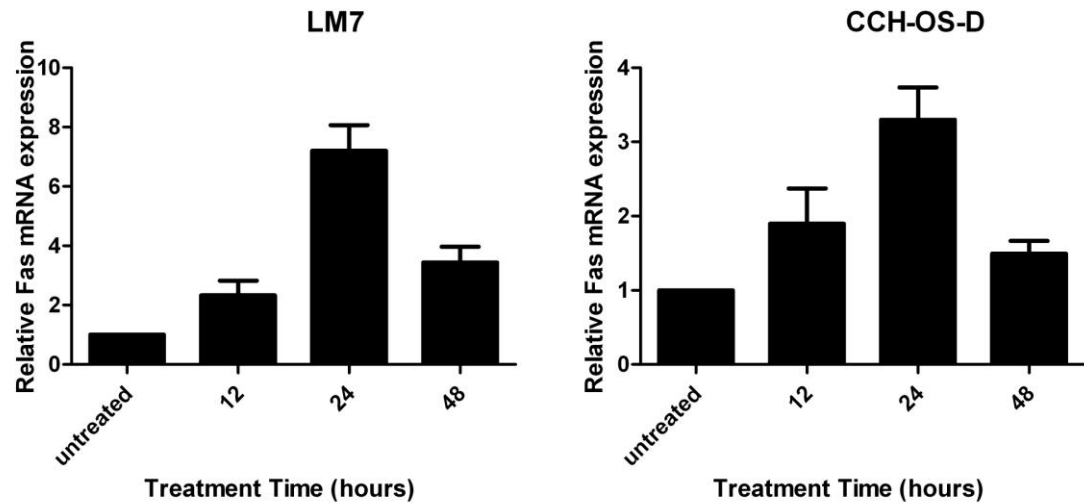


Figure 11. MS-275 increases Fas mRNA expression. OS cells were treated with 2 μ M MS-275 for 24 hours. RNA was isolated from cells using Trizol reagent and quantitative real-time PCR was performed using primers specific for Fas. Data shows an average and standard deviation of five independent experiments. (*) represents p-values comparing treatment with combination and single agents. *Figure (left panel) was originally published in Cancer, February 2011 (107). Permission was obtained from Cancer.*

There was also an increase in Fas protein expression, which was time dependent (Figure 12A). In contrast, the cell surface expression of Fas, as detected by flow cytometry, did not change after addition of MS-275 (Figure 12B). However, MS-275 treatment did result in a significant increase in sFasL binding (Figure 12B). Gemcitabine (GCB) was used as a positive control, since we have previously demonstrate that GCB increases Fas at the cell surface in LM7 cells (35).

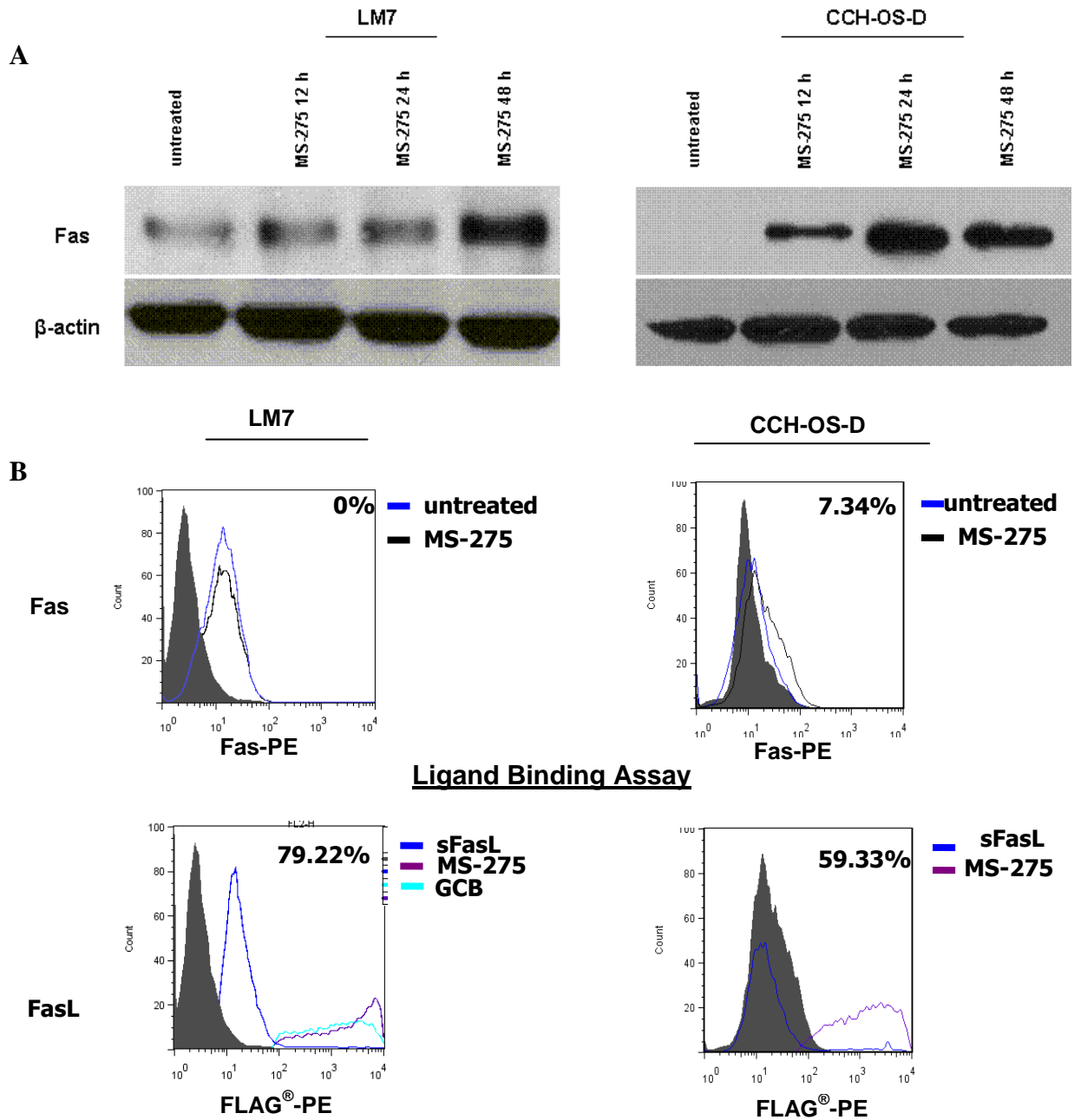


Figure 12. Fas cell surface expression and FasL binding after MS-275 treatment

(a) LM7 and CCH-OS-D cells were treated with MS-275 or control media then lysed using NP40 buffer and subjected to western blot analysis using antibodies for Fas and β -actin. (b) Cells were treated with 2 μ M MS-275 for various time-points, then analyzed by flow cytometry using Fas-PE (top panel) or FLAG-PE to detect sFasL binding (bottom panel). Gemcitabine (GCB) was used as a positive control. A representative example of three independent experiments is shown at 48 hours.

SUMMARY

Our data demonstrate that a functional Fas pathway is important for MS-275 sensitization of OS cells to sFasL. This result was demonstrated by transfecting OS cells with FDN to block formation of the DISC in the Fas signaling pathway. To further investigate the mechanism of action, we measured changes in Fas mRNA by quantitative real-time PCR after MS-275 treatment. An increase in Fas mRNA was found to increase after treatment and peaked at 24 hours. We also observed an increase in Fas protein at 48 hours. Contrary to our initial hypothesis, Fas protein was not significantly altered at the cell surface following treatment. However, treatment did increase the binding to sFasL. Based on our findings that Fas expression does not change on the cell surface after treatment, we hypothesize that MS-275 may effect the expression of a downstream mediator of the Fas signaling pathway. However, since MS-275 treatment did increase sFasL binding, the results also suggest the possibility that MS-275 may increase Fas protein expression in specific membrane compartments, which may not be detectable by flow cytometry.

RESULTS

Chapter 4

MS-275 downregulates the inhibitor of apoptosis, c-FLIP mRNA and protein

RATIONALE

Our data revealed that treatment of OS cells with MS-275 increases Fas mRNA and protein expression. However we were unable to detect increased Fas protein on the cell surface using flow cytometry. We, therefore, hypothesized that MS-275 may sensitize OS cells to FasL by effecting proteins downstream of Fas in the signaling pathway. Several chemotherapeutic agents have been shown to sensitize cells to FasL by the downregulation of inhibitors of the Fas pathway, such as bcl-2 and c-FLIP. In addition, pro-apoptotic factors, such as bax, have been demonstrated to increase following treatment with anti-cancer agents. Therefore, mRNA and protein expression of downstream mediators in the Fas pathway were analyzed following MS-275 treatment of OS cells. Both RT-PCR and western blot analysis was performed to examine bax, bcl-2 and c-FLIP mRNA and protein expression (Figure 13 and 14). Our data suggested the possibility that a mediator directly downstream of Fas may play a role in this mechanism.

RESULTS

Effect of MS-275 on mediators of the Fas signaling pathway

We previously demonstrated that in both LM7 and CCH-OS-D cells, Fas expression was unaltered after treatment with MS-275 (Figure 12A). This suggested that the mechanism by which MS-275 sensitizes OS cells to sFasL is not mediated through induction Fas expression on the cell surface but may involve mediators of the Fas/FasL signaling pathway downstream of the Fas receptor itself. To test this hypothesis, we treated OS cells with MS-275 and performed RT-PCR to examine mRNA levels of bax, bcl-2 and c-FLIP.

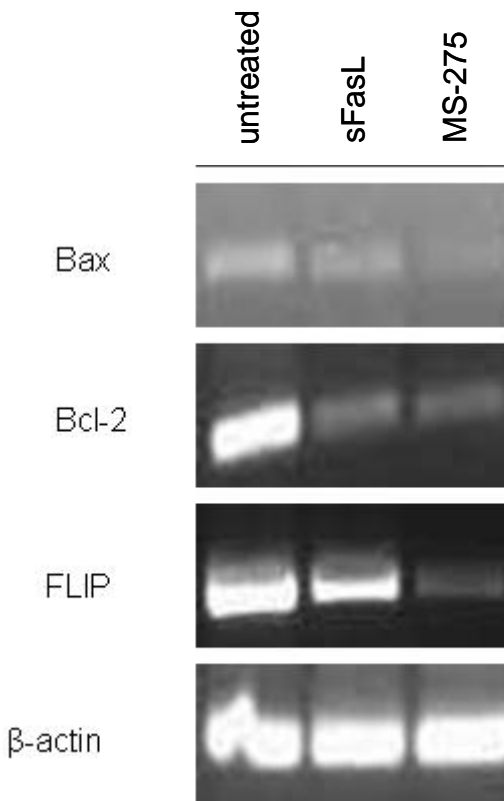


Figure 13. MS-275 decreases bcl-2 and c-FLIP mRNA. LM7 cells were treated with MS-275 for 24 hours. Following treatment, cells were collected, RNA extracted and RT-PCR was performed to examine mRNA expression of bax, bcl-2 and c-FLIP.

Our results demonstrated no change or possibly a decrease in bax mRNA (Figure 13). These intermediates were specifically chosen since MS-275 treatment has been reported to increase bax expression/activity and downregulate the expression of bcl-2 and c-FLIP in other tumor models *in vitro* and *in vivo* (106, 108-110). The expression of bcl-2 and c-FLIP mRNA was significantly decreased following treatment with MS-275 (Figure 13). Therefore, our data demonstrating downregulated bcl-2 and c-FLIP is consistent with previously published findings.

Further, we examined the effect of MS-275 on bax, bcl-2 and c-FLIP at the protein level. A series of western blots were performed in which we demonstrated a downregulation in c-FLIP protein expression following MS-275 treatment (Chapter 4). However, we observed no change in bax and bcl-2 protein in OS cells, which is contrary to what was demonstrated in other tumor cell types (Figure 14). Therefore, we decided to focus on the effect of MS-275 in downregulating c-FLIP expression.

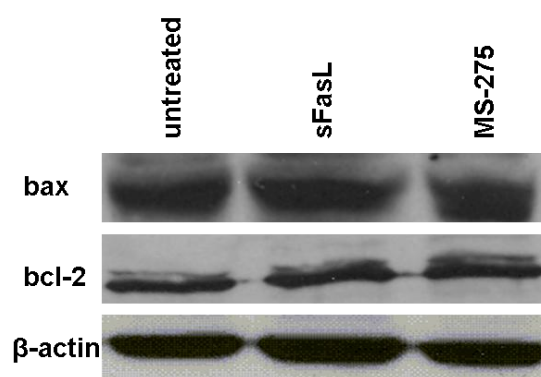


Figure 14. MS-275 does not effect the expression of bax and bcl-2 protein. LM7 cells were treated with MS-275 for 48 hours. Following treatment, cells were collected; lysed and western blot analysis was performed to examine expression of bax and bcl-2.

MS-275 decreases the expression of c-FLIP mRNA and protein

To validate our initial findings that MS-275 decreases c-FLIP mRNA, as examined by RT-PCR, we further quantified c-FLIP expression by quantitative real time-PCR and western blot analysis. Our results demonstrated that cells treated with MS-275 had decreased levels of c-FLIP mRNA and protein (Figures 15A and 15B).

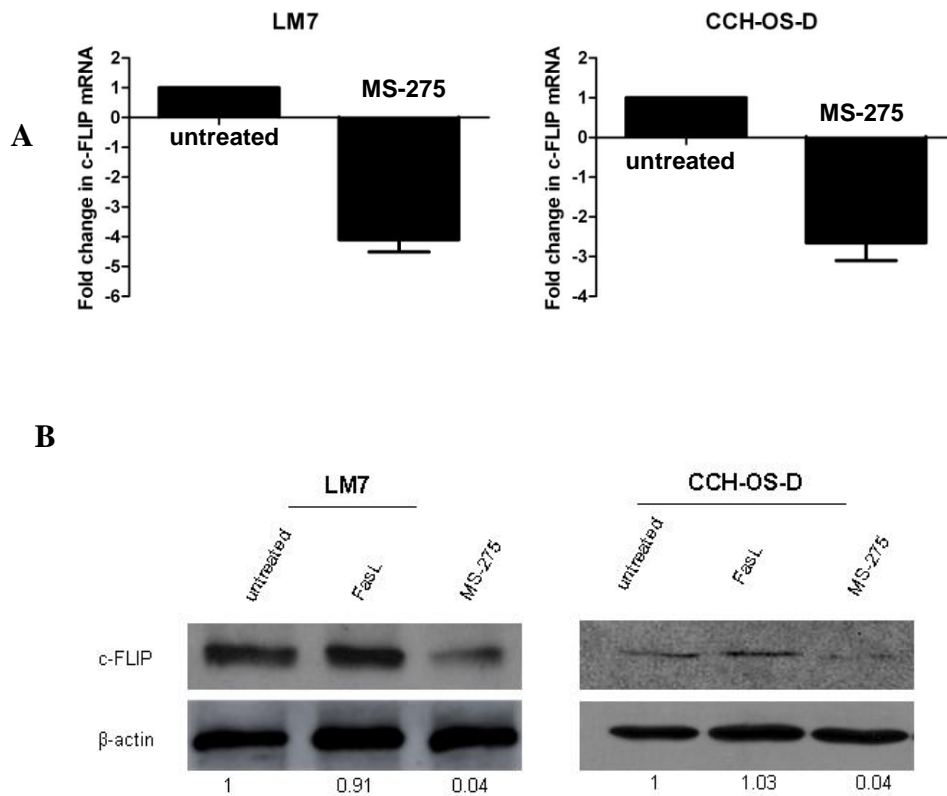


Figure 15. MS-275 downregulates c-FLIP expression in OS cells. (A) OS cells were treated with 2 μ M MS-275 for 24 hours. RNA was extracted and analyzed by quantitative real-time PCR using primers specific for c-FLIP. (B) OS cells were treated with 2 μ M MS-275 for 48 hours. Cells were lysed and analyzed by western blot using an anti-c-FLIP antibody.

SUMMARY

MS-275 treatment of OS cells resulted in the decrease of c-FLIP mRNA and protein. Inhibiting c-FLIP may lower the threshold necessary to induce Fas signaling and subsequent apoptosis in the presence of FasL. Therefore, one mechanism by which MS-275 can sensitize cells to FasL may be through the downregulation of c-FLIP. Our results are consistent with previous findings demonstrating that histone deacetylase inhibitors can downregulate c-FLIP expression in OS cells (79). However, our findings are novel as we demonstrate histone deacetylase inhibitor-induced downregulation of c-FLIP in metastatic Fas⁻ OS cells that are relatively resistant to FasL-induced cell death.

RESULTS

Chapter 5

**MS-275-induced sensitization of OS cells to FasL is mediated by the localization of Fas to
membrane lipid rafts**

RATIONALE

While the mechanism of how MS-275 sensitizes OS cells to FasL may involve the downregulation of c-FLIP expression, an explanation for our data demonstrating increased FasL binding remains unanswered. Recent studies have demonstrated the importance of membrane lipid rafts platforms in Fas receptor signaling (86-88). In fact, few groups have shown that treatment of cells with chemotherapeutic agents resulted in the redistribution of death receptors from non-rafts to rafts, which sensitized cells to ligand-dependent cell death (102). Based on our data and these previous findings, we hypothesize that MS-275 will induce the redistribution of Fas to membrane lipid rafts.

One of the most utilized methods of studying protein localization in lipid rafts is the fractionation method. Lipid rafts have been shown to be highly insoluble in detergents such as Triton-X-100 at low temperatures. When cells are lysed the plasma membrane is completely dissolved while the lipid rafts remain intact. The lysates are then subjected to sucrose gradient centrifugation to specifically extract the lipid rafts fractions from the non-raft. We used this method to isolate lipid rafts and performed western blot analysis to detect Fas, the lipid raft marker CTxB-GM1 and the non-raft marker TfR. In addition, we also utilized fluorescence microscopy to detect localization of Fas within GM1⁺-lipid rafts using a fluorophore-conjugated CTxB-GM1 antibody. Further, to identify the importance of lipid rafts in the mechanism of MS-275-induced sensitization of cells to FasL, we used the cholesterol sequestering agent methyl- β -cyclodextrin (MBC) to inhibit lipid rafts. Cytotoxicity was then assessed by performing clonogenic assays.

RESULTS

MS-275 increases the expression of Fas in lipid rafts

To determine whether there was increased Fas in the lipid rafts following MS-275 treatment, we performed a sucrose-gradient density centrifugation to extract the lipid rafts. Western blot analysis using an antibody for the lipid raft marker, CTxB-GM1, confirmed that fractions 3 to 6 contained lipid raft proteins, while fractions 7 to 9 contained non-raft proteins as determined by expression of TfR (Figure 16).

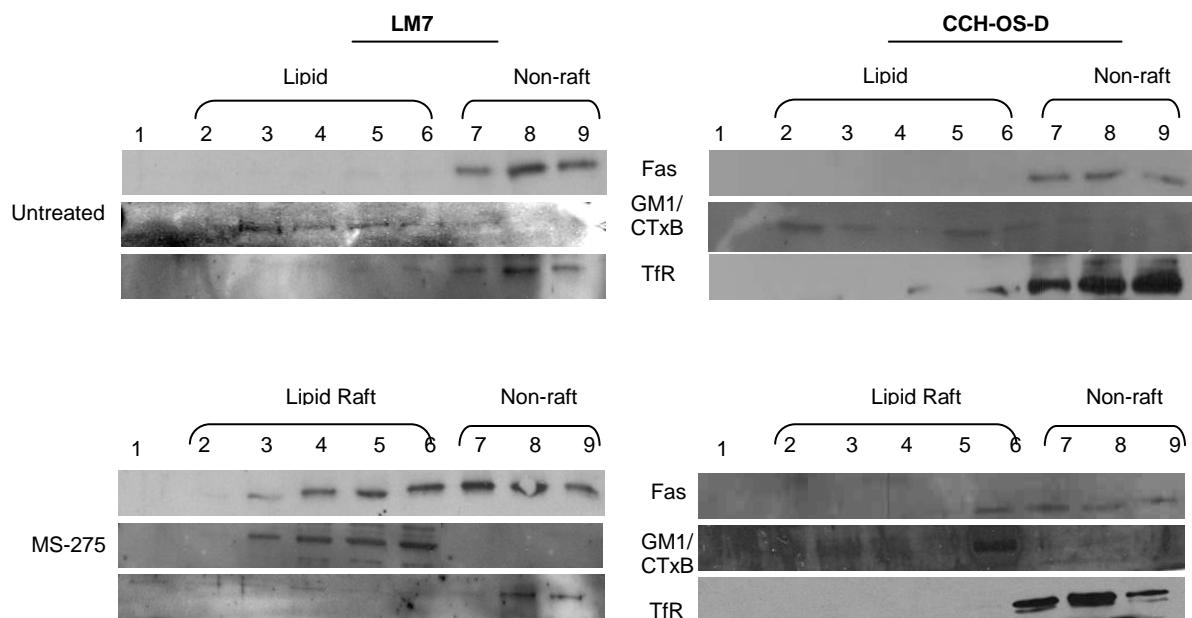


Figure 16. MS-275 increases Fas expression in GM1-positive lipid rafts. LM7 and CCH-OS-D cells were treated with 2 μ M MS-275 or control medium for 48 hours. Cells were lysed with Triton-X-100 and the lysates subjected to sucrose density gradient centrifugation. Each gradient fraction was analyzed by western blotting for Fas, CTxB-GM1 and TfR. Fractions 3 to 6 were confirmed to contain GM1⁺-lipid rafts, while fractions 7 to 9 represented proteins found in the non-raft region.

Further, increased cholesterol content was found in fractions 3 to 6, confirming presence of lipid rafts in these fractions (Figure 17). As compared to untreated control, cells treated with MS-275 displayed increased Fas in the lipid raft fractions (Figure 16). These

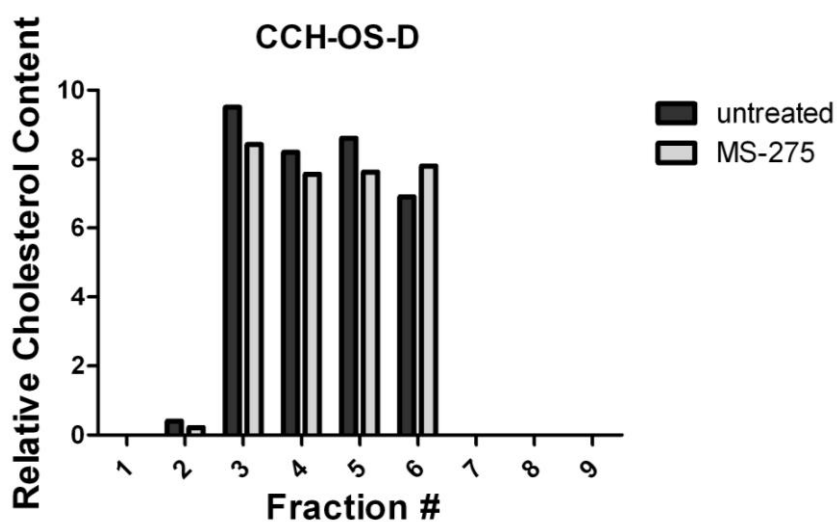
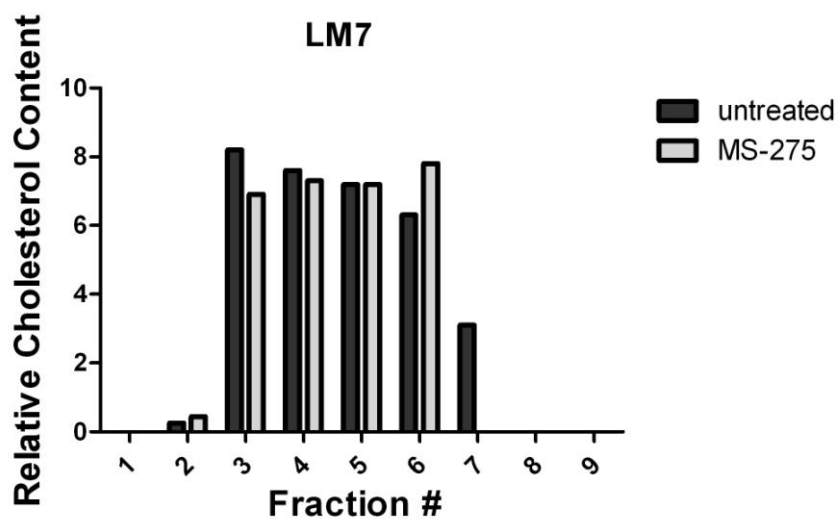


Figure 17. Confirmation of lipid raft fractionation. Amplex red assay was performed to determine cholesterol content following lipid raft fractionation. Fractions 3 to 6 were confirmed to contain higher cholesterol content.

results suggest that the increased Fas protein induced by MS-275 treatment was in lipid raft microdomains rather than the non-raft regions of the cell surface.

Treatment of cells with MS-275 increases the localization of Fas in lipid rafts

To further confirm the localization of Fas in the lipid rafts after MS-275 treatment, immunofluorescence staining of Fas and CTxB-GM1 was performed. Untreated cells had low levels of Fas expression localized outside of lipid rafts and at undetectable levels in lipid rafts. However, cells treated with MS-275 had increased colocalization of Fas within GM1⁺- lipid rafts (Figure 18), confirming our results from the fractionation studies.

Disruption of lipid rafts reduces MS-275-induced sensitization of OS cells to FasL

If localization of Fas in lipid rafts is required for MS-275-induced sensitization of cells to sFasL, then inhibiting lipid raft formation would abrogate this effect. To address this possibility, methyl- β -cyclodextrin (MBC) was used to deplete cholesterol and inhibit lipid raft formation in the cells prior to treatment with MS-275. Reduced cholesterol content was confirmed (Figure 19A). Cells were then treated with MS-275, sFasL or combination and cytotoxicity was assessed using a clonogenic assay. As anticipated, pretreatment of cells with MBC reduced cholesterol content and cytotoxicity associated with the combination of MS-275 and sFasL (Figure 19A, 19B). These results confirm our hypothesis that Fas distribution in the lipid rafts is required for MS-275 induced sensitization to sFasL.

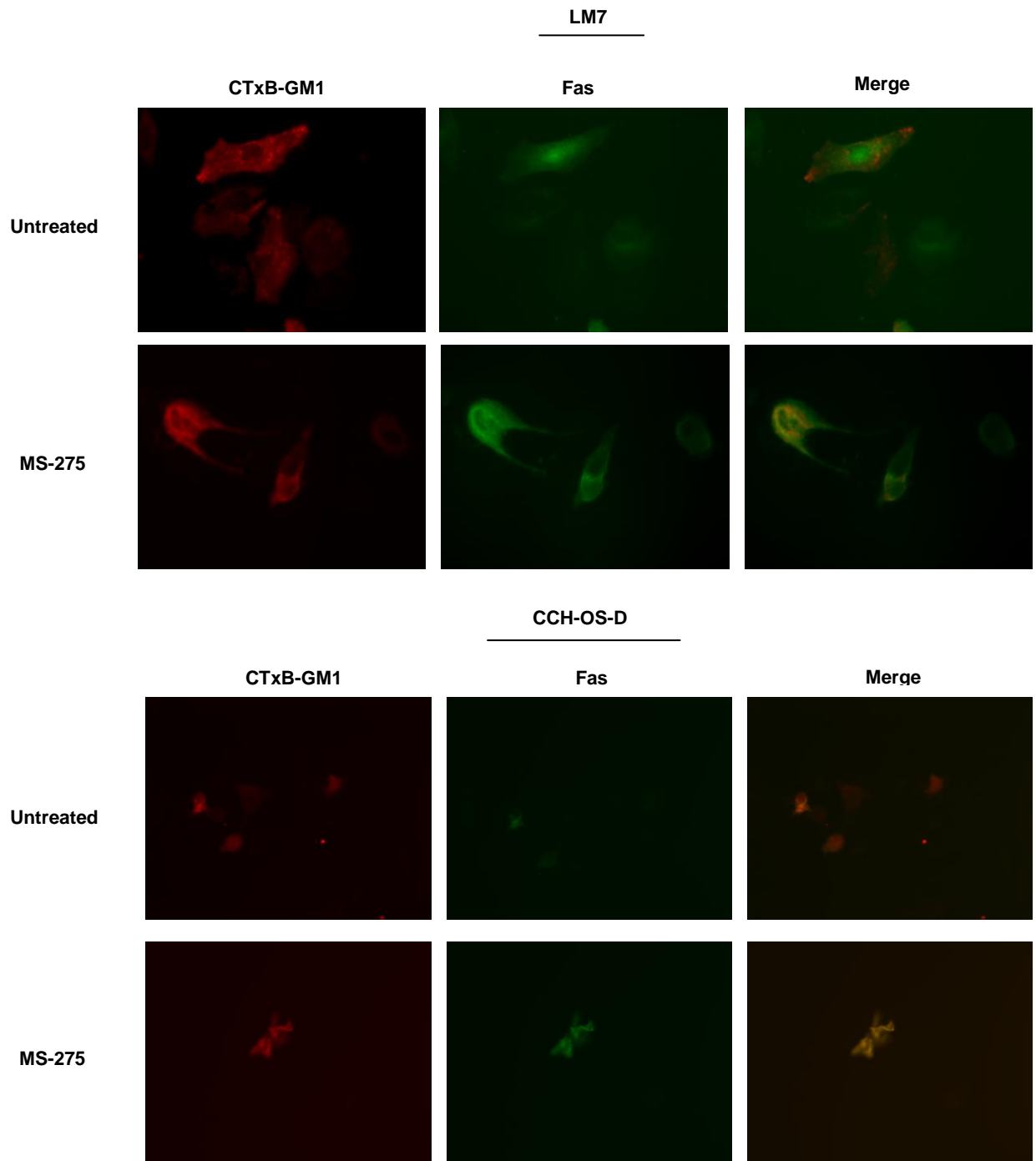
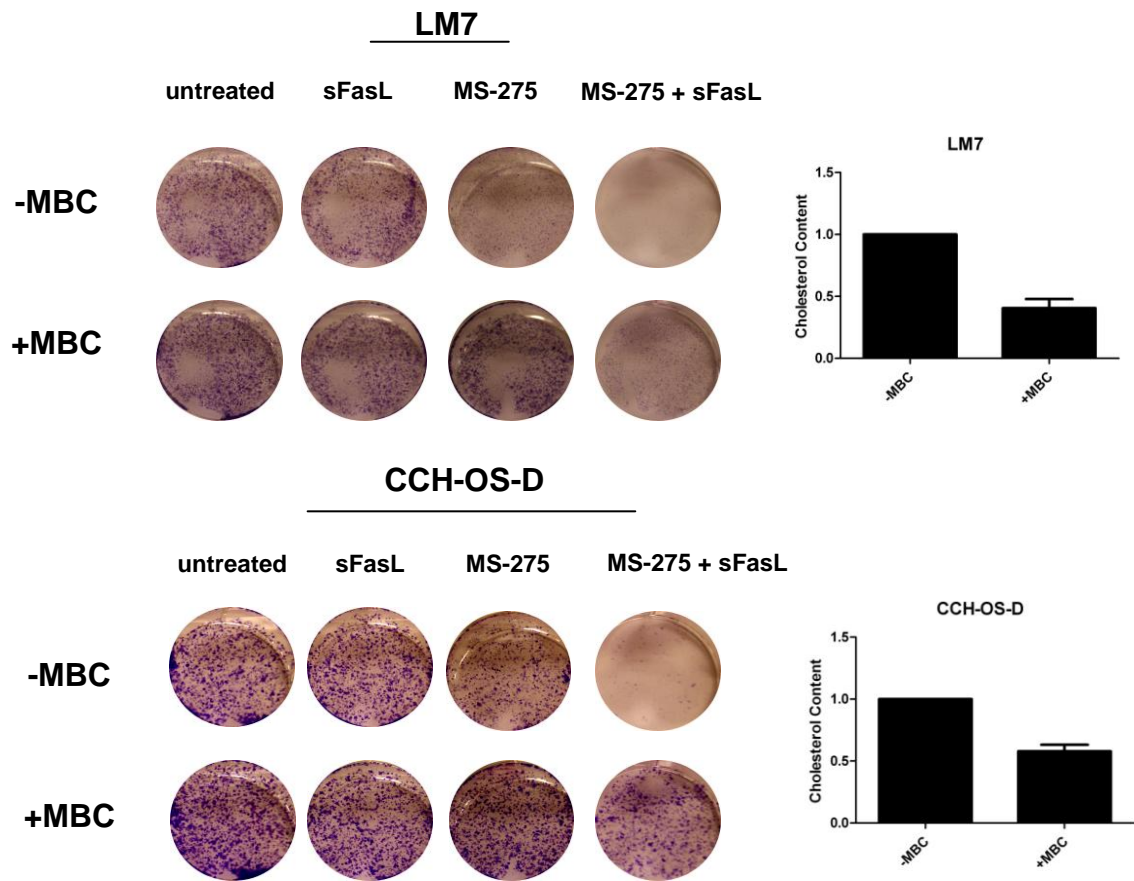


Figure 18. Fas colocalizes with GM1⁺- lipid rafts following MS-275 treatment. Cells were treated with 2 μ M MS-275 for 48 hours, fixed with 4% paraformaldehyde, and then incubated with anti-CTxB-GM1 Alexa 594 or anti-Fas followed by anti-mouse Alexa 488. Stained slides were visualized under a fluorescent microscope. Areas of yellow represent colocalization. All data are representative of three independent experiments.

A



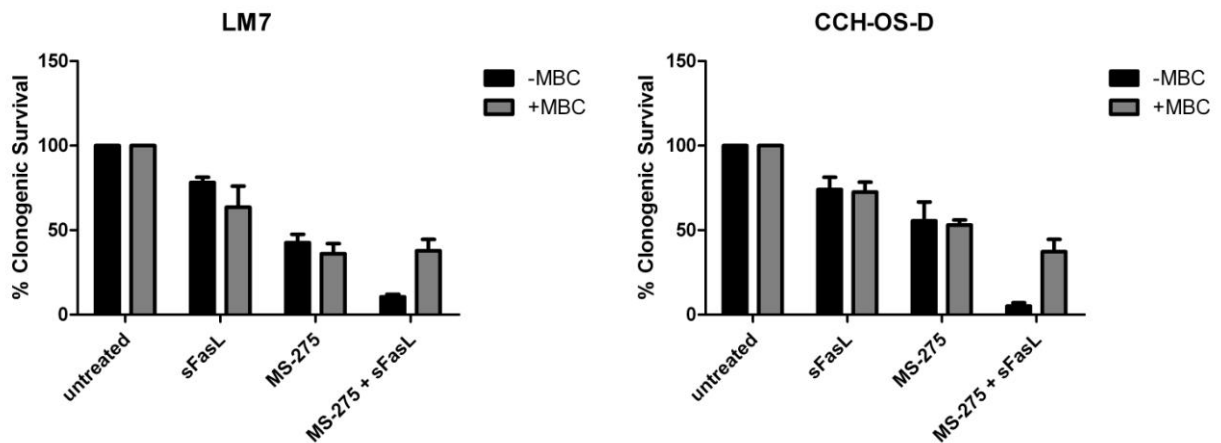
B

Figure 19. Lipid rafts are required for MS-275-induced sensitization to FasL.

MBC protects against MS-275-induced sensitization to sFasL. LM7 and CCH-OS-D cells were pretreated with 20 μ M MBC for 24 hours followed by treatment with either 2 μ M MS-275 for 48 hours, sFasL for 24 hours, or combination. (a) Cytotoxicity with or without MBC was assessed using a clonogenic assay. Cholesterol content following MBC treatment was measured using an amplex red assay. (b) Graph represents average and standard deviation of quantified number of colonies of four independent experiments.

SUMMARY

While chemotherapeutic agents have been shown to effect death receptor localization within these membranes, we are the first to demonstrate that treatment with an HDAC inhibitor results in increased localization of Fas in membrane lipid rafts. We also found that lipid rafts are important for increased susceptibility to FasL after treatment with MS-275. Following sucrose gradient centrifugation to separate raft and non-raft fractions, we observed an increase in Fas expression that was selectively localized to lipid raft fractions in cells treated with MS-275. Further, MS-275-treated cells displayed increased colocalization of Fas within GM1⁺-lipid rafts, as shown by immunofluorescence staining.

If lipid rafts are important for the mechanism of MS-275-induced sensitization of cells to FasL, then blocking lipid raft formation would result in reduced sensitivity. We, therefore, used MBC to inhibit the formation of lipid rafts and performed clonogenic assays to assess cytotoxicity. Pretreatment of cells with MBC significantly inhibited cytotoxicity following MS-275 and FasL treatment. This result underlies the importance of lipid rafts in the ability of MS-275 to sensitize OS cells to FasL.

These data together establish that MS-275 sensitizes OS cells to FasL by increasing Fas expression in lipid rafts. Our results also provide an explanation of MS-275-induced increase in FasL binding (Chapter 2), since receptors in lipid rafts are still exposed at the membrane. While our data demonstrate MS-275 increased Fas protein in these raft domains, we were not able to demonstrate enhanced redistribution of the receptor following drug exposure, as originally hypothesized. This conclusion can be made since the expression of Fas remained unchanged in the non-raft region. Rather, we observed an increase in Fas expression that was selectively localized to the raft region. We, therefore, hypothesize that MS-275 sensitizes OS to FasL by increasing the Fas mRNA and the localization of the newly synthesized Fas protein to lipid raft microdomains.

RESULTS

Chapter 6

Localization of Fas in lipid rafts is dependent on c-FLIP

RATIONALE

Our data has demonstrated that MS-275 increases the expression of Fas in membrane lipid rafts and concurrently decreases the expression of c-FLIP. While it is entirely probable that both events may occur in parallel to increase the sensitivity of cells to FasL, we investigated the possibility that lipid raft localization of Fas may be related to the decrease in c-FLIP. Song et. al. recently identified that downregulation of c-FLIP either by siRNA or treatment with chemotherapeutic agents resulted in the redistribution of TRAIL-R1 and TRAIL-R2 from non-rafts to lipid rafts. They also demonstrated that these events correlated with increased sensitivity of cells to TRAIL-induced apoptosis. We, therefore, sought to determine the relationship between c-FLIP and lipid raft localization of Fas in OS cells following MS-275 treatment.

To determine the potential association between c-FLIP and Fas localization, we downregulated the expression of c-FLIP in OS cells by transfecting cells with shFLIP. We then performed a sucrose gradient centrifugation to separate lipid raft fractions from the non-raft fractions. To confirm our findings we performed immunocytochemistry analysis to determine whether a decrease in c-FLIP would increase the colocalization of Fas within GM1⁺-lipid rafts.

RESULTS

Inhibition of c-FLIP increases the expression of Fas in lipid rafts

We previously demonstrated that MS-275 downregulates c-FLIP expression in osteosarcoma cells and in osteosarcoma lung metastases following oral administration of MS-275 (111). We therefore investigated whether the redistribution of Fas to the lipid rafts induced by MS-275 was mediated by an effect on c-FLIP. Cells with downregulated c-FLIP expression were generated by transfection of cells with shRNA to c-FLIP. Quantitative real-time PCR and western blot analysis confirmed a significant decrease in c-FLIP expression (Figure 20).

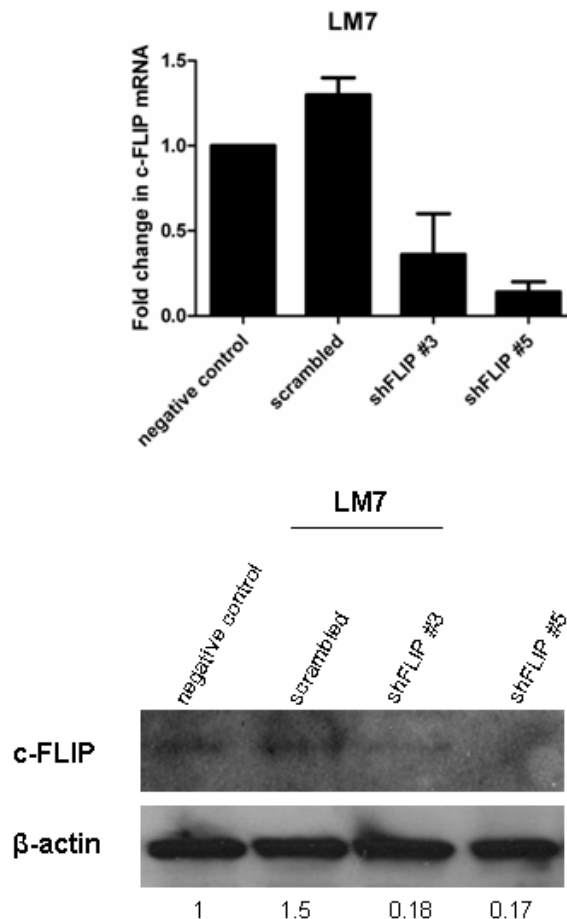


Figure 20. c-FLIP expression is inhibited in cells transfected with shFLIP. Expression of c-FLIP mRNA and protein was determined by quantitative real-time PCR and western blot, respectively in cells transfected with empty vector (negative control), sh-scrambled and shFLIP knockdown clones.

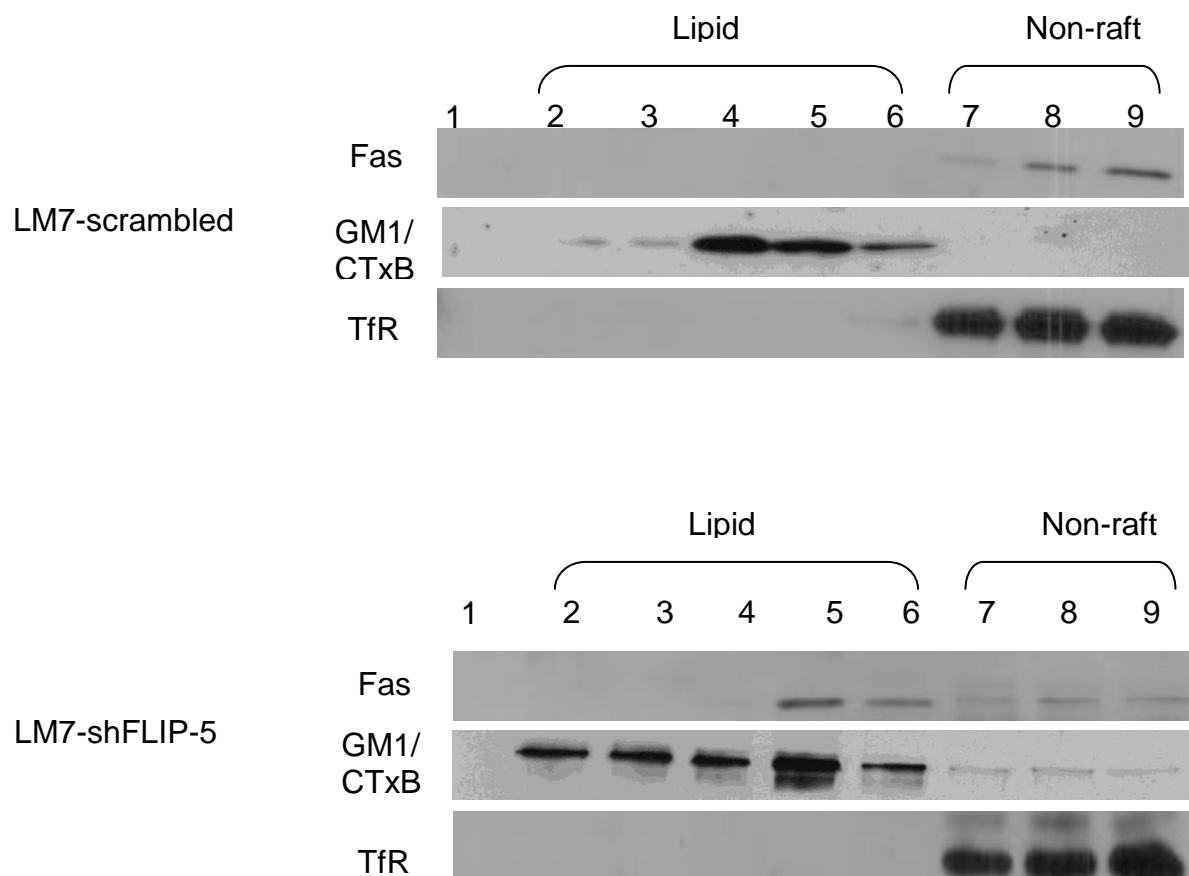


Figure 21. Inhibition of c-FLIP increases localization of Fas in lipid rafts. Cells were lysed with Triton-X-100 and the lysates subjected to sucrose density gradient centrifugation. Each gradient fraction was analyzed by western blotting for Fas, CTxB-GM1 and TfR.

Fas expression in lipid raft and non-raft fractions was then investigated. Inhibiting c-FLIP increased Fas expression in lipid raft fractions when compared to control-sh-scramble-transfected cells (Figure 21). Amplex red assay was utilized to confirm separation of lipid raft fractions (Figure 22). Further, the inhibition of c-FLIP increased the colocalization of Fas in GM1⁺-lipid rafts (Figure 23). These studies indicate that inhibition of c-FLIP can enhance the expression of Fas in lipid raft regions.

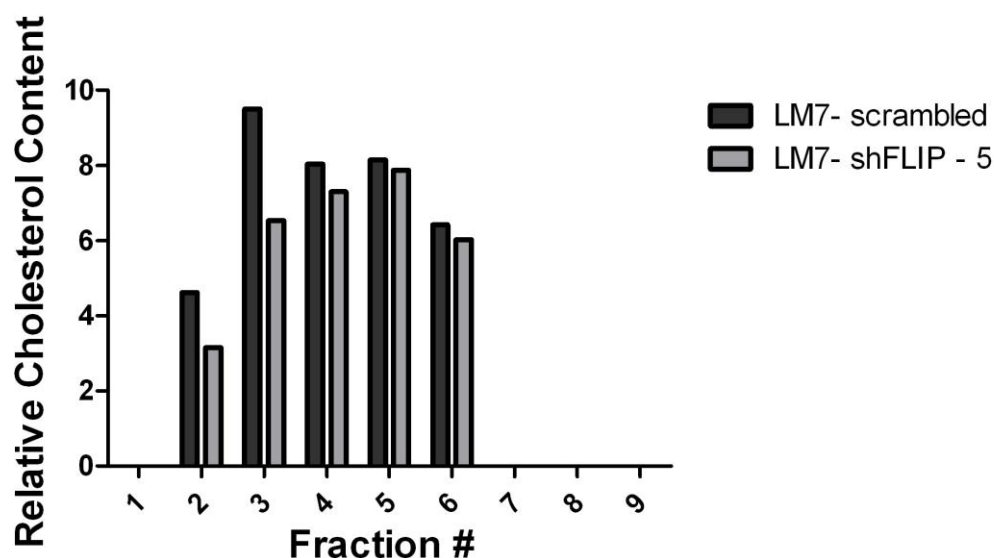


Figure 22. Confirmation of lipid raft fractionation. Amplex red assay was performed to determine cholesterol content following lipid raft fractionation in scrambled and shFLIP transfected cells. Fractions 3 to 6 were confirmed to contain higher cholesterol content.

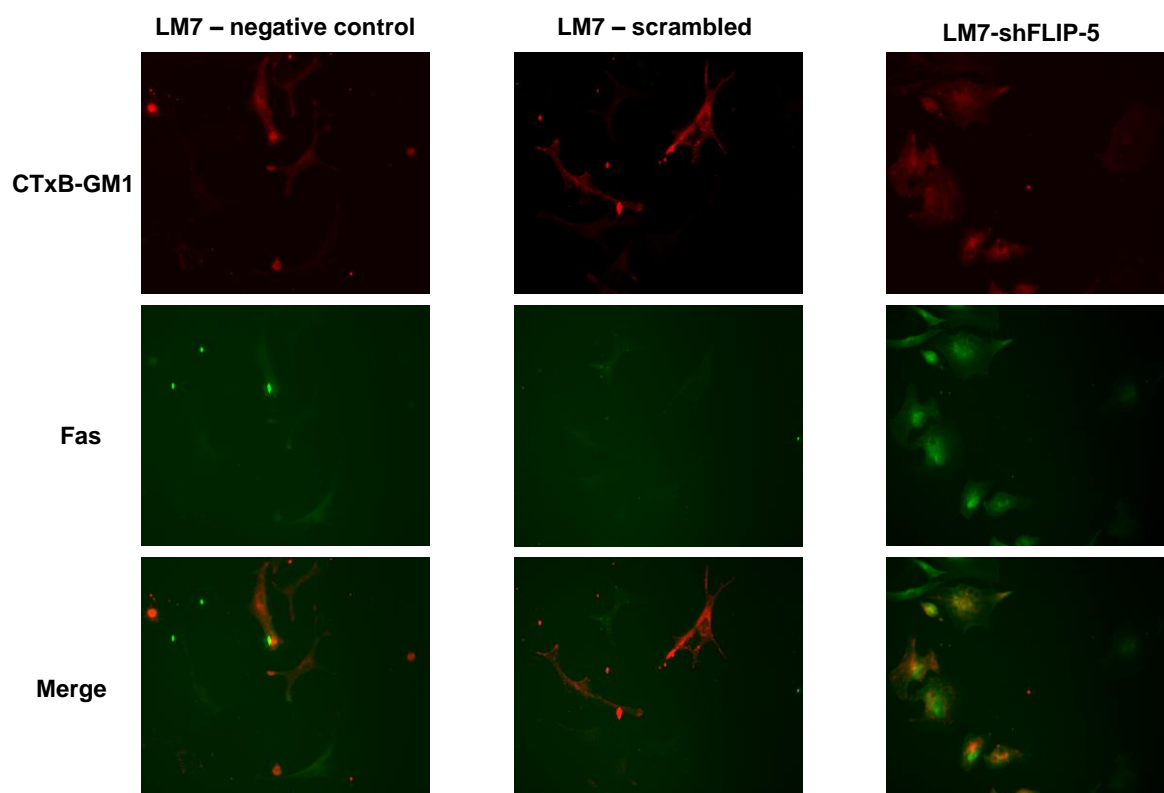


Figure 23. Inhibition of c-FLIP increases the colocalization of Fas in GM1⁺-lipid rafts. Immunofluorescence staining was performed using antibodies for anti-CTxB-GM1 Alexa 594 or anti-Fas followed by anti-mouse Alexa 488. Areas of yellow represent colocalization.

SUMMARY

In this chapter, we hypothesized that Fas localization in lipid raft microdomains may be dependent on the downregulation of c-FLIP. We propose that these effects may contribute to the mechanism of how MS-275 sensitizes OS cells to FasL-induced cell death. To test this hypothesis, we downregulated the expression of c-FLIP in OS cells using the introduction of shFLIP.

Our results confirmed our hypothesis that downregulation of c-FLIP in shFLIP-LM7 resulted in an increase in Fas expression in lipid raft microdomains as compared to sh-scramble-LM7 cells. Further shFLIP-LM7 cells had increased colocalization of Fas in GM1⁺-lipid rafts as compared to either sh-scramble-LM7 or sh-control-LM7 cells. Our findings are the first to demonstrate a correlation between c-FLIP and lipid raft localization of Fas. Additionally, we found that downregulation of c-FLIP also increased the overall expression of Fas in lipid rafts, rather than a redistribution of Fas. Considering that an increase in Fas expression in lipid raft microdomains and a decrease in c-FLIP were also observed in MS-275-treated cells, our data here suggest a correlation between these events.

RESULTS

Chapter 7

Oral administration of MS-275 in mice with osteosarcoma lung metastases inhibits tumor growth and increases survival

RATIONALE

Since our results indicated that MS-275 sensitized OS cells to FasL-induced cell death *in vitro* we investigated the effects of the oral administration of MS-275 *in vivo*. Findings in our lab have shown that chemotherapeutic agents that can upregulate Fas or activate Fas signaling can sensitize OS cells to FasL (16, 17, 33, 35, 104). The lung epithelium constitutively expresses FasL; therefore, we anticipated that MS-275 would induce tumor cell death and the regression of OS lung metastases *in vivo*.

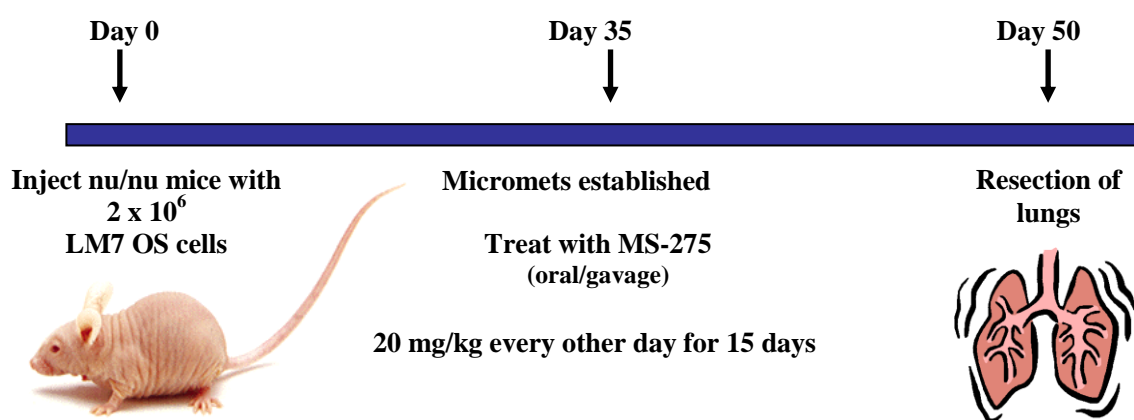


Figure 24. Schematic diagram of *in vivo* model experimental design. Nu/nu mice were injected intravenously via tail-vein with 2 x 10⁶ LM7 OS cells. Presence of pulmonary micrometastases were confirmed by H&E and treatment was initiated on day 35. MS-275 was given at a dose of 20 mg/kg every other day for 15 days by oral gavage. Mice were sacrificed and lung were resected.

We utilized our established OS pulmonary metastasis model, in which LM7 OS cells were injected by tail vein into nu/nu mice. Lung metastases were monitored and detected by sacrificing mice and analyzing resected lungs by H&E. When micrometastases were detectable, treatment was initiated. Oral administration of MS-275 at a dose of 20 mg/kg has been previously published to have antitumor activity *in vivo* (112-115). We therefore began treatment of mice with 20 mg/kg MS-275 given by oral/gavage. Following the treatment period, we sacrificed mice and resected lungs for further analysis (Figure 24).

RESULTS

Oral MS-275 enhances the accumulation of acetylated H3 and downregulates c-FLIP in OS lung metastases

To ensure that the drug was effectively distributed to the metastatic tumors in the lung, tumor sections were analyzed by immunohistochemistry for acetylated histone H3. As shown in Figure 25, tumor sections obtained from MS-275-treated mice had increased acetylated histone H3 expression.

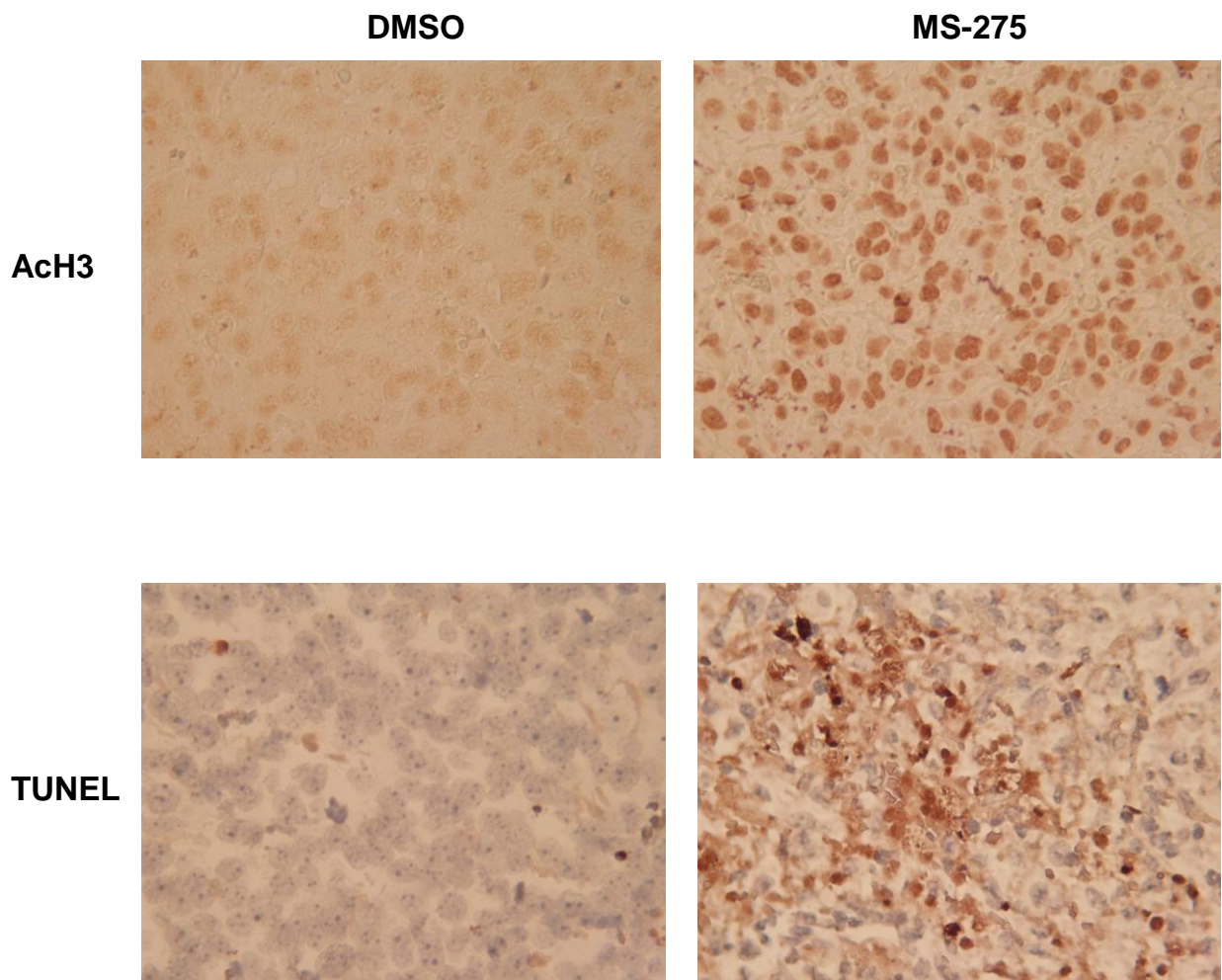


Figure 25. Oral administration of MS-275 in mice with OS lung metastases increases acetylated histones and apoptosis in tumor nodules. Mice were sacrificed, and their lungs were extracted and analyzed using immunohistochemistry. Lung tissue sections were stained with antibodies specific for acetylated histone H3 (AcH3) and TUNEL (magnification, 400x).

Tumor sections from MS-275–treated mice had significantly higher levels of TUNEL positivity and lower levels of c-FLIP expression than did DMSO-treated mice, which were consistent with our *in vitro* data (Figure 26).

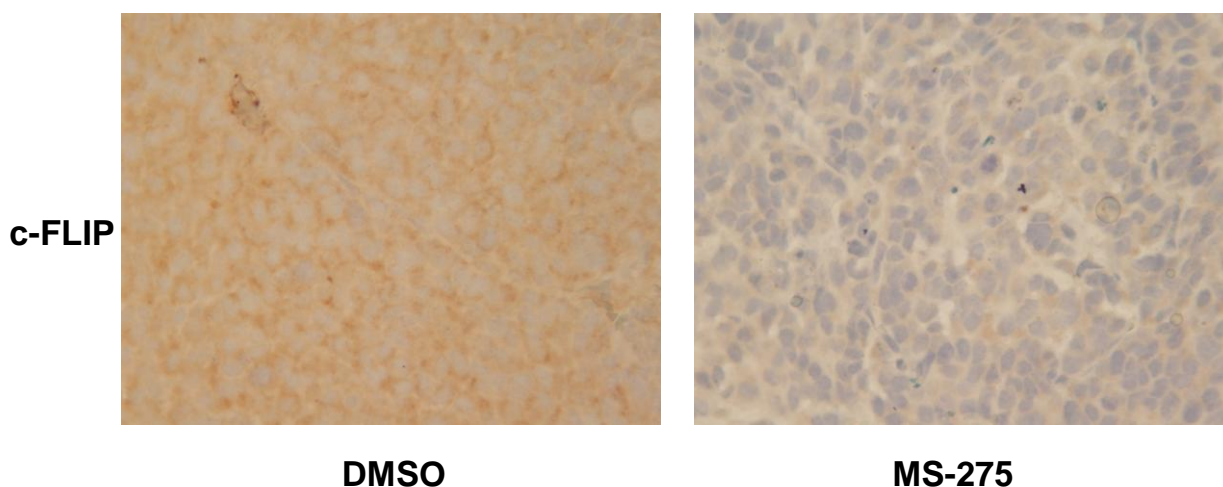


Figure 26. Effect of oral administration of MS-275 on c-FLIP expression in OS lung metastases. Immunohistochemistry of lung tissue sections were performed with an antibody specific for c-FLIP (magnification, 400x).

Toxicity of oral administration of MS-275 in mice with OS lung metastases

There was no evidence of significant organ toxicity observed following MS-275 treatment. MS-275–treated mice did experience intermittent diarrhea during treatment; however, this also occurred in the DMSO-treated mice. Examination of liver and heart tissue sections showed no evidence of irreversible cellular injury. Heart sections were entirely unremarkable. Liver sections from MS-275–treated mice had slightly larger and more metabolically active nuclei than did those from DMSO-treated mice (Figure 27). In addition, liver sections from the MS-275-treated mice showed foci of mild steatosis. These are reversible cellular changes presumed to represent drug effect. Microscopic foci of probable acute

inflammation were observed in liver sections from both treated and control mice with comparably low frequency.

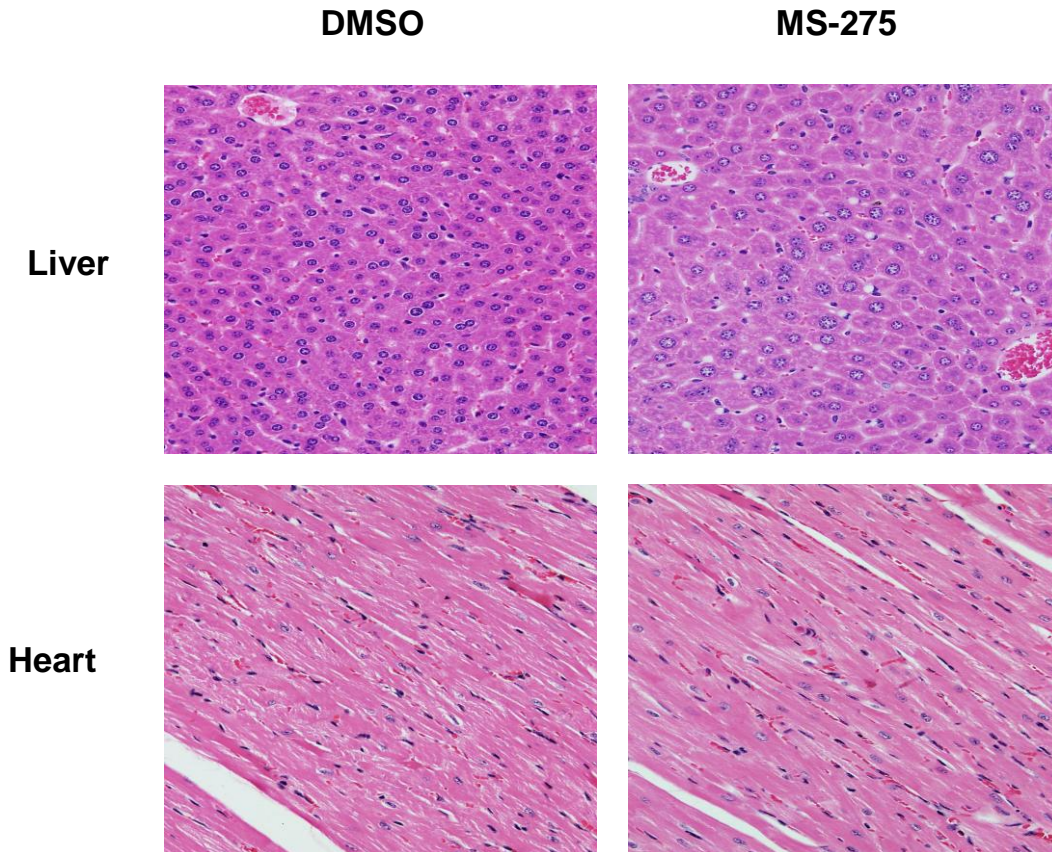


Figure 27. Examination of toxicity following treatment of mice with oral MS-275. Murine liver and heart tissue sections were analyzed using H&E staining at the end of the treatment period of 15 days.

Effect of oral administration of MS-275 on LM7 OS lung metastases *in vivo*.

As anticipated, treatment of mice with LM7 OS lung metastases with MS-275 also resulted in an inhibition of tumor growth (Figure 28). Oral administration of MS-275 significantly reduced the mean tumor surface area ($p = 0.006$), lung weight ($p < 0.05$) and number of micrometastases ($p < 0.05$) (Table 1). The mean number of visible metastases did



Figure 28. MS-275 treatment inhibits OS lung metastases. Mice with LM7 lung metastases were received oral MS-275. Mice were sacrificed at the end of therapy, and their lungs were extracted.

	DMSO	MS-275	p-value
Mean Tumor Surface Area (mm ²)	30.66 ± 1.82	11.81 ± 8.9	0.006
Mean Lung Weight (g)	0.74 ± 0.23	0.24 ± 0.02	0.04
Mean # micrometastases	15 ± 4	9 ± 2	0.04
Mean # of visible metastases	3 ± 2	2 ± 1	0.46

Table 1. Therapeutic response of mice with OS lung metastases following MS-275 treatment. Mice were sacrificed at the end of therapy, and their lungs were extracted, weighed, and assessed for tumor volume.

not differ significantly between the two groups, however. This resulted from the fact that the metastatic nodules in the control DMSO-treated group were extremely large and may have contained multiple smaller tumor nodules.

Oral MS-275 increases the overall survival of mice with OS lung metastases

Given that oral administration of MS-275 was effective in inducing tumor cell apoptosis and inhibiting the growth of OS lung metastases, we investigated whether administration of the same dose of MS-275 affected the overall survival of mice. Mice treated with 20 mg/kg MS-275 every other day had a significantly increased survival when compared to the DMSO-treated control group ($p = 0.026$) (Figure 29). In fact, MS-275 increased 50% survival from 56 days to 83 days.

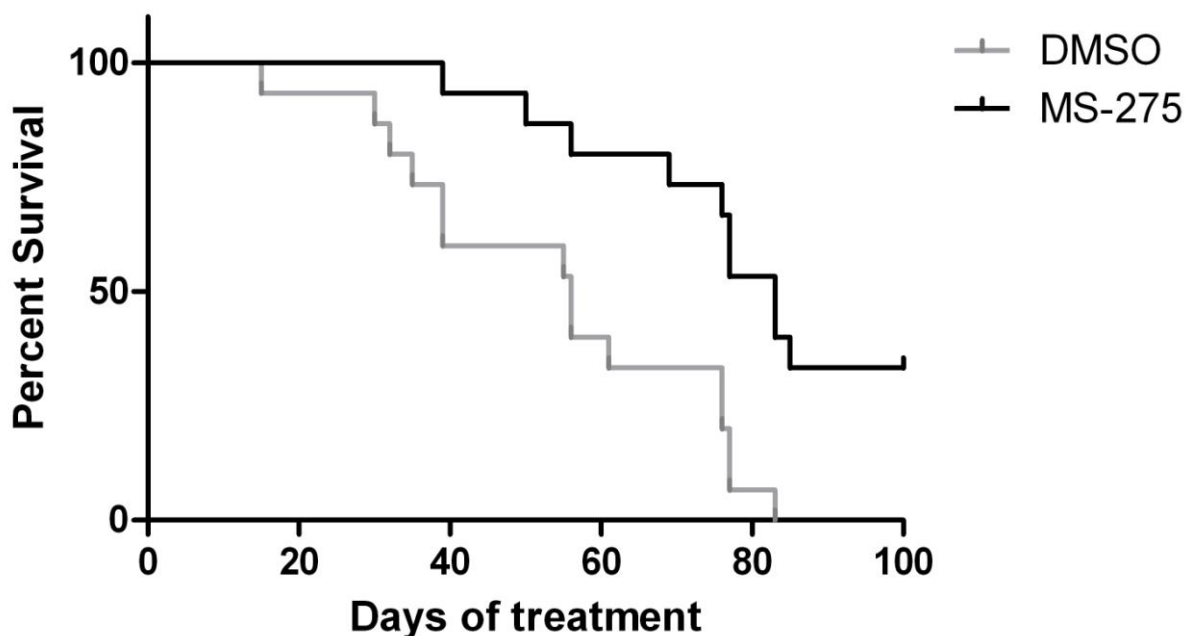


Figure 29. Oral administration of MS-275 increases overall survival of mice with OS lung metastases. Mice (15 mice per group) with LM7 lung metastases received 20 mg/kg MS-275 or DMSO every other day via oral gavage. Long term survival was assessed from the first day of treatment until death. Mice were sacrificed when they became moribund ($p = 0.002$).

SUMMARY

Resected lung tissue was subjected to immunohistochemistry analysis. Tumor tissue exhibited an increase in the accumulation of acetylated histone H3 (AcH3), confirming that MS-275 was effective in penetrating the tumor tissue and functioning as a histone deacetylase inhibitor following oral administration. In addition, c-FLIP expression levels were significantly decreased in tumor tissue following treatment, which correlates with our *in vitro* data demonstrating a downregulation of c-FLIP mRNA and protein following MS-275 treatment.

Since histone deacetylase inhibitors have been associated with cardiotoxicity (116, 117), we sought to determine if treatment of mice with MS-275 resulted in significant toxicities. Mice were sacrificed following treatment and heart and liver tissue were collected. H&E stains were reviewed with John Stewart, MD, Department of Pathology at UT MD Anderson Cancer Center. No significant differences were reported between DMSO-control treated and MS-275 treated mice in heart tissue. There was no significant hepatotoxicity associated with MS-275 treatment. MS-275 treatment resulted in hepatocytes with enlarged nuclei and open chromatin, which is likely related to the general effect of an HDAC inhibitor.

In support of our hypothesis that MS-275 would be effective in inducing the regression of OS lung metastases in mice, we observed an inhibition in the growth of tumors in the lung following treatment with oral MS-275. There was a significant decrease in average tumor surface area, lung weight and number of micrometastases following treatment. MS-275 treatment also increased the overall survival of mice. These results indicate the potential therapeutic activity of MS-275 for the treatment of OS lung metastases.

RESULTS

Chapter 8

c-FLIP is overexpressed in osteosarcoma lung metastases in human patients

RATIONALE

Our data strongly implicate a role for c-FLIP in the mechanism of MS-275-induced sensitization of OS cells and lung metastases to FasL. Considering c-FLIP has been identified as an inhibitor of Fas-mediated apoptosis, is important in drug resistance, and was downregulated by MS-275 both *in vitro* and *in vivo*, we evaluated the expression of c-FLIP in patients with OS.

c-FLIP expression has recently been associated with tumorigenesis in several types of cancers. For example, c-FLIP has been identified to be overexpressed in approximately 60% of patients in a study cohort of 86 patients with non-Hodgkin lymphoma. Expression was found to correlate with tumor progression and poor patient outcome (118). Further, a study in patients with bladder urothelial carcinomas demonstrated c-FLIP positivity in 81% of the cases and found that c-FLIP expression correlated with poor survival (28). Several studies have also demonstrated increased expression of c-FLIP in colon carcinomas, which are often resistant to Fas-mediated apoptosis (28, 119). Additionally, pharmacologic downregulation of c-FLIP has resulted in restored sensitivity to FasL-induced cell death (102). Therefore, an overwhelming amount of data suggests the importance of c-FLIP in tumor progression.

In addition, c-FLIP expression has been associated with drug resistance. One such study demonstrated the correlation of high levels of c-FLIP with resistance to chemotherapy in colon carcinoma. Simultaneous downregulation of c-FLIP-enhanced chemotherapy-induced apoptosis (120). Expression of c-FLIP has also been shown to confer resistance to anti-cancer drugs with various mechanisms of action such as doxorubicin, etoposide, cytosine arabinoside, daunorubicin, chlorambucil and cisplatin in hematological malignancies (121).

To date, c-FLIP expression has not been investigated in OS and OS lung metastasis. Therefore, we evaluated the expression of c-FLIP in human patient samples using

immunohistochemical analysis. In addition, we compared the expression of c-FLIP in primary OS tumors versus OS lung metastasis.

RESULTS

Evaluation of c-FLIP expression in patient samples

To evaluate c-FLIP expression in OS, we obtained patient samples from primary tumors and lung metastases. Immunohistochemistry analysis was performed using a c-FLIP-specific antibody. Our results demonstrate 7/9 of metastatic samples were strongly positive and 1/9 was weakly positive, while 4/4 of the primary samples were weakly positive for c-FLIP expression (Figure 30). We further quantified the expression levels of c-FLIP and found that c-FLIP expression was significantly higher in the lung metastases than in the primary tumor (Figure 31).

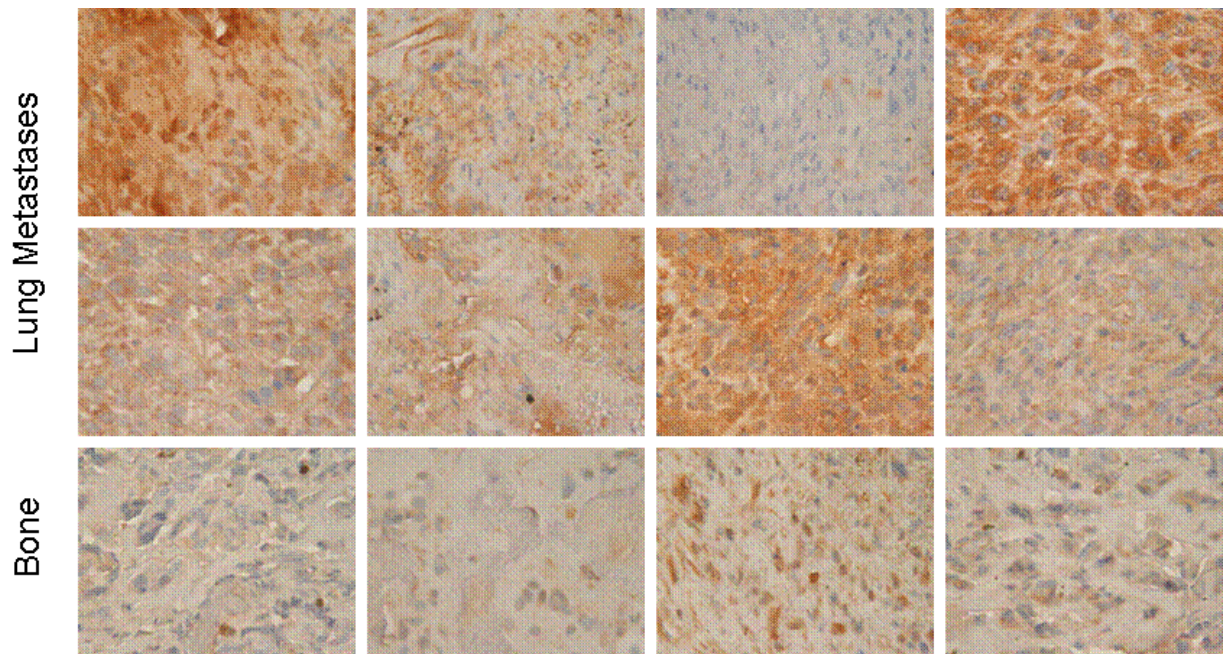


Figure 30. Evaluation of c-FLIP expression in human OS patients. Immunohistochemistry staining was performed on primary bone OS and OS lung metastases patient samples to examine c-FLIP expression.

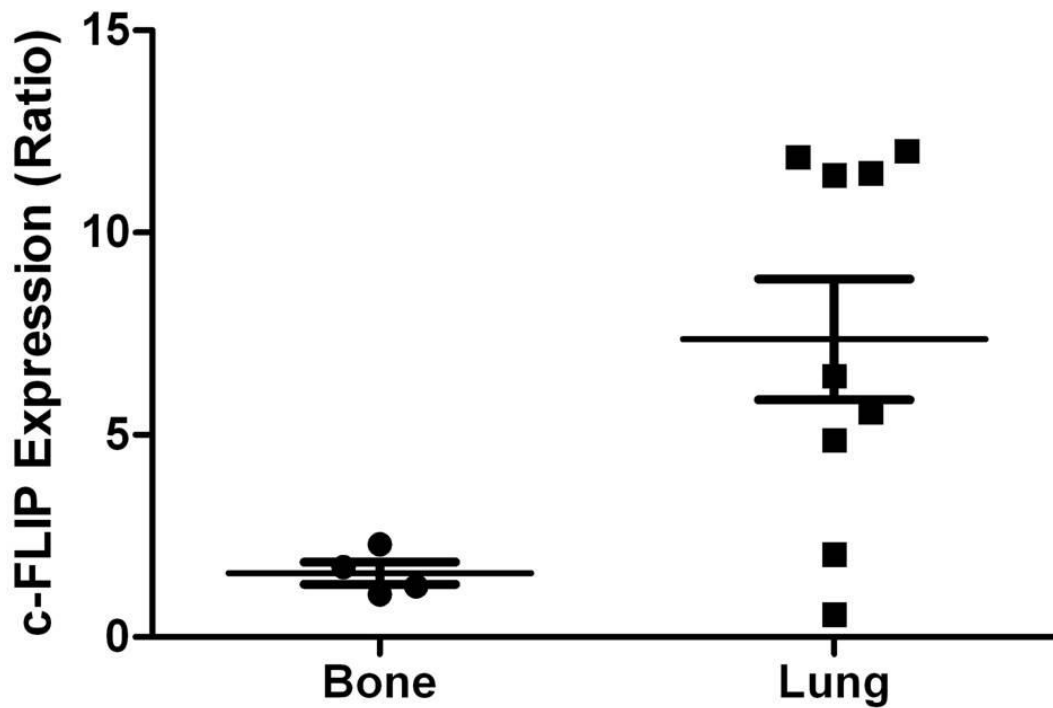


Figure 31. c-FLIP expression in OS patients. Staining for c-FLIP was quantified using SimplePCI software. Intensity of c-FLIP expression was significantly higher in pulmonary metastases than the primary tumor. Whisker bars represent standard error of the mean (p=0.0289).

SUMMARY

Our laboratory has previously published that OS lung metastases from patients were Fas⁻, while the primary tumors in the bone contained both Fas⁻ and Fas⁺ cells. These data support our overall hypothesis that the Fas/FasL pathway is important for the metastatic potential of OS. However, since the downstream mediators of the Fas pathway are also key players in the initiation of the apoptotic cascade, this raises the possibility of the contribution of other Fas-associated proteins to the metastatic potential of OS cells. Therefore, we evaluated the expression of the anti-apoptotic protein, c-FLIP, in OS patient samples.

Our findings demonstrate that OS lung metastases had significantly higher c-FLIP expression compared to the primary bone tumors. While other groups have observed high c-FLIP expression in various cancers, our study is the first to find increased expression in metastatic tumors compared to the primary site. In addition, we identify for the first time that c-FLIP expression is increased in OS patients. These results are significant because our previous data demonstrates that osteosarcoma cells that metastasize to the lung are Fas⁻ and are not sensitive to the FasL that is constitutively expressed in the lung microenvironment. The majority of patient OS lung metastases that we analyzed were Fas⁻; however there was a small population that contained both Fas⁺ and Fas⁻ cells (15). Therefore, the results presented here suggest that high c-FLIP expression may also contribute to the reduced sensitivity of OS to FasL in the lung and may also explain the presence of Fas⁺ cells.

Importantly, our data from this dissertation project demonstrate that the HDAC inhibitor, MS-275, can sensitize metastatic OS cells to FasL via a mechanism involving both the increase of Fas protein in lipid rafts and the downregulation of c-FLIP. Therefore, the evaluation of c-FLIP expression in patients may have important therapeutic implications, as this finding would justify targeting this specific protein. Therapeutic agents that can inhibit the

high expression of c-FLIP in metastatic OS cells, such as MS-275, may help increase the sensitivity to chemotherapeutic drugs and to FasL in the lung.

DISCUSSION

Chapter 9

Discussion: Implications of Results and Future Directions

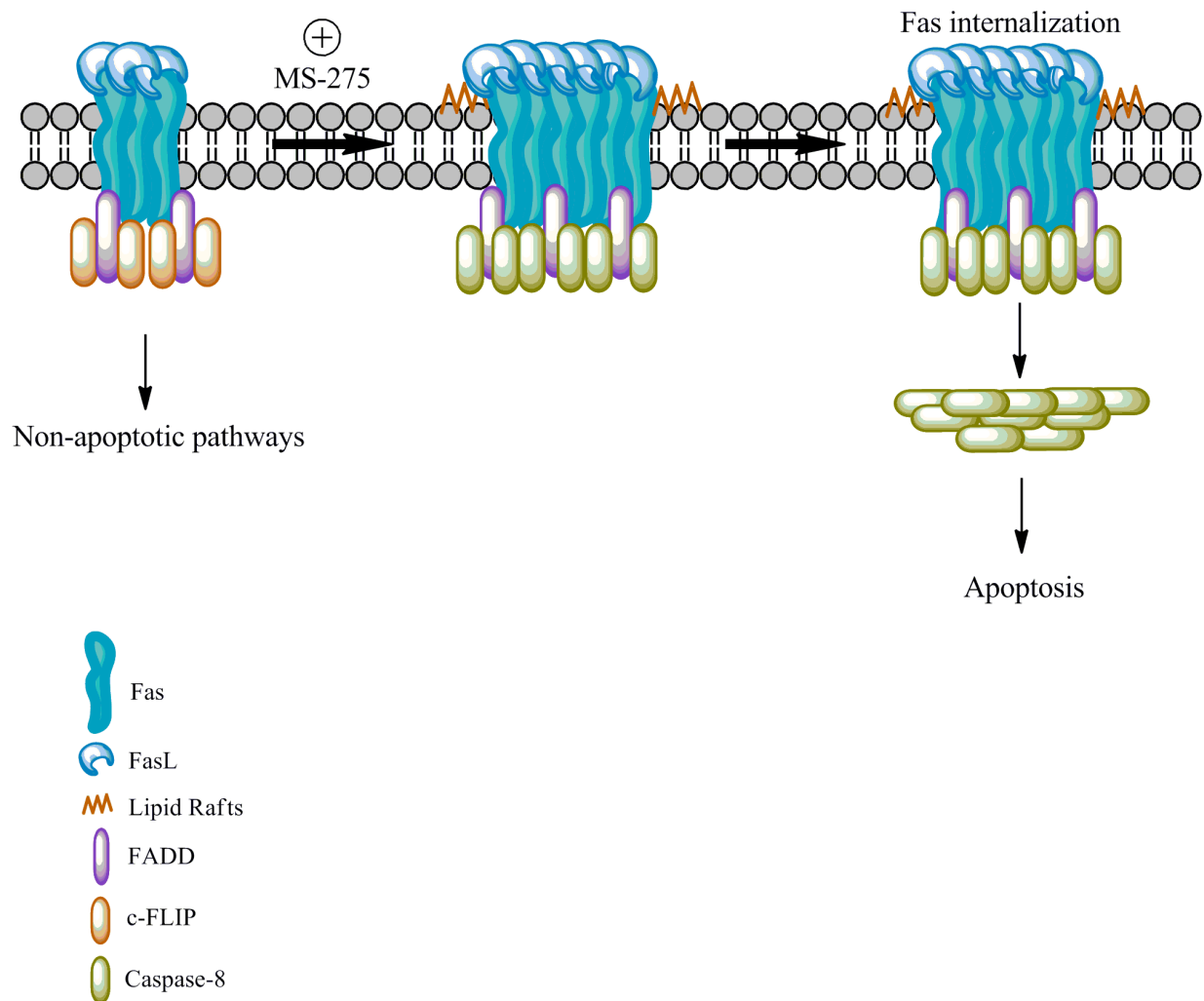


Figure 32. Proposed mechanism of MS-275-induced sensitization of OS cells to FasL-mediated apoptosis. OS cells in the lung microenvironment express low levels of Fas, high c-FLIP and are localized to the non-raft region of the cell surface. This prevents caspase-8 activation even though FasL is present. Treatment of cells with MS-275 decreases c-FLIP, increases Fas and results in the localization of Fas to lipid rafts. Since c-FLIP is inhibited, caspase-8 can readily bind to the death inducing signaling complex (DISC). The presence of FasL promotes the internalization of the DISC, which results in caspase-8 activation and apoptosis.

Accumulating evidence suggests that downregulation of Fas or corruption of the Fas signaling pathway plays a critical role in the formation of osteosarcoma lung metastases (14-16, 19, 20, 122). In contrast to primary tumors, which contain a mixed population of Fas⁺ and Fas⁻ cells, OS lung metastases are predominantly Fas⁻ (20). Our laboratory previously showed that OS lung metastases were Fas negative in both mouse models and patient samples (14, 15, 104). We also have demonstrated that Fas⁺ OS cells are rapidly cleared from the lung microenvironment by the FasL⁺ lung epithelium, leaving only Fas⁻ cells to form metastatic tumors (14, 16). In contrast, OS lung metastases in FasL-deficient mice were comprised of both Fas⁺ and Fas⁻ cells (20). These data provided the basis for our conclusion that Fas expression on OS cells and the FasL⁺ lung microenvironment play a critical role in the metastatic potential of OS. We also demonstrated that inducing the re-expression of Fas using aerosol therapy resulted in the regression of OS lung metastases (16, 33, 35, 104). There was no therapeutic effect, however, in FasL-deficient mice once again underscoring the importance of the lung microenvironment. Since the lung is one of the few organs to express FasL, this implicates the role of the lung microenvironment in the elimination of the Fas⁺ OS cells leaving only Fas⁻ cells to form metastases. We have shown that the upregulation of Fas in established Fas⁻ OS lung metastases resulted in tumor regression (32). Therefore, agents that increase Fas expression may have therapeutic potential in patients with OS lung metastases. The results presented herein extend our previous findings and indicate that the HDAC inhibitor MS-275 sensitizes OS to FasL-mediated cell death and may have potential as a therapeutic agent for the treatment of OS lung metastases.

Effect of MS-275 on the Fas signaling pathway

At subtoxic, therapeutically achievable doses, MS-275 sensitized OS cells to FasL-induced cell death *in vitro* as indicated by a reduction in clonogenic growth and increase in apoptosis (Figure 6). We also observed an increase in caspase cleavage/activity (Figure 8). Further, pretreatment of cells with the caspase inhibitor z-VAD-fmk decreased the sensitivity of cells to FasL following MS-275 treatment, suggesting that the mechanism is caspase-dependent, which is an integral component of Fas signaling (Figure 8). Blocking the Fas signaling pathway using FADD-dominant negative transfection of OS cells also inhibited the ability of MS-275 to sensitize cells to FasL-induced cell death (107) (Figure 10). Taken together, these results implicate a role for the Fas signaling pathway in the mechanism of action of MS-275. Upregulating cell surface Fas is one way to sensitize cells to FasL. Initial studies demonstrated that selective inhibition of histone deacetylase (HDAC)-1 or HDAC-3, resulted in the upregulation of Fas mRNA in OS cells (123, 124). Here we show that MS-275, an HDAC 1/3-specific histone deacetylase inhibitor, sensitized Fas⁺ OS cells to FasL-induced cell death. We also demonstrated that treatment of cells with MS-275 upregulated Fas mRNA and protein levels (Figures 11 and 12). However, we were unable to demonstrate an increase in Fas on the cell surface, as detectable by flow cytometry (107) (Figure 12). Treatment of cells with MS-275 increased the binding of sFasL, which suggested enhanced availability of Fas receptor present on the cell membrane and the possibility that Fas may be localized to specific membrane compartments (Figure 12). We also evaluated the effect of MS-275 treatment on the downstream mediators of the Fas pathway (Figures 13 and 14).

Fas localization in lipid raft microdomains is enhanced after MS-275 treatment

Much of the recent focus in death receptor signaling has been on the importance of lipid rafts as platforms for Fas receptor signaling (86-89). Although we were unable to demonstrate an increase in cell surface Fas following MS-275 treatment, we did show an increase in Fas in membrane lipid rafts (Figure 16). Our results provide the first evidence that treatment of OS cells with a histone deacetylase inhibitor results in increased localization of the Fas receptor in lipid raft microdomains. We determined this by the lysis of cells with Triton X-100, of which lipid rafts are resistant, followed by sucrose gradient centrifugation. Western blotting of isolated fractions revealed the enhanced localization of Fas within lipid raft fractions. We also demonstrated increased colocalization of Fas with GM1⁺-lipid rafts (Figure 18). These findings were supported by the observation that disruption of lipid rafts, using the cholesterol sequestering agent MBC, abrogated the ability of MS-275 to sensitize OS cells to sFasL (Figure 19). Taken together, our data suggest MS-275 increases sensitivity to FasL by increasing transcription of Fas and the localization of Fas protein in lipid rafts. However, the mechanism of how a HDAC inhibitor would alter protein localization or alteration in membrane fluidity is not well understood. Nevertheless, our data are supported by evidence demonstrating that the chemotherapeutic agent, edelfosine, results in the altered distribution of both pro-apoptotic and anti-apoptotic proteins within membrane lipid rafts (125). Further, investigations of the mechanism of edelfosine-mediated lipid raft distribution, identified alterations in cholesterol and lipid raft aggregation (126). Given that MS-275 is a nonpolar benzamide, the possibility of its interaction with hydrophobic lipid moieties in the membrane is likely.

MS-275 results in the downregulation of c-FLIP in OS cells

The inhibitor of apoptosis, c-FLIP, has been shown to regulate Fas-mediated apoptosis (22). Consistent with our findings, HDAC inhibitors such as LAQ824, LBH589, depsipeptide, valproic acid and droxinostat have been shown to decrease c-FLIP expression and increase death-receptor mediated apoptosis in leukemia, breast cancer, pancreatic cancer and hepatoma cell lines (105, 127-130). Sensitization of OS cells to Fas-dependent apoptosis by depsipeptide involved the downregulation of c-FLIP (79). Depsipeptide is a selective class I/II HDAC inhibitor (131). On the other hand, MS-275 is selective for class I HDACs, specifically HDAC 1 and 3 (132). Similar to depsipeptide, we demonstrated that MS-275 induced the downregulation of c-FLIP (Figure 15). Taken together these data support the hypothesis that the downregulation of c-FLIP may be related to class I HDAC inhibition. We report for the first time that OS cells with high metastatic potential and low Fas expression are sensitized *in vitro* to FasL-induced cell death by MS-275 with a concomitant downregulation of c-FLIP mRNA and protein. These findings are consistent with those reported above but are unique in that we demonstrated increased cell death and downregulated c-FLIP with a class I HDAC inhibitor.

The role of c-FLIP in lipid raft localization of Fas

Death-receptor induced apoptosis is inhibited by c-FLIP. Overexpression of c-FLIP can confer resistance to Fas-mediated apoptosis (22, 133-136). In addition, cells that are death receptor-ligand sensitive have been demonstrated to have DISC assembly localized to both nonrafts and lipid rafts. On the other hand, cells that are resistant to ligand display DISC assembly predominantly in the nonraft region, even after ligand stimulation (102). These findings suggest that localization of the DISC may be regulated differently in ligand-sensitive and resistant cells. We have demonstrated that MS-275 treatment results in the downregulation of c-FLIP mRNA and protein in OS cells (Figure 15). c-FLIP has recently been shown to

regulate the distribution of the death receptors, DR4 and DR5 in lipid rafts (102). We, therefore, investigated whether altering c-FLIP affected the localization of Fas in lipid rafts. Using shRNA transfection to inhibit c-FLIP expression, we demonstrated that downregulation of c-FLIP in OS cells increases Fas localization in GM1⁺-lipid rafts. This result was determined by sucrose gradient centrifugation of both sh-scrambled and shFLIP transfected LM7. We examined Fas, GM1 and TfR (control) expression in the different fractions. shFLIP-LM7 cells displayed increased Fas in GM1⁺ fractions, while sh-scrambled LM7 displayed Fas localized to the non-raft region (Figure 21).

Our data are consistent with recent data demonstrating that chemotherapy treatment resulted in an upregulation of TRAIL-R, downregulation of c-FLIP and the redistribution of the DISC in TRAIL-resistant colon carcinoma cells. However, our findings are the first to report a novel role for c-FLIP in mediating the Fas-specific distribution in lipid rafts and the mechanism by which MS-275 increases the localization of Fas to the lipid rafts and sensitizes OS cells to FasL.

The effect of oral MS-275 in mice with OS pulmonary metastases

MS-275 has exhibited preclinical activity in several tumor models, including pediatric cancers (107, 115). However, we observed the novel finding that oral administration of MS-275 in mice with established OS lung metastases resulted in increased tumor histone acetylation, tumor cell apoptosis, and tumor regression (Figures 25 and 28). MS-275-treated mice had fewer and smaller lung metastases compared with control DMSO-treated mice. Oral MS-275 resulted in decreased c-FLIP, an increase in tumor cell apoptosis and most importantly increased the overall survival in mice with established OS lung metastases (Figures 25, 26, 28 and 29). Importantly, the dose of MS-275 that produced antitumor activity did not cause significant toxic effects (Figure 27). The current clinical trials with MS-275 utilize an oral

formulation. Our data showing the activity of oral of MS-275 against established OS lung metastases indicate that this agent may have therapeutic potential for patients with relapsed OS in the lung.

In summary, our data show that oral MS-275 is effective against OS pulmonary metastases as judged by a reduction in tumor size, number of metastases and an increase in survival. MS-275 has demonstrated therapeutic activity in several preclinical models and clinical trials either as a single agent or in combination with chemotherapy (137). The dose of MS-275 used in our *in vivo* studies was comparable to the dose used in other tumor mouse models (115). Consistent with our data, the pan-HDAC inhibitor, depsipeptide, also sensitized OS cells to Fas-mediated apoptosis (79). Our data is unique however, as we show for the first time that in addition to sensitizing OS cells to FasL, a class I-specific HDAC inhibitor affected the expression of c-FLIP both *in vitro* and *in vivo* and had a *therapeutic* effect in metastatic OS. This downregulation of c-FLIP was associated with the sensitization of OS cells to FasL-induced cell death. Our findings indicate that MS-275 may have therapeutic potential for patients with OS lung metastases and also suggest c-FLIP as a possible new therapeutic target.

c-FLIP expression in patients with OS pulmonary metastases

Despite advances in chemotherapy and surgery, patients with OS pulmonary metastases have very poor survival. The 5-year survival rate for patients with lung metastases is less than 20%. Further, the majority of deaths associated with OS are due to the presence of metastatic disease (138). Therefore, understanding the biology behind OS lung metastases is important in the development of novel therapeutic strategies for these patients. Investigating the mechanism of metastases may also uncover methods of drug resistance, which is critical in controlling OS pulmonary metastases.

Work in our laboratory has demonstrated the importance of the Fas/FasL pathway in the metastatic potential of OS. In fact, we previously found that the majority of metastatic tumors in the lung from patients were Fas⁻, while the primary tumor in the bone contained a mixed population of Fas⁻ and Fas⁺ cells. While enhanced Fas expression in tumors has been associated with sensitivity to chemotherapy, some studies have shown that the presence of Fas is not always synonymous with Fas pathway functionality (139, 140). Alterations in the pathway such as downregulation of caspases and upregulation of the anti-apoptotic proteins bcl-2 and c-FLIP have been reported to correlate with resistance to apoptosis (22, 141, 142).

A large body of evidence indicates that c-FLIP is overexpressed in many human cancers. Fas pathway resistance and increased c-FLIP expression has been demonstrated in non-hodgkins lymphoma, bladder urothelial carcinoma and colorectal carcinoma (28, 118, 120). Further, c-FLIP has been shown to be linked with tumor progression, metastases and resistance to chemotherapy (120, 143, 144).

While loss of Fas seems to be clearly associated with OS pulmonary metastases, the role of c-FLIP in metastases has not yet been investigated. The findings in this dissertation project demonstrate a role for c-FLIP in the mechanism of MS-275-induced sensitization of metastatic OS to FasL-induced apoptosis. Further, we found that oral administration of MS-275 in mice with OS lung metastases decreased c-FLIP in metastatic nodules, which correlated with tumor regression. Therefore, c-FLIP may be attractive candidate for therapeutic intervention. In addition, since the Fas/FasL pathway has been shown to be important in OS lung metastases, c-FLIP may be a key regulator in tumor progression. Thus, we evaluated the expression of c-FLIP in patients with OS.

In this study, we obtained 4 samples from primary bone tumors and 9 samples from metastatic sites. Immunohistochemistry analysis was performed on all samples to detect c-FLIP expression. Interestingly, all bone tumors were weakly positive for c-FLIP and 8/9 lung tumors

were strongly positive for c-FLIP (Figure 30). Further, c-FLIP expression was found to be significantly higher in lung metastases than in the primary tumor (Figure 31). Overall, our data along with our previously published findings indicate that OS lung metastases display downregulated Fas along with an upregulation in c-FLIP. These findings correlate with studies in gastric and bladder cancer in which samples exhibited low Fas and high c-FLIP. The results found in this study demonstrate for the first time that c-FLIP is frequently overexpressed in lung metastases as compared to primary bone tumors. This result suggests that downregulation of c-FLIP may contribute to the ability of OS cells to evade FasL in the lung microenvironment. However, the mechanism of how c-FLIP is upregulated in OS remains to be answered. One possibility may involve the clearance of OS cells with low c-FLIP expression in the lung following Fas/FasL interaction. There is also the likelihood that c-FLIP is upregulated during metastasis from the primary site, leaving only c-FLIP-positive cells to form metastases. Studies investigating this process are necessary in understanding the biology of OS and pulmonary metastasis.

These studies also implicate c-FLIP as a biomarker to predict the effectiveness of therapeutic agents that act by the downregulation of c-FLIP expression. Further studies are necessary to explore whether the mechanism of action of other investigational agents, in addition to MS-275, may involve the downregulation of c-FLIP. Since we have found in our laboratory that treatment of mice with OS lung metastases with IL-12, gemcitabine and 9-nitrocamthothecin result in tumor regression, studies identifying whether these agents may also function by downregulating c-FLIP are warranted.

Conclusion

There is an ongoing need for novel therapeutic strategies for patients with osteosarcoma lung metastases. Overall, we have demonstrated that the histone deacetylase inhibitor, MS-275, sensitizes osteosarcoma cells to FasL-induced cell death by increasing the expression of Fas in lipid raft microdomains, which correlated with c-FLIP downregulation. Oral MS-275 administered to mice with OS lung metastases resulted in decreased c-FLIP in the tumor nodules and tumor regression (111). Thus, modulating Fas distribution in lipid rafts and inhibiting c-FLIP, may be novel therapeutic strategies for treating OS lung metastases. Upregulating the expression of Fas in the lipid rafts may induce tumor susceptibility to FasL. Downregulating c-FLIP may also enhance sensitivity to chemotherapeutic agents and to the FasL⁺ lung microenvironment. This is an example of how the tumor microenvironment can be harnessed to eradicate metastatic cells. Therapeutic strategies aimed at altering tumor Fas expression may therefore play a role in the treatment of metastatic OS in the lungs.

FUTURE DIRECTIONS

Determine the molecular mechanism of MS-275-mediated downregulation of c-FLIP

We have demonstrated that treatment of OS cells with MS-275 decreases the expression of c-FLIP. We also found that this downregulation of c-FLIP correlates with increased Fas in lipid rafts. However, how MS-275 results in the decreased expression of c-FLIP is not well understood. Although HDAC inhibitors generally increase transcription of genes, there are various mechanisms of how a HDAC inhibitor can result in downregulation of genes.

In chapter 4, we demonstrated that MS-275 decreases c-FLIP protein expression. Therefore, one possibility is that MS-275 increases the degradation of c-FLIP protein. To test this, we would pretreat cells with the inhibitor of protein biosynthesis, cyclohexamide, followed by treatment with MS-275. We would expect cyclohexamide to downregulate c-FLIP protein, which would be measured by western blotting. To assess the effect of MS-275, we would treat cells with inhibited protein synthesis with MS-275 and measure any change in the protein degradation rate over time. If there is no change in protein half-life even after MS-275 treatment, the mechanism of downregulation may not involve a change in degradation. Similarly, MS-275 may reduce the synthesis of c-FLIP protein. To test this possibility, we could utilize the well-characterized pulse-chase assay. Briefly, we could radioactively label OS cells with [³⁵S]methionine and measure its incorporation over time. A decrease in [³⁵S]methionine, using autoradiography, following MS-275 treatment, would equate to a reduction de novo synthesis of c-FLIP protein.

However, since we demonstrated that MS-275 treatment resulted in a decrease in c-FLIP mRNA, it is more likely that the mechanism may involve a direct effect on RNA. Thus, MS-275 may either induce the degradation or reduce the synthesis of RNA. To test, mRNA degradation we would pretreat cells with actinomycin D, which inhibits transcription. Northern

blot analysis would reveal changes in c-FLIP RNA levels over time. If treatment with MS-275 in actinomycin D-treated cells further decreases the half-life of RNA, then the mechanism may involve RNA degradation. On the other hand, the mechanism of MS-275 may include a reduction in the synthesis of c-FLIP mRNA. To determine this, a nuclear run-assay using radioactively labeled nucleotides and slot blot analysis to measure transcription of c-FLIP. If addition of MS-275 decreases the incorporation of radioactively labeled nucleotide, then the mechanism may involve a decrease in de novo c-FLIP mRNA synthesis.

While the mechanism of how HDAC inhibitors can decrease transcription of genes is not well understood, a study by one group has demonstrated that the HDAC inhibitors depsipeptide, Trichostatin A and sodium butyrate downregulate c-FLIP mRNA by a decrease in mRNA synthesis (79). Although these compounds used are pan-HDAC inhibitors, the data still suggest that the mechanism of the class I-HDAC inhibitor MS-275 may involve a reduction in de novo mRNA synthesis of c-FLIP as well.

Mechanism of Fas upregulation following treatment with MS-275

Our data also demonstrate that concurrently with c-FLIP inhibition, Fas expression also increases following treatment of OS cells with MS-275. However, the mechanism of how MS-275 treatment results in the upregulation of Fas mRNA and protein is not well understood. Similar to c-FLIP, MS-275 may result in the increased stability or increased de novo synthesis of protein or mRNA. Since we observed an increase in Fas mRNA, we would expect that MS-275 may increase either RNA stability or synthesis. The same experiments used to study the mechanism of c-FLIP downregulation may be employed to answer this question.

Since treatment of cells with HDAC inhibitors generally result in increased gene expression, it is possible that targeting specific HDACs, with MS-275, may enhance transcription factor binding to the Fas promoter, thus increasing the transcription of Fas. Recent

studies demonstrated that specific inhibition of HDAC1 and HDAC3 using siRNA resulted in increased Fas mRNA in an OS cell line. Since MS-275 has specificity for HDAC1/3, it is possible that selective inhibition of these HDACs may explain the induction of Fas mRNA. To determine the importance of specific HDAC inhibition in the upregulation of Fas mRNA, we could selectively inhibit HDAC1, HDAC3 and a combination of both using si/shRNA.

Current work in our laboratory is focusing on further understanding this mechanism. Using 6 different Fas promoter deletion constructs, we have demonstrated using a luciferase reporter assay, that there exist two deletion regions that exhibited a 5-fold decrease in luciferase activity following MS-275 treatment (unpublished data, Dr. Thomas Yang). These data suggests two transcription factor binding sites within the Fas promoter that may be important for the upregulation of Fas mRNA following MS-275 treatment. A future experiment would be to identify the specific transcription factors that bind specifically to these sites using a ChIP assay. Further, once transcription factors are identified, a transcription factor-specific luciferase reporter assay (Affymetrix Inc., Santa Clara, CA) can be employed to verify results from ChIP and to measure binding activity of the specific promoters.

Determine mechanism of how MS-275 results in increased localization of Fas in lipid rafts

A recent study reports palmitoylation of Fas is essential for redistribution of Fas into lipid rafts. Cells with palmitoylation-deficient Fas were relatively resistant to Fas-mediated cell death. Further, they found that Fas internalization, a necessary step in the induction of Fas-mediated apoptosis, took place in lipid rafts and Fas palmitoylation was necessary for initiation of this process (87). Protein palmitoylation is a post-translational modification where addition of a covalent lipid moiety directs proteins to lipid raft platforms in the membrane. Interestingly, unlike the other forms of protein lipidation such as prenylation and myristoylation,

palmitoylation is a reversible process. This suggests that palmitoylation of Fas may regulate localization in and out of lipid rafts and non-rafts and may be altered by therapeutic agents.

Therefore, to validate our findings that MS-275 treatment results in the increased localization of Fas in lipid rafts, we can examine the effects of MS-275 on palmitoylation. A novel proteomic approach, known as acyl-biotin exchange (ABE) chemistry, can be utilized to analyze whether palmitoylation of Fas is enhanced after treatment (145). Since the specific residue at which Fas is palmitoylated has been identified, using a site directed mutagenesis approach; we could transfect OS cells with a palmitoylation-deficient mutant. These cells could be treated with MS-275 to determine the importance of palmitoylation and lipid raft distribution in the effect of MS-275. However, the limitation to the approach is that transfecting Fas into a low Fas expressing cell line may itself alter the response to MS-275. Determining cause and effect would be complicated in this approach. Therefore, an alternative approach would be to utilize a pharmacological approach using the palmitate analogs 13-oxypalmitate and 2-Bromo-palmitate to inhibit palmitoylation. If palmitoylation of Fas is necessary for lipid raft distribution and sensitivity, then sensitivity of cells to FasL following MS-275 treatment would be abrogated. In addition, this effect would correlate with the localization of Fas in non-raft regions.

The molecular mechanism of how MS-275 can effect lipid raft localization of proteins is another question that remains to be answered. The possibility exists that HDAC inhibitors could increase overall palmitoylation of proteins by increasing the expression of palmitoyl transferases. This can be investigated by treating cells with MS-275 followed by western blot analysis for palmitoyl transferase expression. No studies, thus far, have investigated the possibility of this mechanism so further studies are merited. Conversely, since HDAC inhibitors increase the acetylation of proteins, it is possible that acetylation of Fas may play a role in directing it to membrane to lipid rafts. To determine this, Fas can be

immunoprecipitated following treatment with MS-275 and analyzed by western blotting for acetylation using an anti-acetylated lysine antibody (Cell Signaling Technology, Danvers, MA).

There has been a growing interest in studying the modulation of protein localization in lipid rafts. Edelfosine was the first anti-cancer agent that was shown to induce apoptosis by the redistribution of proteins to membrane lipid rafts (99). Further investigation into the mechanism of action suggested that edelfosine has a high-affinity for cholesterol. Since lipid rafts are cholesterol-rich membranes, edelfosine accumulates in lipid rafts and alters the proteins that localize there. Edelfosine has been shown to induce the concentration of Fas and other Fas signaling molecules in lipid rafts in leukemic cells, which correlates with induction of apoptosis (90). Given that MS-275 is a nonpolar benzamide compound, this raises the possibility that it may interact with lipid moieties and alter membrane structure. Further, MS-275 could increase the levels of cholesterol in the membrane, which would promote the localization of Fas to in lipid rafts. To determine this, we could fractionate the plasma membrane using a membrane protein extraction kit (Thermo Fisher Scientific, Waltham, MA) and analyze cholesterol content by an amplex red assay. Further, our data using MBC to inhibit lipid raft formation (Chapter 5) strongly supports this hypothesis. We demonstrated that pretreatment of cells with MBC reduced the effects of MS-275-induced sensitization to FasL. Since MBC functions by inhibiting cholesterol synthesis, this suggests the mechanism of MS-275 may involve alterations in cholesterol. However, alterations in membrane structure following MS-275 treatment may not only be restricted to changes in cholesterol, but may effect the expression of other lipid raft associated proteins. Thus, understanding these effects merits further investigation.

Implications of c-FLIP overexpression

Our data demonstrate that c-FLIP may play an important role in the mechanism of MS-275-induced sensitization of OS cells to FasL. We showed that a decrease in c-FLIP expression with shRNA mirrored the effect of MS-275 in increasing Fas expression in membrane lipid rafts. Therefore, to validate our proposed role for c-FLIP we could investigate the effects of introducing c-FLIP overexpression in a cell line that expresses low levels of c-FLIP. First, we would have to determine whether Fas is localized to lipid rafts in c-FLIP low expressing cells. Next, we would identify if reexpression of c-FLIP induces the translocation of Fas from lipid rafts to non-rafts. We would expect, based on our results, that overexpression of c-FLIP would prevent localization of Fas in membrane lipid rafts. We could also examine the expression of Fas mRNA in c-FLIP overexpressing cell lines. Since we demonstrated that decreasing c-FLIP with shRNA induced the expression of Fas mRNA (Figure A7), we would expect that the expression of Fas would be lower in cells with increased c-FLIP.

Further, several groups have found that Fas localization and signaling in non-rafts is associated with activation of NF- κ B and ERK1/2 pathways, which are associated with increased proliferation. In fact, studies with the death receptors, found that knockdown of c-FLIP in cells switched the signal from both FasL and TRAIL-induced activation of NF- κ B and ERK1/2 to apoptosis in T-cells (102, 146, 147). Therefore, understanding whether overexpression of c-FLIP is associated with FasL induced activation NF- κ B is of great interest in cancer cells. Importantly, MS-275 has been reported to inhibit NF- κ B in breast cancer cells, which strongly supports our hypothesis (109). To determine this, we would obtain the cells with c-FLIP overexpression and treat them with sFasL followed by western blot analysis to assess the expression of NF- κ B. To further analyze c-FLIP dependent NF- κ B activation, we can use a luciferase assay by transfecting cells with a NF- κ B luciferase reporter vector in cells that are transfected with different concentrations of c-FLIP. We would predict a dose-

dependent response in NF- κ B activity with the addition of c-FLIP. Finally, we could assess the effect of MS-275 on NF- κ B by transfecting our cell lines with the NF- κ B luciferase reporter vector and determining whether treatment decreases NF- κ B activity. Again, we would hypothesize based on previously published data that MS-275 would decrease the activity of NF- κ B. These investigations would suggest that in addition to activating FasL-induced apoptosis, MS-275 may also inhibit FasL-mediated activation of survival through NF- κ B. Similarly, we can investigate the effects of MS-275 treatment on the ERK1/2 pathway.

Overexpression of c-FLIP in cancer cells has shown to correlate with increased resistance to chemotherapeutic agents (148). In addition, increased c-FLIP is also associated with resistance to Fas-mediated cell death (149). Therefore, we would want to assess the effect of c-FLIP overexpression on the effects of MS-275. To determine this, cells transfected with c-FLIP would be treated with sFasL, MS-275 and the combination. Cytotoxicity assays, such as the clonogenic assay, could be performed to compare the sensitivity of cells in the c-FLIP overexpressing and control cell lines. Since we have shown that the mechanism of MS-275-induced sensitization to FasL correlates with the downregulation of c-FLIP, we would predict that overexpression of c-FLIP would inhibit this response.

Role of c-FLIP *in vivo* models

The body of work in our laboratory strongly suggests the importance of the Fas/FasL signaling pathway in the metastatic potential of OS cells (14-16, 20). This result was demonstrated by the injection of OS cells into mice and examination of Fas expression in OS cells in primary and metastatic sites. We observed high Fas expression in bone tumors, while metastatic tumors in the lung were Fas⁻. Therefore, we hypothesized that Fas⁺ cells would be cleared in the lung upon interaction with FasL, which is constitutively expressed in the lung. To test this hypothesis, cells were transfected with FADD-dominant negative (FDN) plasmid to

functionally block to the Fas pathway and were then injected into mice. Since the Fas pathway was blocked, the metastatic tumors formed were Fas-positive. In fact, tumors were more aggressive and more tumors were formed (14, 20). This result suggests that OS cells can alter the Fas signaling pathway in order to evade host defenses in the lung microenvironment.

In chapter 8, we analyzed OS patient samples for the expression of c-FLIP in primary and metastatic tumor tissues. We demonstrated that lung tumors from metastatic sites display increased c-FLIP expression as compared to the primary bone tumor. We also found in chapter 7 that LM7 lung metastases have high expression of c-FLIP, which can be downregulated with the treatment of mice with MS-275. Our results indicate that in addition to loss of Fas, overexpression of c-FLIP may be an additional mechanism by which OS cells can evade interaction with FasL and form metastases. To test this hypothesis, we could transfect cells with c-FLIP overexpression vector and inject these cells into mice. If the lung metastases formed have high levels of Fas expression and a greater number of highly aggressive tumor nodules as compared to the control, then this confirms that OS cells can upregulate c-FLIP in order to avoid FasL in the lung microenvironment. Conversely, OS cells with decreased c-FLIP by transfection of shRNA can be injected into mice. If c-FLIP is important for the formation of metastases, then shFLIP transfected cells should not be able to form lung metastases since these cells would have a functional Fas pathway and would be cleared upon interaction with FasL. However, these results would be difficult to analyze cause and effect since we have shown cells with decreased c-FLIP also display an increase in Fas expression. Therefore, it would be unclear whether these cells are unable to form tumors because of decreased c-FLIP, increased Fas or the combination of both.

We have demonstrated in chapter 7 that treatment of mice with OS lung metastases with MS-275 induced tumor regression and tumor cell apoptosis. In addition, MS-275 treatment resulted in the decrease of c-FLIP expression in metastatic tumor nodules. Since this is a

correlative finding, a future experiment would be to determine whether the effect of MS-275 *in vivo* is reduced when c-FLIP is overexpressed in metastatic cells. Our lab has previously demonstrated that corruption of the Fas pathway reduced the effect of chemotherapy in inducing the regression of metastatic OS tumors. Therefore, we would expect that treatment of mice c-FLIP overexpressing tumors with MS-275 would have a greater number of metastases, larger tumors and reduced overall survival compared to controls. These experiments would confirm our hypothesis that c-FLIP plays an important role in the effect of MS-275 *in vivo*.

Effects of MS-275 post-treatment and resistance

In our studies, mice were treated with MS-275 for 15 days and sacrificed at the end of the treatment period. Lungs, liver and heart were resected and analyzed by immunohistochemistry. For the treatment period assessed there was no significant tissue toxicity as examined by H&E. However, studies involving the effects of MS-275 on long term toxicity may be necessary for pre-clinical studies.

HDAC inhibitors, such as vorinostat, depsipeptide and panbinostat, have been reported to be associated with cardiotoxicities. In particular, these HDAC inhibitors have been coupled to QTc prolongation, a risk factor for sudden cardiac death (116, 150, 151). On the other hand, studies with MS-275 in phase I clinical trials, reported fatigue, nausea and diarrhea as dose-limiting toxicities (71). This result suggests that MS-275 is relatively well-tolerated, but further studies assessing toxicities long after cessation of treatment in mice is warranted.

Survival studies demonstrated that MS-275 significantly increased the overall survival of mice with OS pulmonary metastases. Treatment increased 50% survival by 27 days. While this effect is impressive, only 2/15 mice survived after 100 days, suggesting the possibility that 9 mice may have developed resistance to treatment or experienced rebound tumor growth. To assess this concern, we could treat mice with OS lung metastases with MS-275 and monitor the

growth and number of tumors in the lung over time. This experiment would be done by sacrificing mice and measure the volumes and quantity of tumors at different time points. The setback to this approach is that not all tumors are the same size across all animals when the experiment is started. Therefore, an alternative is to use luciferase-labeled or near-infrared (NIR)-labeled cells and monitor changes over time to determine any possibilities of rebound growth.

Another possible issue is the development of resistance to MS-275 treatment. Our clonogenic assays demonstrated that treatment of OS cells with MS-275 and sFasL in combination inhibited clonogenic growth and induced apoptosis. However, approximately 5-10% of cells still survived after treatment. In addition, while treatment of mice with OS lung metastases with MS-275 induced the regression of tumors, treatment did not eradicate tumors completely. These results suggest that some OS cells may be resistant to MS-275. Understanding mechanisms of resistance *in vitro* and *in vivo* may eventually translate to the clinic. To study mechanisms of resistance, a clonogenic assay can be performed and cells that survive after treatment with MS-275 and sFasL can be collected and cultured. This resistant population can then be further examined. Cancer cells have been shown to develop resistance to the HDAC inhibitors by drug efflux mechanisms. To assess this in response to MS-275, resistant cells can be analyzed for p-glycoprotein and multidrug resistance protein 1 (MDR1) by western blotting. The HDAC inhibitors vorinostat, belinostat, AN9, and romidepsin have all been associated with upregulation of both of these proteins, however benzamides such as MS-275 have yet to be examined (152). We have identified that the mechanism of MS-275 induced sensitization to FasL is through an effect on the Fas/FasL pathway itself. This raises the possibility that changes in protein expression of mediators of the Fas pathway may confer resistance to MS-275. For example, an increase in the inhibitors bcl-2, bcl-x_L and XIAPs or a decrease in the pro-apoptotic factors FADD, bid or caspases may result in the resistance of cells

to MS-275 induced sensitization to FasL. To examine this, we could perform western blot analysis of resistant cells and specifically measure the expression of these proteins as compared to the original population. Further, autophagy has been linked to increased resistance to chemotherapeutic agents (153). Therefore, increased autophagy may be a mechanism of resistance to MS-275. This mechanism can be determined by evaluating the expression of the autophagy markers LC3-II and p62 in the resistant population. If this resistant population has increased autophagy, then we would observe an increase in LC3-II conversion and a decrease in p62. Understanding the molecular mechanisms of resistance can help identify responders and predict response to treatment if MS-275 is translated to pediatric populations.

Combination therapy

HDAC inhibitors have shown promise *in vitro* and in several preclinical models of cancer. Unfortunately, they have not been as successful as single agents in clinical trials. However, in combination these agents have shown a promising response. In fact, the majority of ongoing clinical trials with HDAC inhibitors have included a combination with other chemotherapeutic agents. Our goal therapeutically is to use a combination of agents that can target metastatic OS in the lung with a two prong approach.

One such approach is to use two agents that both target the death receptor pathway. The possibility exists that alterations in mediators of the Fas pathway, such as upregulation of bcl-2, may contribute to MS-275 resistance. Therefore, using agents that target bcl-2 can counter this effect. ABT-737 is a small molecule bcl-2 inhibitor that has been shown to synergize with HDAC inhibitors (154). Following *in vitro* analysis of synergy, we could treat mice with OS pulmonary metastases with a combination of MS-275 and ABT-737 to assess its effectiveness. We would measure tumor area, lung weight and number of metastases following treatment. This would be compared to each as a single agent. Additionally, since we have demonstrated

that treatment with MS-275 significantly increased the overall survival of mice with metastases, we could compare the combination to MS-275 as a single agent in extending the survival.

Proteasome inhibitors have been shown to function synergistically with HDAC inhibitors (155). In fact, a study with the proteasome inhibitor, PS-341, demonstrated sensitization to death receptor pathways by decreasing the expression of c-FLIP. We have demonstrated in this dissertation that MS-275 downregulates c-FLIP both *in vitro* and *in vivo*. This result suggests that proteasome inhibitors that decrease c-FLIP expression may work in combination with MS-275 to sensitize OS to FasL-induced cell death.

There are several clinical trials assessing the efficacy of HDAC inhibitors and DNA methyltransferase (DNMT) inhibitors in combination (156). In fact, phase I and II trials have been conducted with MS-275 and azacitadine and have demonstrated safety and efficacy (157, 158). The rationale of using these in combination is that DNA methylation is often aberrant in cancer cells. This process is generally catalyzed by the enzyme DNA methyltransferase. When CpG islands are hypermethylated, this is associated with transcriptional repression. For this reason DNMT inhibitors are used to induce transcriptional activation in cells. Further, the recruitment of HDACs to DNA is also controlled by DNA methyltransferases (156). This suggests that together HDAC inhibitors and DNMT inhibitors would both prevent the recruitment of HDACs and inhibit the functionality of HDACs. We could rationalize that in combination MS-275 and azacitadine would have a synergistic response in sensitizing OS cells to FasL. However, our previously published data demonstrated that treatment of OS cells with azacitadine did not enhance the sensitivity of cells to FasL (36). This result implies that the combination of MS-275 and azacitadine may not prove to be synergistic. However, we cannot completely rule out that alternative DNMT inhibitors, with different specificities and effects,

may work well in combination with MS-275. These may include zebularine, procaine and EGCG, which have been shown to have various mechanisms of action *in vitro* (159).

While the aforementioned combinations target similar pathways and mechanisms, an alternative would be to utilize a strategy with diverse targets. Our group has demonstrated that OS cells and lung metastases from patients express IL-11R α . Therefore, in collaboration with Dr. Laurence Cooper (Department of Pediatrics, UT MD Anderson Cancer Center), we have engineered IL-11R α .CAR T-cells that were shown to selectively target IL-11R α -positive OS cells *in vivo* (Drs. Gangxiong Huang and Ling Yu, manuscript in revision). However, we have found that not all OS cells express IL-11R α , suggesting that some cells would escape therapy. Here we have demonstrated that MS-275 is effective in inducing tumor regression of OS lung metastases. Therefore, an ongoing approach in our lab is the combination of MS-275 and T-cell targeted therapy. Here, MS-275 would be used to target IL-11R α ⁻ OS cells and sensitize them to FasL-mediated apoptosis *in vivo*. We could assess tumor volume, number of metastases and overall survival comparing the combination to each of the single agent effects. According to our findings with the single agents, we hypothesize using two approaches with diverse mechanisms would be promising in targeting OS lung metastases.

MATERIALS AND METHODS

Chapter 10

Materials and Methods

Cell lines and reagents

Human osteosarcoma LM7 and CCH-OS-D cells were maintained in complete Dulbecco's Modified Eagle's Medium (Whittaker Bioproducts Inc., Walkersville, MD) supplemented with 10% heat-inactivated bovine serum (Intergen, Purchase, NJ). The human LM7 OS lung metastatic cell line was created in our laboratory by the repeated intravenous recycling of its parent cell line, SAOS-2, through the lungs of nude mice (12). CCH-OS-D cells were a kind gift from Dennis Hughes, MD, PhD (Division of Pediatrics, The University of Texas MD Anderson Cancer Center). All cell lines were mycoplasma-negative and validated by STR DNA Fingerprinting using the AmpFLSTR Identifier Kit (Applied Biosystems, Carlsbad, CA). The STR profiles were compared with known ATCC fingerprints (ATCC.org), to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 and to the MD Anderson fingerprint database. The STR profiles matched either known DNA fingerprints or were unique. The authenticity of cells was determined by the Characterized Cell Line Core at The University of Texas MD Anderson Cancer Center. Super FasL (human recombinant soluble FasL; sFasL) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). All *in vitro* experiments conducted in this project utilized a dose of 10 ng/ml sFasL. MS-275 was a generous gift from Syndax Pharmaceuticals, Inc. (Waltham, MA). The lipid raft inhibiting agent, methyl- β -cyclodextrin (MBC) was purchased from Sigma Aldrich, Inc (St. Louis, MO).

Cytotoxicity and apoptosis assays

Clonogenic assays were performed to assess cytotoxicity of OS cells after addition of sFasL, MS-275 or the combination. OS cells were seeded into six-well culture plates and allowed to attach overnight. Cells were then treated with 10 ng/ml sFasL for 24 hours, 2 μ M MS-275 for 48 hours or a combination of both. The medium was then removed and cell were

allowed to grow for 12 days, washed with PBS and fixed with formalin. Cells were then stained with 0.4% crystal violet for 30 minutes and colonies were counted. The colonies were counted using the Leica DLMS light microscope by averaging eight fields per well. One colony was defined as 50 cells. For lipid raft inhibition studies, cells were pre-treated with MBCD for 1 hour. Media was removed and replaced. 10 ng/ml sFasL for 24 hours, 2 μ M MS-275 for 48 hours, or a combination of both. Cells were then treated with Clonogenic assays were performed in triplicate.

Acridine Orange/Ethidium Bromide (AO/EB) assay is a viability stain that specifically detects apoptotic cells. This assay determines the membrane integrity of a cell based on the uptake or exclusion of specific dyes from the cells. Ethidium bromide (EB) has the ability to pass through the membrane of a dead or dying cell, while acridine orange (AO) is a membrane-permeable dye that will stain all cells. AO staining causes a cell to fluoresce green and EB staining fluoresces red. Therefore, cells that fluoresce orange (combination of green and red) is considered apoptotic. Cells were plated in 4-well slides and were treated with 10 ng/ml sFasL for 24 hours, 2 μ M MS-275 for 48 hours or combination of both. The conventional AO/EB staining protocol was performed following the guidelines from Current Protocols of Immunology (160). An AO/EB dye mix, containing 100 μ g/ml acridine orange (Sigma-Aldrich, Inc.) and 100 μ g/ml ethidium bromide (Sigma-Aldrich, Inc.), was added to each well and cells were visualized using a fluorescent microscope. Apoptosis was determined as percentage of apoptotic cells out of 100 cells counted. Assay was performed in triplicate and data was averaged.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the viability of cells after pretreatment with the caspase inhibitor Z-VAD-fmk (Enzo Life Sciences Inc., Farmingdale, NY) followed by treatment with MS-275 and sFasL. In addition, the MTT assay was utilized to determine cytotoxicity in FDN-transfected cells as

compared to controls. Briefly, 3000 cells were plated on 96-well plates and pre-treated with 20 μ M Z-VAD-fmk for 2 hours followed by treatment with 10 ng/ml sFasL for 24 hours, MS-275 for 48 hours or a combination of both MS-275 and sFasL. Untreated cells and cells treated with a single agent served as controls. After treatment, MTT was added to the cells at a concentration of 0.08 mg/ml for 4 hours followed by lysis with 0.1 ml of dimethyl sulfoxide (DMSO). Using a microtiter plate reader, the absorbance was measured at 570 nm to calculate the cytotoxicity. These experiments were performed in triplicate and repeated three times.

Caspase-activity assays

Colorimetric caspase-8 and caspase-3 activity assays were performed using IETD-pNA and DEVD-pNA substrates, respectively, according to manufacturer's instructions (BioVision Inc., Palo Alto, CA). The colorimetric assay involves the detection of the chromophore pNA when the substrate is cleaved. Briefly, cells were treated with sFasL, MS-275 and the combination. Following treatment, cells were lysed and incubated with either IETD-pNA or DEVD-pNA for 2 hours at 37°C. Samples in a 96-well plate were then read on the microplate reader (SpectraMax Gemini EM, Molecular Devices Corporation, Sunnyvale, CA) at 405 nm and were performed in triplicate. The assays were repeated three times.

Establishment of stable FADD dominant-negative clones

LM7 cells were transfected with FDN plasmid using Fugene 6 (Roche Applied Biosystems, Indianapolis, IN), according to manufacturer's protocol. The vector constructs for human FADD dominant negative (FDN) and neomycin-control were a gift from Drs. Rokhlin and Taghiyev (University of Iowa, Iowa City, Iowa) (161). Transfected cells were cultured in neomycin-containing media for selection. Transfection was confirmed by western blotting using an anti-FADD antibody (Millipore Corporation, Billerica, MA).

<u>Gene Target</u>	<u>Annealing Temperature (°C)</u>	<u>Forward Primer</u>	<u>Reverse Primer</u>
Fas	59	GGCTATAGATCAC CTTCATGTA	GCAGTTAACTCAGGGA CCAAG
c-FLIP	57	ACCGAGACTACGA CAGCTTTGTG	CAATGTGAAGATCCAG GAGTGGG
bax	53	GTTTCATCCAGGAT CGAGCAG	CATCTTCTTCCAGAT GGTGA
bcl-2	59	TGTTTTGCAGCTTT GCATTC	ATTGCCAGAAGGCACC ATAC
β-actin	57	ATCATGGTTTGAGA CCTTCAACA	CATCTCTTGCTCGAAG TCCA

Table 2. Primer sequences and annealing conditions. Table displays conditions used for detection of Fas pathway signaling molecules using PCR or quantitative real-time PCR.

Quantitative real-time PCR and reverse transcriptase-PCR

Total RNA was isolated from OS cells using Trizol reagent (Life Technologies, Inc, Gaithersburg, MD). RNA was then reverse-transcribed using a Reverse Transcription System (Promega Corporation., Madison, WI). For RT-PCR, 2.5 μ L of the resulting cDNA was used for PCR amplification with Taq polymerase (Promega Corporation) using specific primers for bax, bcl-2, c-FLIP or β -actin (Table 2). For quantitative real-time PCR, the resulting cDNA was used for PCR amplification with SYBR green buffer (Bio-Rad Laboratories, Inc., Hercules, CA) and specific primers for Fas, c-FLIP and β -actin. The PCR reaction mixture contained 100 ng of reverse-transcribed total RNA, 50 nM forward and reverse primers, and 12.5 μ L of SYBR green buffer (Bio-Rad Laboratories Inc., Hercules, California) in a final volume of 25 μ L. Quantitative real-time PCR was carried out in triplicate using the Bio-Rad 105 Real-time PCR detection system. Primer sequences and cycling conditions are described in Table 2. All experiments were repeated three times.

Western Blotting and antibodies

For determination of acetyl-histone H3 (AcH3) and histone H3 expression, nuclear fractionation was performed by lysing cells with nuclear extraction buffer containing 50 mM HEPES-K pH 8.0, 140 mM NaCl, 1 mM EDTA pH 8.0, 0.40% Igepal CA-630, 0.20% Triton X-100, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 ng/ml pepstatin and 0.5 mM PMSF. Nuclei were pelleted via centrifugation at 3000 x g for 3 minutes. Pellets were resuspended in sodium dodecyl sulfate (SDS) lysis buffer and sonicated for 15 minutes using the Misonex Sonicator 3000 at a power of 1. Lysates were then centrifuged for 10 minutes at 15,000 x g and the supernatant was collected as the nuclear extract. Equal amounts of protein were separated on 10% SDS- polyacrylamide gel electrophoresis (PAGE) gels and transferred onto a

nitrocellulose membrane. Immunoblot analysis was performed using antibodies specific for AcH3 (Millipore Corp., Billerica, Massachusetts) and histone H3 (Abcam, Cambridge, UK),

For detection of c-FLIP and caspases, LM7 and CCH-OS-D cells were lysed using radioimmunoprecipitation buffer. Equal amounts of protein were separated on a 10% SDS-PAGE gel and blotted to the nitrocellulose membrane (Amersham Biosciences Corp., Piscataway, NJ). The membranes were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20. Loading was confirmed by probing membranes with an anti- β -actin antibody (Sigma Aldrich, Inc.). Membranes were then incubated with antibodies against caspase-8 (C15) c-FLIP (NF6) (Enzo Life Sciences Inc., Farmingdale, NY), cleaved caspase -9, -3 and -7 (Cell Signaling Technology, Danvers, MA). Western blot analysis was repeated three times, and the consistency of results was verified. Densitometric analysis on immunoblots was performed using the ImageJ software program (version 1.42q; National Institutes of Health).

For detection of Fas protein expression, OS cells were collected and lysed using NP-40 buffer and then incubated with either anti-Fas antibody (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. Western blot analysis was performed using the protocol mentioned above. Antibodies and conditions are shown in Table 3.

Flow cytometry and ligand binding assay

OS cells were treated with 2 μ M MS-275 for 30 minutes, 1, 2, 6, 12, 24, 48 and 72 hours (data shown is a representation of 48 hours). To detect expression of cell surface Fas, one million OS cells were suspended in FACS buffer (PBS, containing 2% fetal calf serum and 0.1% sodium azide). Cells were then incubated with 1 mg/ml PE-conjugated mouse anti-human Fas antibody (BD Transduction LaboratoriesTM, Lexington, KY) for 45 minutes. PE-conjugated

<u>Antibodies</u>	<u>Indication</u>	<u>Mono/Poly</u>	<u>Dilution</u>	<u>Company</u>
Acetyl H3	WB	poly	0.05 µg/ml	Millipore
Histone H3	WB	poly	1:2000	Abcam
Fas	WB	poly	1:200	Santa Cruz Biotechnology
FADD	WB	poly	1:500	Millipore
c-FLIP	WB	mono	1:500	Enzo Life Sciences
bax	WB	poly	1:1000	Cell Signaling
bcl-2	WB	poly	1:200	Abbiotec
caspase-8	WB	mono	1:500	Enzo Life Sciences
cleaved caspase-9	WB	poly	1:1000	Cell Signaling
cleaved caspase-3	WB	mono	1:1000	Cell Signaling
cleaved caspase-7	WB	poly	1:1000	Cell Signaling
Fas-PE	FC	mono	1:50	BD Biosciences
FLAG-PE	FC	mono	5 µg/ml	Prozyme
CTxB-GM1- HRP	WB	---	15 µg/ml	Sigma-Aldrich
TfR	WB	mono	1 µg/ml	Invitrogen
Fas	IF	mono	1:50	BD Biosciences
CTxB-GM1- Alexa 594	IF	---	10 µg/ml	Molecular Probes
Acetyl H3	IHC	poly	1:100	Millipore
c-FLIP	IHC	poly	1:100	Abbiotec

Table 3. Antibody conditions. Table lists antibodies and conditions used for western blotting (WB), flow cytometry (FC), immunofluorescence (IF) and immunohistochemistry (IHC).

isotype-control IgG antibodies were used as negative controls (BD Transduction LaboratoriesTM). Samples were analyzed with the FL-2 channel using FACScan (Becton Dickinson, Mountain View, CA).

OS cells were treated with 2 μ M MS-275 for 48 hours. Human recombinant soluble FasL contains the extracellular domain of FasL fused at the N-terminus to a linker peptide and a FLAG[®]-tag (Enzo Life Sciences Inc.). Therefore to quantify sFasL binding, cells were incubated with 5 μ g/ml PhycoProTM PE-conjugated mouse α -FLAG[®] M2 monoclonal antibody (ProZyme, Hayward, CA) for 45 minutes. Samples were analyzed using FACScan (Becton Dickinson). Flow cytometry analysis and percent expression of Fas and FLAG was determined using FlowJo v. 7.5.

Lipid raft fractionation

Lipid raft fractions were isolated using the Caveolae/Rafts isolation kit (Sigma-Aldrich Inc.) and was performed according to the manufacturer's instruction. Briefly, samples were centrifuged and the pellet was extracted containing 1% Triton X-100 and incubated on ice for 30 minutes. Lysates were then mixed with cold OptiPrep Density Gradient Medium and overlaid with 35, 30, 25, 20 or 0% OptiPrep in an ultracentrifuge tube. Samples were then centrifuged at 200 000 x g using a fixed angle rotor for 4 hours at 4 °C. Nine (1 ml) fractions were collected from the top to the bottom of each tube. Western blotting was performed with antibodies for Fas (Santa Cruz Biotechnology, Santa Cruz, CA), HRP-conjugated cholera toxin B subunit (CTxB, Sigma-Aldrich) as a lipid raft marker or transferrin receptor (Invitrogen Corporation, Carlsbad, CA) as controls.

Amplex Red assay for cholesterol detection

An Amplex Red assay kit was used to measure cholesterol content in samples according to manufacturer's protocol (Invitrogen Corporation). The assay quantifies cholesterol by incubating lysates with peroxidase, which breaks down cholesterol and leaves H_2O_2 as a byproduct. Amplex red solution interacts with H_2O_2 to produce resorufin, which can then be measured spectrophotometrically. Briefly, 50 μ l of cell lysate was added to the amplex red mix, containing 10 mM amplex red stock solution, 20 mM H_2O_2 stock solution and 1X reaction buffer in a 96-well plate. As a positive control, 10 U/ml HRP stock solution was used in 1X reaction buffer, while 1X reaction buffer alone was used as a negative control. Reaction was incubated for 30 minutes at room temperature in the dark. Absorbance was measured using a microplate reader at 560 nm (SpectraMax Gemini EM, Molecular Devices Corporation).

Immunofluorescence staining

Cells were cultured in 4-well chamber slides and were either untreated or treated with MS-275 at conditions reported above. Cells were fixed with 4% paraformaldehyde in PBS for 30 minutes. Samples were then incubated with anti-CTxB conjugated to Alexa 594 (Molecular Probes, Eugene, OR) at 4 °C overnight followed by incubation with anti-Fas (BD Biosciences, Franklin Lakes, NJ) at 4 °C overnight and anti-mouse IgM (Alexa Fluor 488; Jackson ImmunoResearch, West Grove, PA) for 37 °C for 2 hours. After washing with PBS, samples were mounted and visualized with a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) and images were captured using a Hamamatsu C5810 camera (Hamamatsu Photonics, Bridgewater, NJ). Pictures were analyzed using Optimas imaging software (Media Cybernetics, Bethesda, MD).

Inhibition of lipid raft formation

Methyl- β -cyclodextrin (MBC; Sigma-Aldrich Inc.), which removes cholesterol from cultured cells, was used to disrupt lipid rafts in cells. OS cells were treated with 5 μ g/ml of MBC followed by treatment with sFasL, MS-275 or the combination. Clonogenic assay was used to determine the effect of MBC (lipid raft disruption) on MS-275-induced sensitization of cells to sFasL.

Generation of c-FLIP short hairpin RNA-expressing clones

To stably knockdown c-FLIP expression, OS cells were transduced with retroviruses expressing shRNA-cFLIP, shRNA-scrambled or vector pGFP-V-RS (Origene Technologies, Inc., Rockville, MD). Cells were grown in media containing puromycin to select for stably transfected clones. shRNA constructs were tested for knockdown using western blot analysis using an antibody against c-FLIP (NF6; Enzo Life Sciences Inc., Farmingdale, NY) and by quantitative real time-PCR using primers against c-FLIP. The pGFP-V-RS vector containing the 29-mer shRNA sequence TGCACAGTT CACCGAGAAGCTGACTTCTT (ID: GI355835) exhibited more than 80% c-FLIP knockdown.

Animal studies

Female nu/nu mice were purchased from the National Cancer Institute (Bethesda, MD). All animals used for *in vivo* experiments were housed in standard cages, at five mice per cage and provided with food and water *ad libitum*. Animal experiments were performed in accordance with the MD Anderson Institutional Animal Care and Use Committee and approved by the American Association for Laboratory Animal Science (AALAS). Mice were injected with 2×10^6 LM7 cells via the tail vein. Formation of microscopic and macroscopic lung metastases was verified within 5 - 6 weeks by observation and hematoxylin and eosin (H&E)

staining of resected lung tissue. The mice were randomly divided into 2 groups (10 mice/group) and received 20 mg/kg MS-275 or DMSO (as a control) in 0.2 ml by oral gavage every other day for 15 days. Animal studies were repeated three times to verify results. Data shown is representative of one experiment. Survival studies were conducted using 15 mice per group using the same method described above to establish lung metastases. MS-275- and DMSO-treated mice were observed to assess their survival. Survival study was repeated two times to verify results. Data is representative of one experiment.

Immunohistochemistry

Lung tissue sections were deparaffinized in xylene, rehydrated, and antigen retrieval was performed using sodium citrate. Sections were incubated with 3% H₂O₂ for 12 minutes to block exogenous peroxidase and then incubated with PBS containing 10% normal horse serum. Antibodies against AcH3 (Millipore Corp., Billerica, Massachusetts) and FLIP (Abbiotec, San Diego, CA) were applied and left overnight at 4°C. Secondary antibodies labeled with horseradish peroxidase were then applied for 2 hours at room temperature. Slides were then developed with 3,3'-diaminobenzidine (DAB) as a substrate and counterstained with hematoxylin. Negative controls were prepared via omission of the primary antibodies. Paraffinized sections of murine liver and heart tissue were subjected to H&E staining and then pathological analysis to identify any drug-induced toxic effects.

Apoptosis was measured using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Lung tissue sections were deparaffinized as described above, incubated with 20 mg/mL proteinase K (Sigma Aldrich, Inc.) for 10 minutes, 3% H₂O₂ for 12 minutes, and terminal deoxynucleotidyl transferase buffer for 2 minutes at room temperature. Tissue sections were then incubated with terminal transferase (Boehringer-Mannheim Corp., Mannheim, Germany) and biotin-160 (Roche, Indianapolis, IN) in a humidity chamber at 37°C

for 1 hour. Following incubation, sections were incubated with 2% bovine serum albumin (BSA) for 10 minutes followed by horseradish peroxidase-conjugated streptavidin at 37°C for 1 hour. The tissue sections were washed twice with double-distilled water, stained with DAB, and counterstained with hematoxylin, as described above.

Analysis of Patient Samples

Thirteen paraffin-embedded tissue patient samples were obtained from Mayo Clinic (Rochester, MN), Children's Hospital and Regional Medical Center (Seattle, WA), or The University of Texas M. D. Anderson Cancer Center (Houston, TX) (15). Nine out of the thirteen samples were from metastatic lesions and four from primary tumors. The study protocol was approved by the institutional review board (IRB) at the UT MD Anderson Cancer Center.

Tissue sections were deparaffinized in xylene, rehydrated, and antigen retrieval was performed using sodium citrate. Sections were then incubated with 3% H₂O₂ for 12 minutes to block exogenous peroxidase and then incubated with PBS containing 10% normal horse serum. An antibody against FLIP (Abbiotec) was applied and left overnight at 4°C. Secondary antibody labeled with horseradish peroxidase was then applied for 2 hours at room temperature. Slides were then developed with 3,3'-diaminobenzidine (DAB) as a substrate and counterstained with hematoxylin. Negative controls were prepared via omission of the primary antibodies. Staining for c-FLIP was qualitatively assessed for positivity.

In addition, quantitative analysis was performed using four random sections that were randomly selected on each slide. Positive c-FLIP staining was measured using Simple PCI software (Hamamatsu, Sewickley, PA). Mean pixel area was measured for c-FLIP and nuclei. Mean positive pixel:nuclei ratio was calculated for each sample.

Statistical Analysis

Statistical comparisons of groups were performed using student *t*-test and Kaplan-Meier curves were generated using the GraphPad Prism 5 software program. Statistical significance of survival studies was determined using a Mann-Whitney test. A p-value of less than 0.05 was deemed as statistically significant.

APPENDIX

Chapter 11

Appendix

Characterization of LM7 and CCH-OS-D cell lines and xenograft models

Our group has previously demonstrated that Fas expression in OS cells is inversely correlated with metastatic potential (19). This was determined by quantifying the expression of Fas by flow cytometry in human OS cell lines. A highly metastatic subline, LM7, was created by recycling SAOS-2 cells through the lungs of nude mice. The Fas mRNA and protein expression in the highly metastatic LM7 subline was significantly greater than the parental SAOS-2 (19).

CCH-OS-D are primary OS cells obtained from UT MD Anderson Cancer Center which were cultured in the laboratory of Dr. Dennis Hughes (Department of Pediatrics). These cells were found to make spontaneous lung metastases when administered via intratibial injection.

Prior to beginning work on this project, we examined Fas expression in SAOS-2, LM7 and CCH-OS-D cells. According to our previous findings, the metastatic cells LM7 and CCH-OS-D would have lower Fas expression as compared to SAOS-2. This was confirmed by flow cytometry analysis in which we found SAOS-2 to express 76.48% Fas. On the other hand, LM7 and CCH-OS-D cells displayed 22.98% and 9.4% Fas expression, respectively (Figure A1).

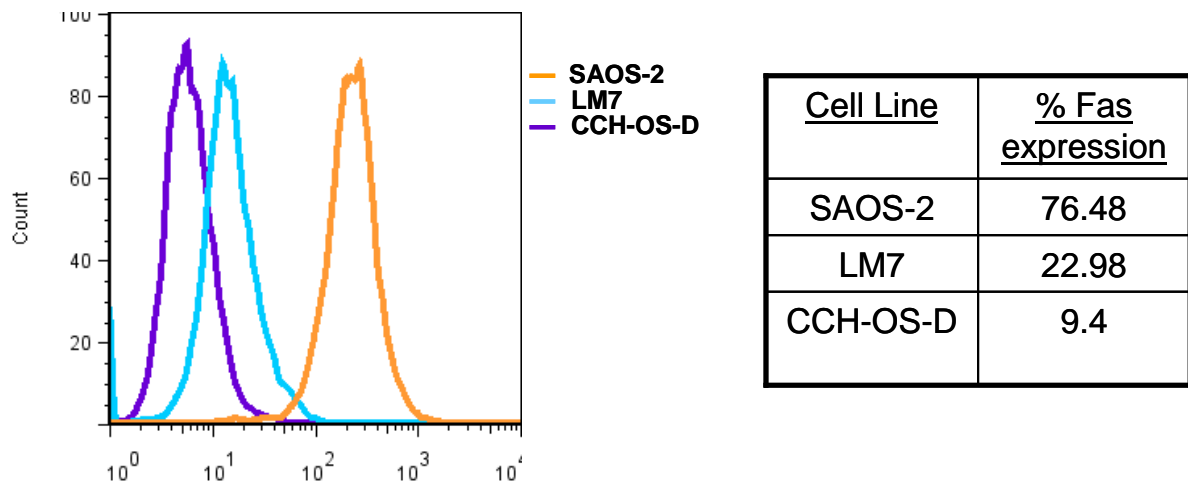


Figure A1. Fas expression profile of OS cell lines. Flow cytometry analysis was performed to measure Fas expression in cell lines using a Fas-PE antibody. LM7 (22.98%) and CCH-OS-D (9.4%) cells have lower Fas expression as compared to SAOS-2 (76.48%) cells.

While the LM7 lung metastases model is a fairly well established in our laboratory, we wanted to confirm this model before beginning treatment with MS-275. We, therefore, injected LM7 cells intravenously in nude mice and confirmed formation of pulmonary metastases by H&E staining at 6 weeks post-injection (Figure A2).

The CCH-OS-D lung metastases model was established by Dr. Dennis Hughes (Department of Pediatrics, UT MD Anderson Cancer Center). Dr. Ling Yu in our laboratory confirmed this model (in collaboration with Dr. Hughes's lab) by the intratibial injection of CCH-OS-D cells into NOD/SCID/IL-2R γ -deficient mice. Lungs were extracted and presence of lung metastases was demonstrated by H&E staining at ~60 days post-injection.

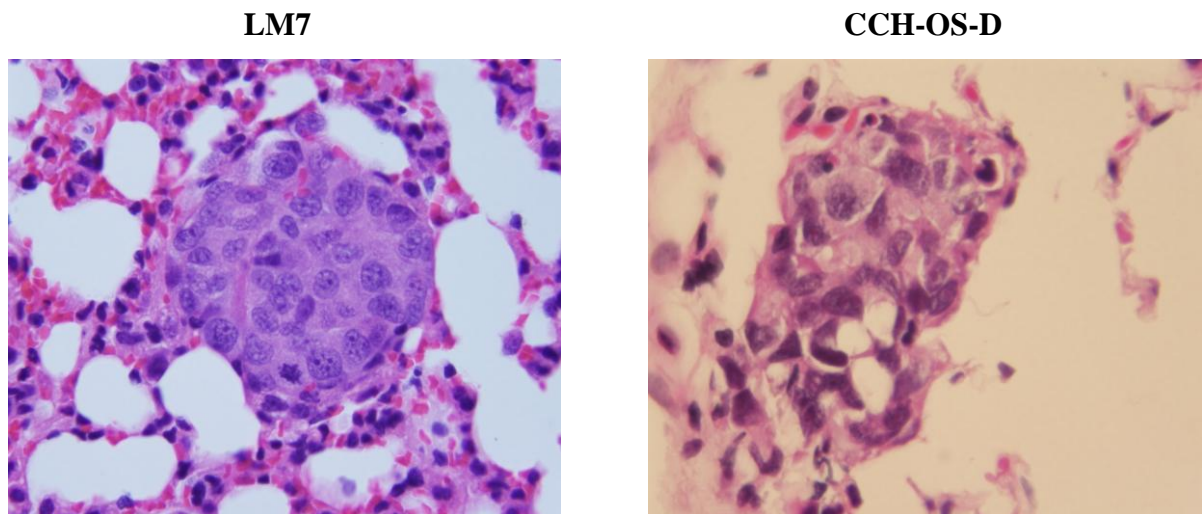


Figure A2. LM7 and CCH-OS-D cells form pulmonary metastases in mouse models. LM7 cells were injected via tail-vein injection in nu/nu mice. CCH-OS-D cells were injected via intratibial injection into NOD/SCID/IL-2R γ -deficient mice. Lungs were extracted and H&E staining was performed to confirm presence of metastases.

Comparison of SAHA (Vorinostat) versus MS-275

Vorinostat or SAHA was the first HDAC inhibitor to be FDA approved for the treatment of cutaneous T-cell lymphoma (42). It is currently in clinical trials either as a single agent or in combination for various types of cancers. Due to the translational relevance of this HDAC inhibitor, we initially investigated the effects of SAHA on our OS cell lines. Cytotoxicity assays were performed in which OS cells were treated with increasing doses of SAHA alone or in combination with sFasL. Our results demonstrated that relatively low doses of SAHA were toxic to OS cells. Further, treatment with SAHA did not significantly alter the sensitivity of cells to sFasL (Figure A3). In comparison, OS cells treated with increasing doses of MS-275 increased the sensitivity of cells to FasL-induced cytotoxicity (Figure A3). To investigate the difference between SAHA and MS-275 further, we examined Fas expression following treatment. OS cells were treated with 0.03 μ M (IC₅₀) SAHA for 12, 24 and 48 hours. Quantitative real-time PCR was performed using primers for Fas. The results demonstrated that Fas mRNA was not altered following treatment with SAHA at the time-points tested (Figure A4). In contrast, 2 μ M (IC₅₀) MS-275 resulted in a 5-fold increase in Fas mRNA and significantly sensitized cells to sFasL-induced cell death (Figure 6 and 11). Based on our initial findings, MS-275 may be a more promising agent than SAHA in sensitizing OS cells to FasL.

In support of this hypothesis, previously published studies demonstrated that specific inhibition of HDAC1 and HDAC3 in OS cells resulted in the upregulation of Fas mRNA. This data correlates with our findings that MS-275, an HDAC1/3 specific inhibitor, upregulated Fas mRNA and protein and sensitized cells to FasL. Although SAHA is a pan-HDAC inhibitor, the specificity for HDAC1 and HDAC3 may be less as compared to MS-275. This may be particularly important if the upregulation of Fas in our cell lines is dependent on the inhibition of these specific HDACs.

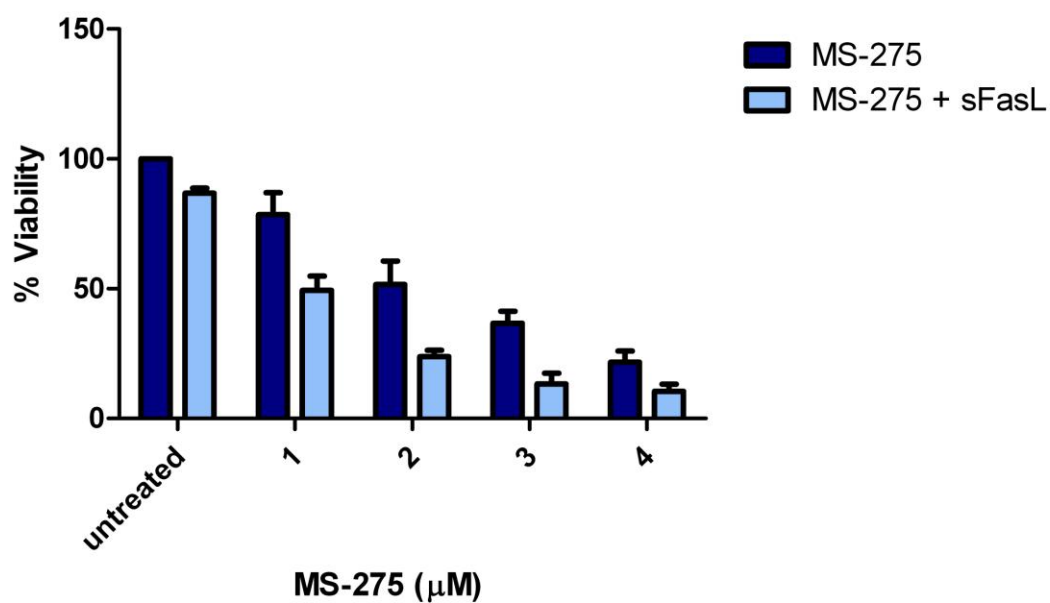
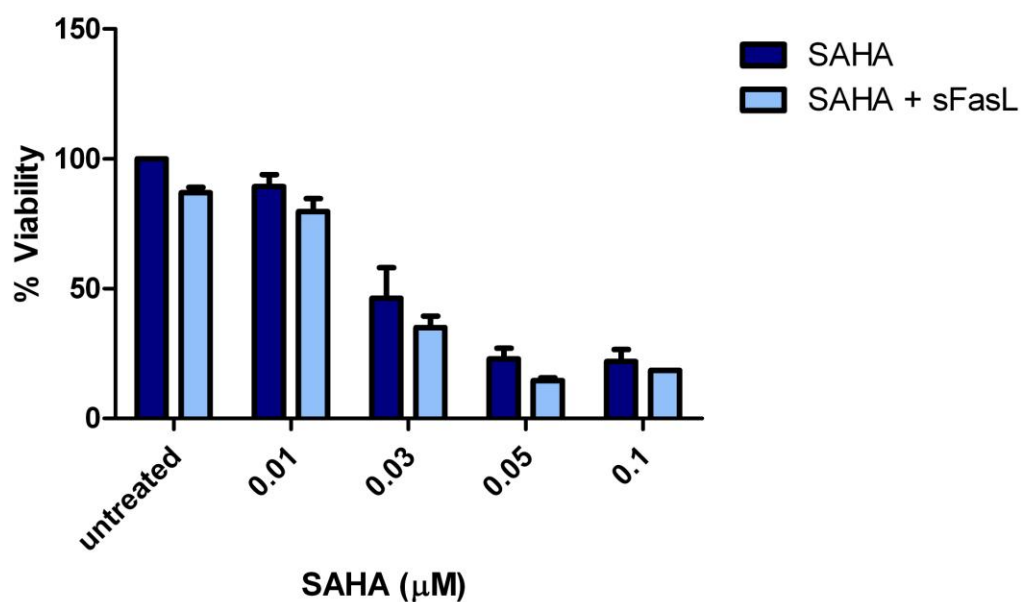


Figure A3. Dose-response curves of SAHA and MS-275. LM7 cells were treated with increasing doses of either SAHA or MS-275 for 48 hours. MTT assay was performed in triplicate. Data represents average and standard deviation of two independent experiments.

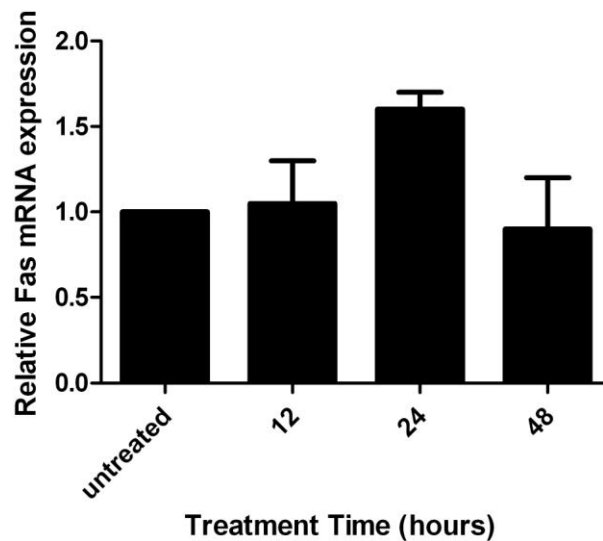


Figure A4. SAHA does not significantly increase Fas mRNA expression. LM7 cells were treated with 0.03 μ M SAHA for 12, 24 and 48 hours. RNA was isolated from cells using Trizol reagent and quantitative real-time PCR was performed using primers specific for Fas. Data shows an average and standard deviation of two independent experiments. When comparing treatment groups with untreated, $p > 0.05$.

MS-275-induced sensitization to FasL is caspase-dependent

In chapter 2, we demonstrated that treatment of OS cells with MS-275 sensitizes cells to FasL-induced cell death. We found this effect to be caspase-dependent. This was determined by increase in caspase-8 and -3 activity following treatment with MS-275 and FasL. In addition, blocking caspase activity with the pan-caspase inhibitor z-VAD-fmk, partially inhibited this effect. To confirm our findings we examined presence of cleaved caspase products following treatment. OS cells were treated with sFasL, MS-275 or the combination and subjected to western blot analysis using antibodies specific for cleaved caspase-9, -3 and -7 (Figure A5).

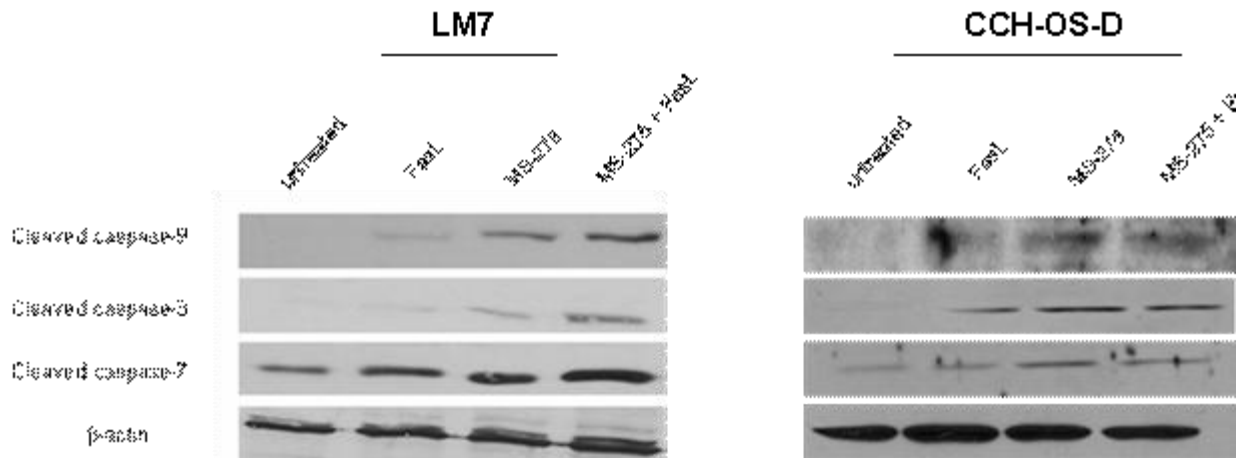


Figure A5. Treatment of LM7 and CCH-OS-D cells with MS-275 and sFasL increases caspase cleavage. OS cells were treated with control media, sFasL, MS-275 or combination. Cells were then lysed and subjected to western blot analysis using antibodies to detect cleaved caspase-9, -3 and -7.

Increased cleavage products of caspase-9, -3 and -7 were observed in MS-275 and sFasL treated cells as compared to either agent alone (Figure A5). These findings correlate well with our caspase activity assay data. Interestingly, cleaved caspase-9 is also significantly increased in the combination treatment group, which suggests involvement of the mitochondrial pathway.

MS-275 treatment does not effect the secretion of soluble Fas

A recent study demonstrated that the HDAC inhibitor, valproic acid (VPA), sensitized OS cells to agnostic anti-Fas antibody. Interestingly, the mechanism of action did not involve upregulation of the expression of cell-surface Fas. Rather, VPA treatment resulted in a decrease in secretion of soluble Fas. It has been previously published that many tumor cells secrete soluble Fas, a splice variant of cell surface Fas, as a decoy receptor to block FasL binding (162, 163). In fact, OS has been shown to produce soluble Fas as a means to resist FasL interaction and apoptosis (164, 165).

In our study, we found that MS-275 increases the sensitivity of OS cells to FasL without increasing the levels of cell-surface Fas, as detectable by flow cytometry. In understanding the mechanism of action, we explored the possibility that, similar to the reported findings, MS-275 could effect the secretion of soluble Fas. To determine this, we treated OS cells with sFasL, MS-275 or combination. Following treatment, we performed an ELISA assay to detect soluble Fas in the media. Our results demonstrate that, unlike VPA, MS-275 did not change the secretion of soluble Fas in LM7 and CCH-OS-D OS cells (Figure A6). This data suggests that alternative mechanisms may be involved in MS-275-induced sensitization of OS cells to FasL. It is also important to note that VPA is a pan-HDAC inhibitor, while MS-275 is a class I-specific HDAC inhibitor. This demonstrates that inhibition of specific HDACs or acetylation of specific proteins may yield different mechanisms of action.

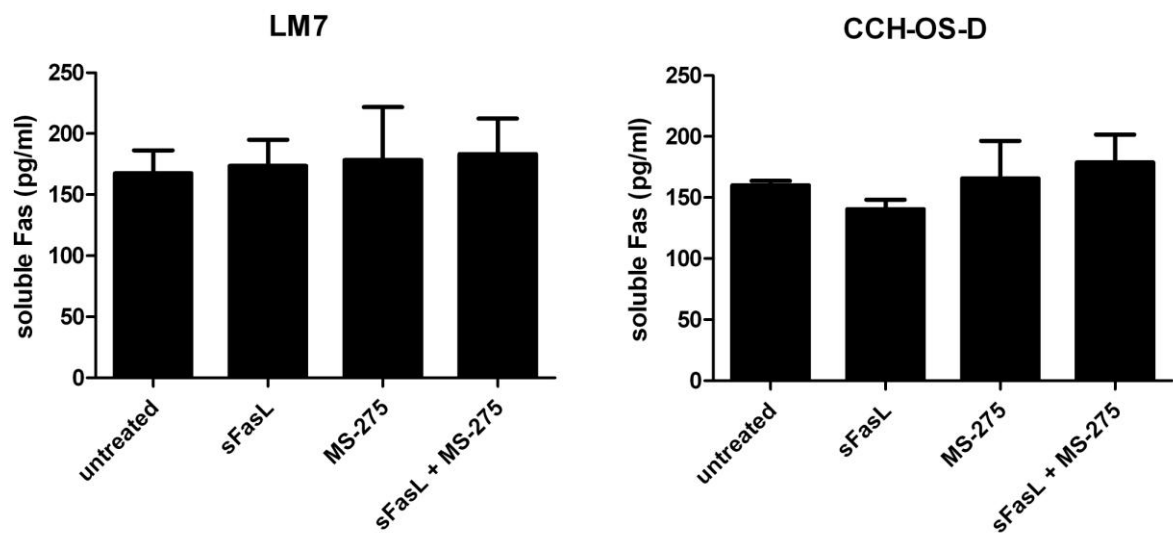


Figure A6. Effect of MS-275 on the secretion of soluble Fas. LM7 and CCH-OS-D cells were treated with control media, sFasL, MS-275 or combination. Amount of soluble Fas was measured in media using ELISA. Data represents average of two experiments and standard deviation. Secretion of soluble Fas was not significantly different between treatment groups ($p > 0.05$).

Downregulation of c-FLIP increases Fas expression in a caspase-8 dependent manner

We observed in chapter 6 that cells with downregulated c-FLIP by shRNA transfection resulted in increased colocalization of Fas within GM1⁺ lipid rafts (Figure 21). We also observed an increase in overall Fas expression in shFLIP-LM7 cells when compared to sh-control and sh-scrambled LM7 cells (Figure 20-21). To confirm this observation, we performed quantitative real-time PCR and western blot to measure Fas expression in these cells. Consistent with our previous data, we demonstrated an increase in Fas mRNA and protein in shFLIP-LM7 cells as compared to controls (Figure A7). We further hypothesized that this c-FLIP-dependent increase in Fas expression may be due to a positive feedback loop in which downregulated c-FLIP may result in activated Fas signaling and subsequent increase in Fas expression. To test this, we blocked the pathway directly downstream of c-FLIP by utilizing a caspase-8 specific inhibitor. We found that caspase-8 inhibition partially prevented the increase in Fas mRNA and protein (Figure A7). However, additional studies are necessary to understand the details of this mechanism.

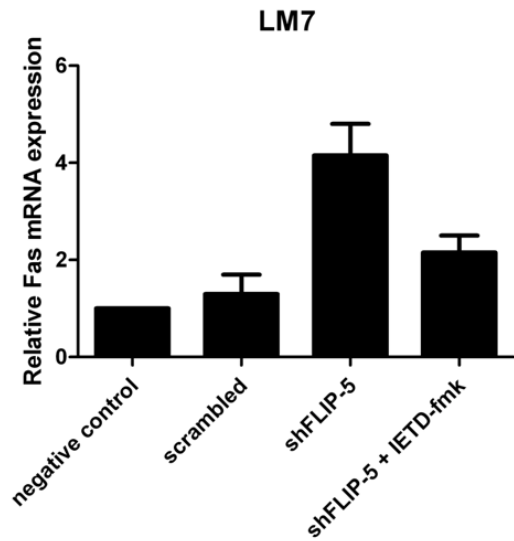
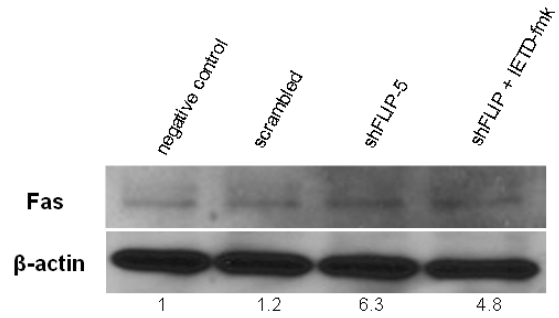
A**B**

Figure A7. Downregulation of c-FLIP increases Fas mRNA and protein. LM7 cells were transfected with sh-negative control, sh-scrambled or shFLIP. Quantitative real-time PCR and western blot analysis was used to detect Fas mRNA (A) and protein expression (B). Increase in Fas was partially inhibited in the presence of a caspase-8 inhibitor.

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VITA

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