THE EFFECT OF COMBINED CHEMOTHERAPEUTIC TREATMENT AND INHIBITION OF HMGB1 SIGNALING IN MALIGNANT MESOTHELIOMA

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MOLECULAR BIOSCIENCES AND BIOENGINEERING

DECEMBER 2013

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LIST OF ABBREVIATIONS

MM	Malignant Mesothelioma
HMGB1	High-Mobility Group Box 1
DAMP	Damage-associated Molecular Pattern
RAGE	Receptor for Advanced Glycation Endproducts
TLR	Toll-like Receptors
TNF-α	Tumor Necrosis Factor alpha
NF-κB	Nuclear Factor kappa-light-chain enhancer of activated B cells
HM	Human Mesothelial
SCID	Severe Combined Immunodeficiency
EP	Ethyl Pyruvate
WA	Withaferin A
ROS	Reactive Oxygen Species
HUVEC	Human Umbilical Vein Endothelial Cell
IL-6	Interleukin 6
IκBα	Nuclear Factor of kappa-light polypeptide gene enhancer in B-cells inhibitor alpha
TNF-R1	Tumor Necrosis Factor Receptor 1
SV40	Simian Virus 40
PP2A	Protein Phosphatase 2
AP-1	Activator Protein 1
IGF-1	Insulin-like Growth Factor 1
P53	Tumor Protein 53
Rb	Retinoblastoma Protein
pRb	Phospho Retinoblastoma Protein
BAP1	BRCA1 Associated Protein-1
HCF1	Host Cell Factor 1
YY1	Ying Yang 1
ASXL1	Additional Sex Combs Like 1
AXL2	Additional Sex Combs Like 2
OGT	O-linked N-acetylglucosamine (GlcNAc) Transferase

FOXK1	Forkhead Box K1
FOXK2	Forkhead Box K2
UVM	Uveal Melanoma
EPP	Extrapleural Pneumonectomies
P/D	Pleurectomy/Decortication
MARS	Mesothelioma and Radical Surgery
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet-Derived Growth Factor
PDGFR	Platelet-Derived Growth Factor Receptor
CD13	Aminopeptidase N
TCA	Tricarboxylic Acid
COX-2	Cyclooxygenase-2
LPS	Lipopolysaccharide
ERK	Extracellular Signal-Regulated Kinase
MAPK	Mitogenactivated Protien Kinase
H_2O_2	Hydrogen Peroxide
JAK	Janus Kinase
STAT	Signal Trasnducer and Activator of Transcription
Bcl-2	B-cell CLL/lymphoma 2
Par-4	Prader-Willi/Angelman region gene 4
Hsp90	Heat Shock Protein 90
FOXO3a	Forkhead Box O3
EMT	Epithelial-mesenchymal Transition
STS	Soft Tissue Sarcomas
IKK	IκB-kinase
GBM	Glioblastoma Multiforme
mTOR	Mammalian Target of Rapamycin
VCAM-1	Vascular Cell Adhesion Molecule 1
ICAM-1	Intercellular Adhesion Molecule 1
Bax	BCL2-associated X protein
PARP	Poly (ADP-ribose) polymerase family

CARP-1	Cell Cycle and Apoptosis Regulatory Protein 1
CCAR1	Cell Division Cycle and Apoptosis Regulator 1
TUNEL	Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
PBS	Phosphate Buffered Saline
FBS	Female Bovine Serum
DMEM	Dulbecco's Modified Eagle Medium
SD	Standard Deviation
SEM	Standard Error of the Mean
DMSO	Dimethyl Sulfoxide

CHAPTER 1. Introduction

1.1. Overview

Malignant mesothelioma (MM) arises from the transformation of the mesothelial cells lining pleural, peritoneal and pericardial cavities. The incidence of MM has sharply increased over the past 50 years. Currently, MM causes about 3,000 deaths per year in the US and over 100,000 deaths per year worldwide. Most individuals develop pleural MM due to inhalation of asbestos or other carcinogenic mineral fibers. From initial fiber exposure and diagnosis of MM, there is a long latency period, typically 20-40 years[1]. In most MM patients, diagnosis frequently occurs at a late stage of disease progression. At that point, the tumor is unresectable and chemotherapy is only palliative. Life expectancy upon diagnosis is usually 8-14 months, although some patients may survive significantly longer. Several combination chemotherapy regimens have been shown to improve survival, when compared with single agent treatment, and have become the main standard of care[2]. The combination of cisplatin and pemetrexed has yielded the best effect in malignant pleural mesothelioma with a median survival of 12.1 month, a median time to disease progression of 5.7 months, and a response rate of 41% in a phase III study[3]. Despite the current advances, MM prognosis remains dismal, with almost 90% of patients dying within 2 years from diagnosis. Therefore, there is a significant need for alternative interventions.

1.2. Malignant Mesothelioma

Asbestos alone does not transform mesothelial cells[4]. However, results from Dr. Yang's group suggest that High-Mobility Group Box 1 (HMGB1) is a key regulator of MM transformation upon asbestos exposure[5]. HMGB1 is a damage-associated molecular pattern (DAMP) protein that is released during necrosis and is a key regulator of inflammation[6, 7]. The binding of HMGB1 to its cell surface receptors, including the receptor for advanced glycation endproducts

(RAGE) and Toll-like receptors (TLRs 2 and 4), initiates the inflammatory process by causing the differentiation of local monocytes to macrophages. The macrophages phagocytize asbestos and release inflammatory cytokines, including Tumor Necrosis Factor-alpha (TNF-α). This leads to activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway, promoting cell proliferation and inhibiting cell death. This process enhances survival of human mesothelial (HM) cells that have accumulated DNA alterations following asbestos exposure, thus facilitating malignant transformation[5].

1.3. HMGB1

Recent data from Dr. Yang's group reveal that HMGB1 is also critically involved in MM progression. HMGB1 is highly expressed in MM cells in both the nucleus and cytoplasm. Inhibition of HMGB1 signaling impairs the motility, survival, and anchorage-independent growth of HMGB1-secreting MM cells *in vitro*. An HMGB1 blocking monoclonal antibody partially reduced tumor growth in SCID mouse MM xenografts, extending animal survival. These results indicate that the sustained release of HMGB1 by MM cells, along with its secretion by surrounding inflammatory cells, supports the malignant phenotype of MM[8].
The significance of the proposed work derives from the general principle that inflammation is thought to be intimately involved in the neoplastic transformation, growth, and metastatic processes of many types of cancer[9]. HMGB1, as an inflammation messenger, is known to be a significant driver of neoplastic transformation and chemoresistance[10]. Furthermore, HMGB1 is found in different redox states, possibly reflecting the redox state of the cell[11]. The equilibrium between the different redox forms of HMGB1 effects its subcellular distribution and function[10]. One of the receptors of HMGB1 is the receptor for advanced glycation end products (RAGE). RAGE is aberrantly expressed by various tumors, and binding of HMGB1 to RAGE stimulates cancer cell proliferation, invasion, chemoresistance, and metastasis[12].

Concurrently, HMGB1 upregulates autophagy, and as a consequence, promotes chemoresistance in osteosarcoma and leukemia[13, 14]. Signaling initiated by HMGB1 binding to RAGE results in the activation of the NF-κB pathway. This results in upregulation of the expression of leukocyte adhesion molecules and the production of pro-inflammatory cytokines and angiogenic factors in both hematopoietic and endothelial cells, thereby promoting inflammation and angiogenesis[15]. The effect of HMGB1 is abrogated when cells are treated with ethyl pyruvate (EP) or when RAGE is downregulated by antisense RNA[16, 17]. Moreover, antibodies targeting HMGB1 inhibit angiogenesis *in vitro* and *in vivo*[18].

1.4. Ethyl Pyruvate

Ethyl pyruvate (EP) is a simple aliphatic ester derived from the endogenous metabolite, pyruvic acid. It has been found to be an effective anti-inflammatory agent in many *in vitro* and *in vivo* models and to inhibit secretion of HMGB1 in murine colitis. EP ameliorates organ damage and improves survival in animal models of mesenteric ischemia and reperfusion, hemorrhagic shock, endotoxemia, and sepsis. While inhibiting HMGB1, it has been found to simultaneously attenuate NF-κB activation with concentrations of 10mM and above in lipopolysaccharide-stimulated RAW 264.7 murine macrophage-like cells and embryonic kidney 293 cell lines through inhibition of NF-κB DNA binding and gene transcription, targeting the p65 unit of the transcription factor[16, 19, 20]. The pharmacological effects of EP also include the downregulation of proinflammatory cytokine expression and inhibition of proinflammatory cytokines secretion[21-23].

1.5. Cisplatin

Cis-diamminedichloroplatinum (II), commonly known as cisplatin, is a platinum-based compound that has been used as a single agent in phase II trials and in combination with pemetrexed against mesothelioma[24]. Cisplatin has also

been found to have clinical activity against a wide spectrum of solid cancers including ovarian, testicular, bladder, colorectal, lung, and head and neck tumors. Cisplatin spontaneously changes from its inert state and undergoes an aqueous reaction, resulting in the replacement of one or both *cis*-chloro groups with water leading to the generation of highly reactive mono- and bi-aquated cisplatin forms, which avidly bind to DNA causing protein-DNA complexes and DNA-DNA inter- and intra-strand adducts. In the cytoplasm it also targets glutathione, causing oxidative stress in addition to generating superoxide anions and hydroxyl radicals. The use of cisplatin is limited due to acquired or intrinsic chemo-resistance of tumors and the cumulative nephrotoxicity, as a result of ROS production inducing apoptosis[25].

1.6. Withaferin A

Withaferin A is a steroidal lactone purified from leaves of the medicinal plant *Withania somnifera.* Withaferin A is has been found to exert anti-inflammatory, pro-apoptotic, anti-angiogenic, and anti-proliferative properties, making it a viable potential drug candidate for the treatment of different types of cancer[26]. It was originally used as a botanical extract with multiple mechanisms of action as a chemotherapeutic agent[27, 28]. Withaferin A has previously been shown to reduce tumor growth and metastatic potential in a malignant mesothelioma both *in vitro* and *in vivo* (mouse model), and is an attractive potential cancer treatment in human patients due to its potential for oral administration and use as a preventative agent[29]. In HUVEC cells stimulated with HMGB1, WA has been found to inhibit NF-κB activation and production of IL-6 and TNF-α through a decrease in phospho-IκBα[27]. This same NF-κB inhibition was found in malignant mesothelioma through an increased activation of IκBα, an inhibitor of NF-κB[29]. These insights suggest WA had the potential to be an inhibitor of HMGB1, preventing the phosphorylation of IκB and its subsequent degradation, or preventing NF-κB activation in the nucleus. The cytotoxicity of WA through oxidative stress-mediation in glioblastomas, breast

cancer, and ovarian cancer, ultimately inducing apoptosis in these cells has been suggested[30-32].

1.7. Justification and Significance

Based on these findings, we hypothesize that blocking the HMGB1 pathway may reduce tumor growth and prevent chemoresistance, thereby improving chemotherapeutic responses in combination with other chemotherapeutic agents. Using an *in vitro* model of MM, we propose here to investigate the therapeutic efficacy of ethyl pyruvate in MM and to determine whether inhibition of HMGB1 signaling may sensitize MM to chemotherapy. We also propose to evaluate the therapeutic efficacy of the novel natural compound, Withaferin A, to investigate its mechanism of action in MM and determine its effects in combination with chemotherapeutic agents. Using this approach, we postulate that signaling by HMGB1 will be impaired, resulting in abrogation of the activation of intracellular signaling pathways, such as NF-κB. We further hypothesize that chemotherapeutic sensitivity will be increased in MM cells.

CHAPTER 2. Literature Review

2.1. Etiology and Pathogenesis

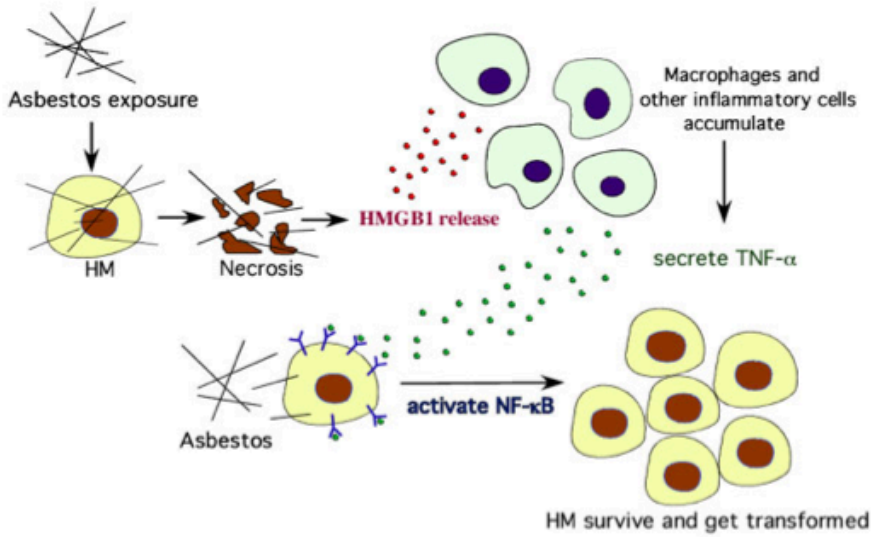
Malignant mesothelioma is an aggressive tumor that arises from the transformation of mesothelial cells that line the pleural, peritoneal, and pericardial cavities. This disease is typically associated to exposure to asbestos. Recently, the disease has also been linked to erionite exposure. This mineral fiber has no commercial applications, but it is present in the rocks of many world geographic areas, thus being a widespread environmental carcinogenic fiber. Other co-factors to MM pathogenesis include SV40 infection, genetic predisposition, and radiation exposure.

2.1.1. Asbestos and Erionite Exposure

Asbestos refers to a family of silicate minerals either in the serpentine form, i.e. chrysotile, or in the amphibole form, which includes crocidolite, anthophyllite, actinolite, amosite, and tremolite. Asbestos has been found to cause MM, but also other diseases, such as, asbestosis, pleural fibrosis/plaques, lung cancer, and laryngeal cancer[1, 6]. Crocidolite is considered the most oncogenic type of asbestos causing about 2,500 deaths per year in the US. In Syrian hamster embryonic cells asbestos can cause malignant transformation by altering chromosomal morphology and ploidy, through mechanically interference with mitotic segregation. Asbestos fibers are very cytotoxic to primary human mesothelial cells *in vitro*. It has been clarified that asbestos exposure induces an inflammatory reaction, contributed by macrophages that phagocytize asbestos, then releasing numerous cytokines and reactive oxygen species that are mutagenic[33-35]. The most prevalent of these cytokines, TNF-α, has been linked to asbestos pathogenesis[36]. High exposure to asbestos has been correlated to a high amount of TNF-α release as well as the induction of TNF-R1 expression in HM. The necrotic cell death of HM upon asbestos exposure causes the release of HMGB1, which attracts macrophages, in turn inducing the secretion of TNF-α.

TNF-α promotes the activation of the NF-κB pathway in HM, allowing for their survival and transformation[4].

Erionite is a potent carcinogenic mineral fiber, capable of causing pleural and peritoneal MM. It belongs to a group of minerals called zeolites. Unlike asbestos, which has been related to MM and other diseases and cancers[37], erionite has been found to specifically cause only MM in both humans and in animals[38, 39]. The carcinogenic potential of erionite is much greater than asbestos. When animals were injected either with erionite or asbestos, it was found that 48% of animals injected with asbestos developed MM compared to 100% MM incidence in animals injected with erionite[6].



Molecular Mechanisms of asbestos-induced carcinogenesis[6].

2.1.2. Simian Virus 40

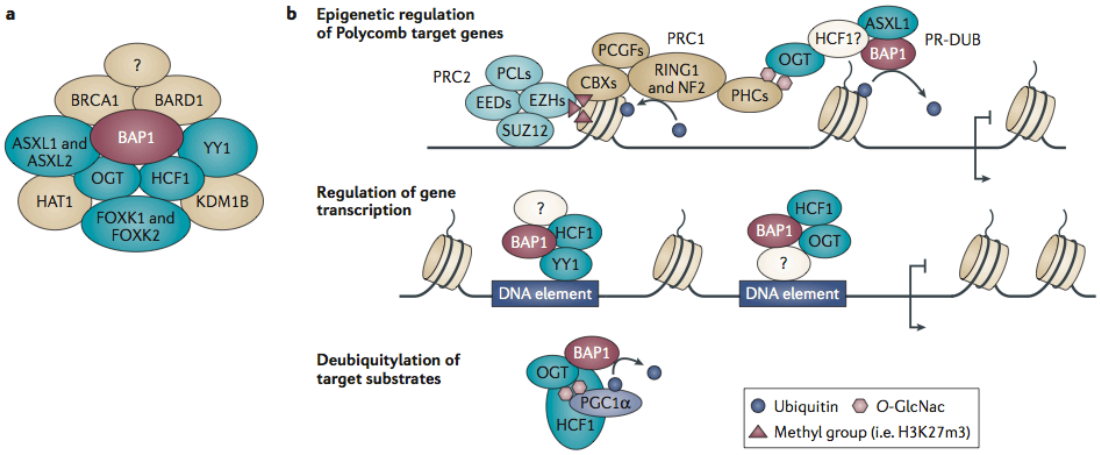
Exposure to the simian virus 40 is a co-factor that can increase the incidence of mesothelioma when combined with asbestos exposure[6]. SV40 belongs to a group of double-stranded DNA-containing viruses called polyomavirus. It encodes two transforming proteins, the large tumor antigen (Tag) and the small tumor antigen (tag), and three capsid proteins (VP1-3). Brain and bone tumors, lymphomas, and MM tumors have been found to contain

SV40 sequences. SV40 carcinogenesis is accompanied by Tag binding and inactivation of the tumor suppressor genes Rb and p53 proteins, while tag inhibits PP2A, causing the upregulation of the transcription factor AP-1. However, Dr. Carbone's group demonstrated that Tag can also form a multi-protein complex with p53 and other proteins like pRb and p300, which acquires transcriptional activity on the promoter of the IGF-1 (Insulin-like Growth Factor-1) gene. This increases the secretion of IGF-1 and activation of its downstream pathway, involved in malignant cell growth. These data provide a novel model for DNA tumor virus transformation, where p53 acts not as a passive inactive partner of Tag, but actively contributes to the activation of an oncogenic pathway[40]. SV40 has been detected specifically in the MM cells and not with stromal or adjacent lungs tissues, indicating that SV40 is not an artifact or contamination. Additionally, the virus induces specific biological changes in the infected MM cells, such as the activation or upregulation of the Met and Notch 1 oncogenes and of insulin-like growth factor 1. In HM cells, the high endogenous levels of p53 make them extremely susceptible to SV40-mediated transformation[41, 42].

2.1.3. Genetic Predisposition

Genetic predisposition is another co-factor that makes some individuals in familial cohorts more susceptible to develop malignant mesothelioma than others, when exposed to minimal fiber exposure. This susceptibility derives from an autosomal dominant mutation. BAP1 has been identified as the gene that is mutated and associated with high rates of mesothelioma in two families from Louisiana and Wisconsin. BAP1 is a member of the ubiquitin C-terminal hydrolase subfamily of deubiquitylating enzymes. In these two families, BAP1 mutation correlated with individuals with mesothelioma, uveal melanoma, and other cancers. These germline BAP1 mutations are indicative of a novel cancer syndrome characterized by the onset of benign melanocytic skin tumors at an early age, and high incidence of mesothelioma, uveal melanoma, cutaneous

melanoma and other cancers later in life. BAP1 and its relation to MM pathogenesis have yet to be elucidated in mesothelioma and other cancers. Putative BAP1 protein partners that form the BAP1 core complex have been identified by co-immunoprecipitation and affinity-capture mass spectrometry as HCF1, YY1, ASXL1, ASXL2, OGT, FOXK1, and FOXK2. Other hypothetical complexes that bind to this BAP1 core complex have varied from cell types and conditions[43, 44].



BAP1 protein partners and proposed functions[44].

2.1.4. HMGB1

High-mobility group box-1 (HMGB1) is a DNA-binding nuclear protein released actively in response to cytokine stimulation or passively during cell death[45]. In healthy cells, HMGB1 is found primarily in the nucleus, where it stabilizes chromatin and is involved in multiple roles, such as, DNA replication, transcription, and recombination[46]. During programmed necrosis, HMGB1 translocates to the cytosol and the extracellular space, due to the increase of PARP, where it binds to several proinflammatory molecules and triggers inflammatory responses[47, 48]. Secreted HMGB1 stimulates RAGE, TLR2, TLR4 expressed on macrophages and other inflammatory cells, inducing the release of several inflammatory cytokines, such as TNF-α and IL-1B. HMGB1 also enhances NF-κB activity, which promotes tumor formation, progression, and

metastasis. Overexpression of HMGB1 and its receptors have been found in many cancers to promote cancer cell proliferation and decrease apoptosis[49, 50]. Overexpression of HMGB1 has also been linked to the increase of oncoproteins cyclin D/E, which regulate cell proliferation, while decreasing p53 and other tumor suppressor proteins that regulate cell apoptosis[51].

In mesothelioma, HMGB1 functions as a master switch initiating inflammatory responses that cause malignant transformation in asbestos- or erionite-damaged human mesothelial cells[52]. Asbestos exposure in HM cells causes DNA damage, creating a pool of aneuploidy mesothelial cells. These events initiate a process of programmed cell death, which leads HMGB1 to translocate from the nucleus to the cytosol and to the extracellular space. This release of HMGB1 originates an inflammatory response, with increased secretion of TNF-α in macrophages. TNF-α release protects HM from asbestos-induced cell death by activating the NF-κB survival pathway and promoting transformation of aneuploidy HM cells into cancer cells[5].

In established MM tumors HMGB1 and its receptor RAGE are highly upregulated, with HMGB1 being highly expressed in MM tissues and sera of MM patients. HMGB1 was found to induce migration, cell motility, invasiveness, anchorage-dependent growth, and proliferation in MM cells and is responsible for its viability. The release of HMGB1 by MM also acts as an autocrine circuit, influencing its proliferation and survival[8].

2.2. Epidemiology

2.2.1. Asbestos and Erionite Exposure

The incidence of MM in the US is estimated to be between 1-2 million in states with minimal exposure and 10-15 million in states where large amount of asbestos were used. The commercial use of asbestos in the early and mid 20th century has been associated to the development of MM. Before the 1950s, MM tumors were very rare, but today, there are approximately 3,000 deaths per year in the US and 5,000 deaths per year in Western Europe. The incidence rates are

higher for males than females with a ratio of 5 to 1, respectively. This has been typically associated to a work-related higher exposure to asbestos occurring in men. The latency of MM varies between 25 and 71 years. It is thought that the latency correlates with the amount of exposure to asbestos, as trades workers with higher exposure to asbestos appeared to develop MM earlier compared to workers exposed to lower amount of these carcinogenic fibers[53]. However, the relationship between latency period and amount of exposure is not clear. In fact, in the Turkish villages of Karain, Sarihidir, and Tuzkoy, where homes were built from stones containing erionite, nearly 50% of the individuals died of MM, with incidence and latency periods which were similar among individuals who left their villages and those who continued to live in their villages with prolonged exposure to erionite. This evidence suggests that, once a sufficient amount of asbestos or erionite has been inhaled, MM will be initiated, and additional exposures will not increase the risk significantly. Additionally these finding indicate that some individuals may be more susceptible to asbestos or erionite exposure than others[37, 39]. This difference in susceptibility can depend on an individual's genetics, exposure to other cofactors (e.g., SV40), and the type of mineral fiber inhaled. Currently about 100% of those who develop MM die because of this cancer. It has been estimate that more than 100,000 US citizens are expected to die MM in the next 40 years. Due to MM's long latency period, MM mortality rates will continue to increase by 5-10% per year in industrialized countries for the next 2-3 decades despite asbestos removal efforts[54, 55].

2.2.2. Simian Virus 40

Simian Virus 40 (SV40), a DNA tumor virus endemic to Asia macaque monkeys, was exposed to millions of people between 1954 and 1978 due to contaminated Salk and Sabin polio vaccines. Currently the epidemiological evidence to support that SV40-contaminated viruses caused mesothelioma in humans is debated, however, analysis of human MM biopsies revealed that SV40 sequences were present in about 50% of the tumors[6].

2.2.3. Genetics and Malignant Mesothelioma

The first evidence of genetic predisposition in MM came from the Cappadocian region of Turkey in the villages of Karain, Sarihidir, and Tuzkoy where the inhabitants build their homes from stones containing erionite. This has caused the villages to have 50% of their deaths be due to MM. Differently from Europe and US, the incidence of MM in this region is almost equal in both sexes. It was also observed in Cappadocia that MM was prevalent in certain families and less other families. This susceptibility was found to be autosomal-dominant in those families with high incidence of MM[56].

This study led for the search of mesothelioma susceptibility gene or genes. In this search, two unrelated families from Louisiana and Wisconsin (L family and W family) were found to have a high incidence of mesothelioma with minimal exposure to asbestos[43]. Two members in one of these families were found to have developed uveal melanoma (UVM). One of these two members later developed mesothelioma. The likelihood of both diseases developing in more than one individual in the same family is 36 per trillion, indicating a genetic denominator. Microarray and genetic linkage analyses on the L and W families implicated the 3p21 chromosome region, which is a locus that frequently altered in UVM and MM. Sequencing of this region led to identification of BAP1. In these two families, there is a 69.74% cancer incidence among 76 mutation carriers, with some individuals who developed malignancies at an earlier age than observed in the United States. The age range at diagnosis for mesothelioma in these families is between 37-85 (with a mean of 55.2 years), compared to the median age of 74 years in the US[6, 44].

2.2.4. Radiation

Exposure to radiation has been found as a co-factor to developing MM[57]. In a study of 5 female MM patients with a previous history of radiation exposure, one woman was not exposed to asbestos but was given radiation therapy for the treatment of a Wilm's tumor. The other four women had household exposure to

asbestos, but pathology showed no pleural plaques or asbestosis, suggesting that their exposure to radiation therapy for other malignancies is the causal factor[6].

2.3. Diagnosis

Currently, mesothelin, a tumor antigen highly expressed in mesothelial pleural cells of the epithelial subtype, is used as a serum biomarker for tentative diagnosis of mesothelioma. Although it is highly specific, mesothelin has poor sensitivity, which limits its ability as a screening marker. Since most MM tumors are heterogenetic, immunohistochemistry helps to distinguish the difference between MM and metastatic carcinoma[58]. More recently a novel, promising blood and pleural effusion marker, fibulin-3, has been characterized and validated[59].

2.4. Treatment of Mesothelioma

2.4.1. Surgery

Surgery for the majority patients that are diagnosed with MM is not an option due to the diffuse spreading growth of the tumor and if the tumor is unresectable. Currently, surgery techniques that are used to resect tumors include extrapleural pneumonectomies (EPP), pleurectomy/decortication (P/D), and lung-sparing cytoreductive surgery. According to Mesothelioma and Radical Surgery (MARS), which published a multicenter randomized controlled trial that compared P/D versus EPP, found that patients treated with P/D had an equal to better outcome than those treated with EPP. Lung-sparing cytoreductive surgery is usually combined with chemotherapy and radiation. The use of surgery depends on the disease stage, the patient's cardiopulmonary reserve, surgeon's experience, and the extent of planned adjuvant therapy. Often the use of surgery as a single-modality therapy is not used rather multimodality strategies are used such as EPP combined with adjunctive therapies such as immunotherapy,

chemotherapy, and radiotherapy. The type of multimodality strategy is determined on a case by case basis[60].

2.4.2. Chemotherapy

Currently, there are no curative chemotherapy regimens to combat MM. However, the combination of pemetrexed and cisplatin has shown promising results. Pemetrexed plus cisplatin is a combination of a multi-targeted antifolate and a platinum compound. This combination has shown in a phase III consisting of 448 patients to improve overall survival by about 3 months and improve the response rate by 41% compared to cisplatin alone[24].

Other combinations have included, gemcitabine plus cisplatin, which has shown to have similar responses to the pemetrexed plus cisplatin combo, presenting symptomatic improvement, higher quality of life, and a response rate of 48% in 74 patients[61].

Currently, the standard of care for first line palliative chemotherapy is pemetrexed and cisplatin in patients with a good performance status along with a prophylactic dexamethasone plus supplemental folic acid and vitamin B12. The second line of chemotherapy drugs is gemcitabine plus cisplatin[24].

2.4.3. Novel Treatment for MM

Novel treatment of MM includes multimodality therapies that include anti-angiogenic drugs, immunotherapy, and drugs that affect growth factor pathways. The use of anti-angiogenic drugs has been investigated in the treatment of MM, since mesothelioma cells secrete angiogenic factors such as VEGF, PDGF, and PDGFR. However, bevacizumab, a VEGF-blocking monoclonal antibody has shown in phase II and phase III trials to show minimal improvements compared to cisplatin and pemetrexed alone as a first line treatment. In a phase II trial, the addition of bevacizumab to cisplatin and pemetrexed in advanced MM showed an overall survival of 14.8 months and a median progression-free survival of 6.9

months, which did not meet the minimal primary endpoint of a 33% improvement compared to chemotherapy alone.

The use of immunotherapies in MM includes inhibiting TNF-α and mesothelin. NGR-hTNF selectively binds to CD13 overexpressed on the epithelial cells of solid tumors. In a phase II trial that evaluated the use of NGR-hTNF every three weeks versus weekly in 43 and 14 patients, respectively, showed 2.8 months of progression-free survival and 46% of patients achieved disease control. SS1P, an immunotoxin-linked antibody against mesothelin was used as a front-line treatment in 19 patients with cisplatin plus pemetrexed. The use of 45 mcg/kg of SS1P along with cisplatin plus pemetrexed was well tolerated and achieved partial responses of 50%. Additionally, serum mesothelin response in 63-83% of patients correlated with radiological responses in all patients who obtained a partial response. Another antibody used against mesothelin is amatuximab, which is a high affinity chimeric monoclonal antibody. A phase II trial using amatuximab with cisplatin plus pemetrexed and as a maintenance therapy in 89 patients showed an overall survival of 14.5 months and progression-free survival of 6.1 months[62].

2.4.4. Clinical Trials

Current clinical trials include the use of imatinib mesylate in combination with cisplatin and pemetrexed, against mesothelioma. Pemetrexed and cisplatin is also being studied after surgery and radiation therapy. New drugs targeting different signaling pathways that have been found to be activate in mesothelioma, are being studied as a second line of treatment. These include the combination of bevacizumab and eriotinib, Tivantinib, or Dasatinib in previously treated MM. PTK787/ZK 222584, Cediranib and other VEGF Receptor family inhibitors are currently tested in patients with unresectable MM. Another Phase II clinical trial investigates the use of everolimus, an inhibitor of mammalian target of rapamycin (mTOR) in patients with loss of Merlin/NF2 loss as a biomarker to predict sensitivity. (www.clinicaltrials.gov).

2.5. New Molecular Targets

2.5.1. EP

2.5.1.1. Derivative

Pyruvate, the anionic form of 2-oxo-proprionic acid, plays a central role in intermediary metabolism. It is the final product of glycolysis and stands at the junction between anaerobic and aerobic pathways. In anaerobic respiration, pyruvate is used as the starting point for fermentation, yielding lactate. For aerobic respiration, pyruvate is transported to the mitochondria to be used in the tricarboxylic acid (TCA) cycle. Pyruvate functions as an endogenous scavenger of reactive oxygen species (ROS) and has been reported as a treatment for organ injury or dysfunction in myocardial, intestinal, and hepatic ischemia/reperfusion-induced injury in animal models. Pyruvate-containing solutions have also been shown to be beneficial in animal models of galactose, stroke, hemorrhagic shock, and pressure-induced retinal damage. Despite its positive effects, the usefulness of pyruvate as a therapeutic is limited due to its poor stability in solution. It can go through a spontaneous adol-like condensation reaction to form parapyruvate, an inhibitor in the TCA cycle.

To combat problems of stability in pyruvate, ethyl pyruvate, the simple derivative of the endogenous metabolite, pyruvic acid, was created. Ethyl pyruvate has been found to be more stable and more effect than pyruvate[63].

2.5.1.2. Molecular Targets of EP

Treatment with EP has been found to downregulate the expression of various inflammatory genes, including inducible nitric oxidase synthase (iNOS), tumor necrosis factor (TNF), cyclooxygenase-2 (COX-2), and interleukin 6 (IL-6). It has also ben found inhibit the pro-inflammatory transcription factor NF-κB and act as an anti-inflammatory agent[19].

The anti-inflammatory effect of EP has been found in RAW 264.7 macrophage-like cells and lipopolysaccharide (LPS)-induced BV2 microglial cells by suppressing the activation of NF-κB, extracellular signal-regulated kinase

(ERK), and p38 mitogenactivated protein kinase (MAPK) pathways[64]. EP inhibits NF-κB-mediated-signaling in a dose-dependent manner through covalently modifying the NF-κB subunit, p65 inhibiting its DNA-binding and allowing for gene transcription[21].

2.5.1.2.1. Relation to HMGB1

EP inhibits secretion of HMGB1 by preventing the nuclear-to-cytoplasmic translocation of HMGB1, which may contribute to the anti-inflammatory effects of EP[20]. In cancer cells, inhibition of HMGB1 release by ethyl pyruvate led to apoptosis and decreased autophagy in stressed cancer cells, increasing tumor cell sensitivity to anticancer agents, such as melphalan, paclitaxel, oxaliplatin, and Adriamycin[10].

2.5.1.3. Clinical Relevance

The pharmacological effects of ethyl pyruvate include inhibition of redox-mediated cellular damage, inhibition of inflammation, cytoprotection, promotion of apoptosis, and inhibition of cancer cell growth. EP inhibits redox-mediated cellular damage through nonenzymatic scavenging of H_2O_2 and other reactive intermediates. Inflammation is inhibited through decreasing NF-κB dependent signaling and JAK/STAT-dependent signaling due to scavenging of ROS. Cytoprotection of EP is also due to scavenging of ROS and augmented ATP production. Finally EP inhibits cancer cell growth through promotion of cancer cell apoptosis and inhibition of HMGB1[10, 21].

2.5.2. WA

2.5.2.1. Origin and Uses

Withaferin A (WA) is steroidal lactone purified from the leaves of the medicinal plant *Withania somnifera*, also known as Ashwagandha, Indian ginseng, or Indian Winter cherry. This plant is found mainly in the dryer regions in India, the Middle East, North Africa, and the Mediterranean regions. In

Ayurvedic medicine, the roots are used to treat chronic fatigue, dehydration, and rheumatism. The berries and leaves are traditionally used as a topical treatment for tumors and ulcers. In clinical trials, *W. somnifera* was to be beneficial in inflammation and immune modulation, periodontitis, reducing anxiety, and reducing arthritis pain.

2.5.2.2. Derivative

Many analytical approaches have been used to study the bioactive entities in *W. somnifera.* From the plant, steroidal lactones, alkaloids, flavonoids, and tannins are among the chemical constituents identified and extracted. The major chemical constituents are withanolides of which there are different structures. The first compound from the group to be isolated was WA. It was found to be a highly oxygenated withanolide and one of the most biologically active. It has been found to be anti-inflammatory, tumor preventive, cell death inducing, anti-tumor, radiosensitizing, and anti-angiogenic. The chemical and molecular mechanisms through which WA exerts all these effects is still speculative. Proposed chemical mechanisms include acylation or alkylation of critical macromolecules or enzymatic active sites by covalent attachment[26].

2.5.2.3. WA's Molecular Mechanisms of Action

The exact molecular mechanisms of WA are still elusive. Several biological functions have been influenced by WA including induction of apoptosis through inactivation of Akt and NF-κB, decrease of pro-survival protein Bcl-2, induction of Par-4, inhibition of Hsp90 and Notch-1, G2/M cell cycle arrest, vimentin degradation, FOXO3a and Bim regulation, generation of ROS, and inhibition of the proteasomal chymotrypsin subunit β5[29, 30].

2.5.2.3.1. WA's Signaling Pathway Targets

Vimentin, a type III ubiquitous mesenchymal intermediate filament, supports the structural integrity in quiescent cells and participates in adhesion,

migration, survival, and cell-signaling processes via dynamic assembly and disassembly in activated cells. Vimentin along with microtubules and actin microfilaments make up the cytoskeleton. It is an organizer of critical proteins involved in attachment, migration, and cell signaling, regulated by a complex phosphorylation pattern. Enhanced expression of vimentin has been correlated in cancerous cells to the induction of epithelial to mesenchymal transition or EMT, leading to an increase in metastatic disease, poor prognosis, and reduced patient survival. It has been shown that WA binds to vimentin and alters the distribution of its intermediate filaments (IF) in cultured endothelial cells. It has been proposed that WA exerts its anti-migratory and anti-apoptotic functions through disruption of IF organization, which is required for normal cell locomotion as well as cell division. In fact, WA was shown to be ineffective in vimentin knockout mice or knockout cells. In a model of soft tissue sarcomas (STS), WA induced apoptosis in a time and dose-dependent manner and was found to induce vimentin degradation. Using an anti-vimentin siRNA to knockdown vimentin in STS cells, the authors showed that vimentin knockdown cells blocked WA-induced apoptosis, suggesting that vimentin degradation is necessary for enhanced therapeutic effect. This group also demonstrated that WA- induced vimentin degradation is caspase-dependent. Since vimentin degradation is a result of caspase cleavage, Z-VAD, a pan-caspase inhibitor, was used to pre-treat STS cells and was found to significantly abrogate WA-induced apoptosis. Finally, this group demonstrated that epithelial cancers expressing high levels of vimentin and exhibited EMT were significantly more sensitive to WA than the epithelial cancers that do not express vimentin[26, 65].

NF-κB is a transcription factor, which regulates many processes, including cell survival, growth, angiogenesis, susceptibility to apoptosis, as well as inflammatory responses. Altered regulation of NF-κB signaling has been linked to cancer and inflammatory diseases. NF-κB signaling is triggered by several inflammatory stimuli including Tumor Necrosis Factor (TNF), Interleukin-1 (IL1), and microbial pathogens, which activates their cognate receptors and receptor-

specific signaling pathways. These pathways converge in the activation of NF-κB. NF-κB is normally present in the cytoplasm in its inactive state, as a heterodimer, composed of a 50- and 65-kDa subunits, complexed with the inhibitory sub-unit IκB. Upon stimulation, the IκB-kinase (IKK) complex is activated, and phosphorylates IκB, which in turn is ubiquinated and degraded; NF-κB then can translocate into the nucleus, where it binds to consensus sequences in the promoter region of target genes, altering their expression[26]. The IKK complex is composed of the catalytic kinase subunits IKKα and IKKβ, and the adaptor protein IKKγ (aka NEMO). *In silico* molecular docking analysis has revealed that WA disrupts the complex of NEMO and IKKβ, by forming strong intermolecular interactions with NEMO. This has been proposed to prevent phosphorylation and degradation of the inhibitory protein IκBα, leading to inhibition of NF-κB activation and its nuclear localization[66].

WA was also found to exert anti-proliferative effects via induction of a heat shock response and altering Akt/mTOR and MAPK signaling pathways in an *in vitro* model of glioblastoma multiforme (GBM). In this model, WA induced a dose-dependent shift in cell cycle arrest from the G0/G1 checkpoint to G2/M arrest at doses less than 2 μM. The shift to G2/M cell cycle arrest was accompanied by induction of a heat shock stress response through upregulation of HSP70, HSP32, and HSP27, and downregulation of HSF1. A dose-dependent inhibition of Akt and mTOR was also observed, while AMPKα and the tumor suppressor tuberin/TSC1 were upregulated in these cells[31].

2.5.2.3.2. WA and HMGB1

The relationship between the HMGB1 pathway and WA has not been thoroughly elucidated. To date, only one study has investigated this relationship in a model of HMGB1-induced inflammation, where WA exerts a barrier protective effect in human umbilical vein endothelial cells (HUVECs) *in vitro* and in a mouse model. In this study, the authors found that WA inhibited LPS-induced HMGB1 release in a dose-dependent manner. WA was also found to

protect HUVEC cells from either LPS- or HMGB1-mediated barrier disruption. These effects were confirmed *in vivo*, where an inhibition of vascular permeability was observed upon WA treatment in conjunction with suppression of HMGB1-mediated VCAM-1, ICAM-1, and E-selectin protein expression. A decreased binding of monocytes to HMGB1-stimulated endothelial cells was also observed in the presence of WA. Finally, this study indicated that WA was able to significantly inhibit HMGB1-mediated activation of NF-κB, via inhibition of NF- κB and IκBα phosphorylation. WA also significantly decreased the pro-inflammatory cytokines stimulated by HMGB1, TNF-α and IL-6. These effects were achieved at WA concentrations around 1 and 2 μM[27].

2.5.2.4. WA in Mesothelioma and Other Cancers

The anti-cancer effects of WA have not been thoroughly investigated yet. In breast cancer, WA was shown to inhibit metastasis and epithelial to mesenchymal transition[67], while in prostate cancer cells it induced mitotic catastrophe and growth arrest[68]. To date, only one group has studied WA's effect on mesothelioma[29]. In this study, the authors found that WA inhibits MM cell proliferation at doses up to 5 μM WA. At 10 μM WA, WA inhibited proteasomal chymotrypsin-like activity as early as 2 hours of WA exposure. Western blot analysis showed an increase of ubiquitinated proteins after 2-24 hours of 10 μM WA treatment in human and murine MM cells and the accumulation of proteasomal targets p21 and Bax, indicating inhibition of the proteasome by WA. The cell inhibitory protein p21, the pro-apoptotic protein Bax, and the inhibitor of NF-κB, IκBα, showed a time-dependent increase after WA treatment (10 μM). Increase of these proteins indicates that WA may inhibit MM cell growth partly through the induction of apoptosis. This was confirmed by the increase of PARP cleavage in a dose dependent manner from 2.5-10 μM WA. There was also a 3.5-4.7 fold increase in caspase 3/7activity from 2.5 and 5 μM WA. This study also observed apoptotic nuclei in MM cells treated with 10 μM WA. Gene-array based analyses revealed that WA suppressed a number of

cell growth and metastasis-promoting genes including c-myc and c-jun. WA treatments also stimulated expression of CARP-1/CCAR1, a biphasic transducer of cell growth and apoptosis signaling. Finally, they found that female Balb/c mice with AB12 murine MM cell-derived tumors exhibited inhibition of tumor growth after intra-peritoneal administration of 5 mg/kg WA for up to 17 days of treatment. Proteins from the tumor remnants indicated inhibition of proteasomal chymotrypsin-like activity and accumulation of ubiquitinated proteins and Bax. Immunostaining also revealed increased apoptotic cells through TUNEL staining, increased CARP-1 and p27, and decreased c-myc expression. This indicates that inhibition of MM tumor growth *in vivo* is in part due to inhibition of the proteasome and stimulation of apoptosis[29].

2.5.2.5. Clinical Relevance of WA and Current Clinical Trials

In vitro and *in vivo* data have shown that WA is able to inhibit molecular targets involved in anti-inflammatory, pro-apoptotic, anti-angiogenic, and anti-proliferative functions, both in cancer cells and endothelial cells. WA has also been shown to inhibit epithelial to mesenchymal transition. These biological activities make WA an ideal drug candidate for the treatment of different types of cancer, including mesothelioma[26]. The effects of WA is currently being studied in osteosarcoma, stress, inflammation, and immune cell activation, periodontics, generalized anxiety disorder, bipolar disorder, schizophrenia, non-alcoholic steatohepatitis, and memory impairment (www.clinicaltrials.gov.).

CHAPTER 3. Results

3.1. Ethyl Pyruvate decreases the survival of MM cell lines.

We have previously shown that most MM cells secrete HMGB1 and that HMGB1 is required for their survival[8]. Since it is known that EP inhibits HMGB1 release, we tested whether EP could decrease survival of MM. MM cells were treated for 24 hours and cell viability was assessed by MTS assay. After 24 hours, all three MM cell lines showed a decreased in cell survival in a dose-dependent manner (Fig. 1).

These results indicate that EP is able to decrease cell survival possibly via inhibition of HMGB1, which is critical to MM survival.

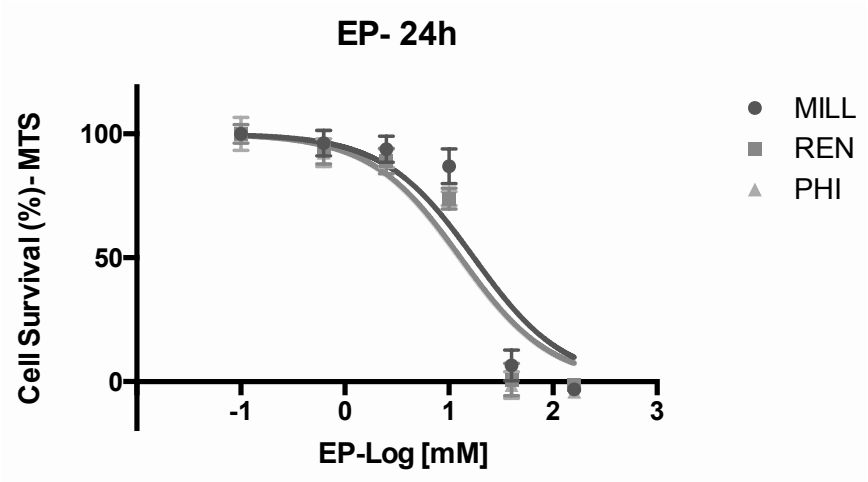


Figure 1. EP decreases the survival of MM cell lines. A. MM cells were treated with either vehicle or indicated doses (0-160mM) of EP for 24 hours. Cell survival was determined using the Promega MTS assay. EP decreased cell survival in all MM cell lines (MILL, REN, and PHI) in a dose-dependent manner. IC50 for MILL, REN, and PHI was 17.36 mM, 12.95 mM, and 12.51 mM, respectively. Data depicts one representative experiment out of three preformed with six replicates per each concentration.

3.2. Ethyl Pyruvate inhibits the motility of MM cells.

HMGB1-specfic antagonists (BoxA, anti-HMGB1, and anti-RAGE antibody) have previously shown to significantly reduce directional cell motility in wound healing assays of HMGB1-secreting MM cell cultures[8]. We therefore wanted to investigate whether EP may exert a similar effect. Our results show

that pre-treatment with EP before wounding the cell monolayer, significantly ($P<0.05$) delayed wound healing in PHI and REN cells, compared to vehicle (PBS)-treated cells (Fig. 2A,B).

We have also previously shown that targeting HMGB1 with specific antagonists decreases MM cell migration rate[8]. Here we show that treatment with EP significantly ($P<0.05$) abrogated the migration of REN and HMESO cells in a Transwell migration assay (Fig. 3A, B).

These results indicate that EP is efficient in reducing both directional cell motility (wound healing) and non-directional motility (migration) of HMGB1-secreting MM cells.

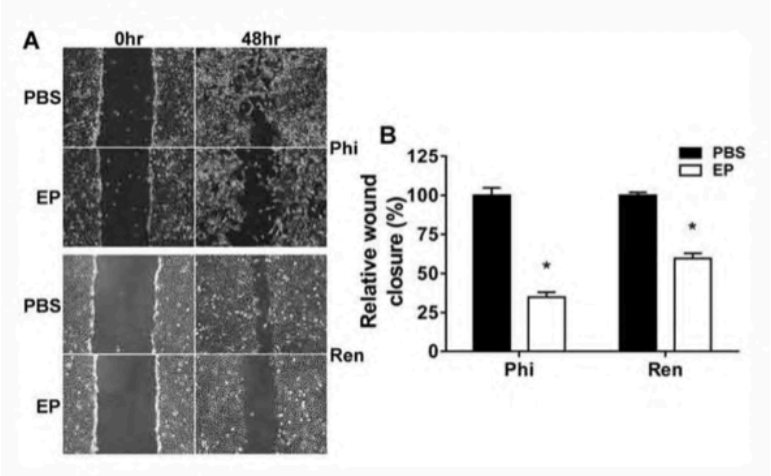


Figure 2. EP inhibits the directional migration of MM cells. A. Wound healing assay. MM cells were seeded in 6-well plates and grown to 80-90% confluence in DMEM plus 1% FBS. One hour before wounding, the cells were treated with EP (5mM) or vehicle (PBS). The cell monolayer was wounded with a P200 pipette tip, and wound closure was observed after 48 hours. B. For quantification of wound closure, cells migrated into the scratched area were quantified using ImageJ software and normalized to control. EP reduced wound healing in PHI and REN cells. Original magnification, x40. Figure shows representative images of wound healing from 3 experiments done in duplicate. *$P<0.05$; EP versus vehicle (PBS). Error bars represent SEM.

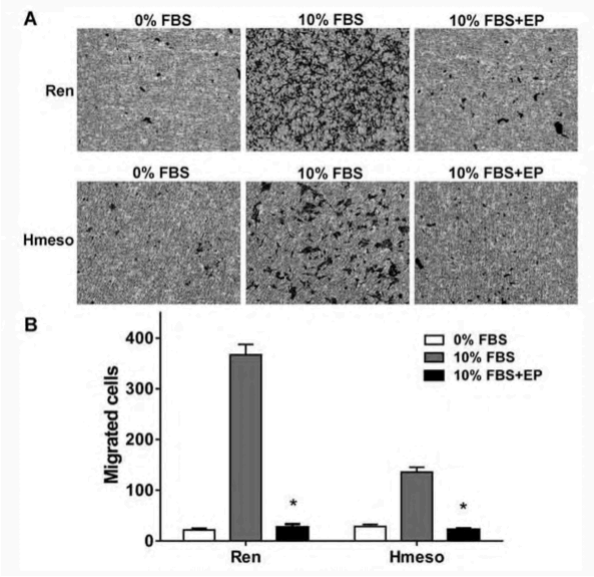


Figure 3. EP inhibits the migration of MM cells. A and B. Transwell migration assay. Cell migration of REN and HMESO MM cells is inhibited by EP (5mM). Original magnification, x40. A. Figures show representative images from three experiments done in duplicate. B. Migrated cells were counted using ImageJ software and represent mean values per field from at least three fields. *P<0.05; 10% FBS+EP versus 10% FBS (positive control). Error bars represent SEM.

3.3. Ethyl Pyruvate impairs the anchorage-independent growth of malignant mesothelioma cells.

The HMGB1 inhibitors BoxA, anti-HMGB1, and anti-RAGE antibodies have been shown to effectively reduce anchorage-independent growth of MM cell lines[8]. Based on these data, we tested whether EP affected anchorage-dependent growth of HMESO and REN cells by soft agar assay. Compared to vehicle, EP caused a significant (P<0.05) reduction of anchorage-independent growth in both cell lines. EP reduced both the size (Fig. 4A) and number (Fig. 4B) of colonies.

These results reveal that EP interferes with a key aspect of the malignant phenotype of human MM cells, anchorage-independent cell growth.

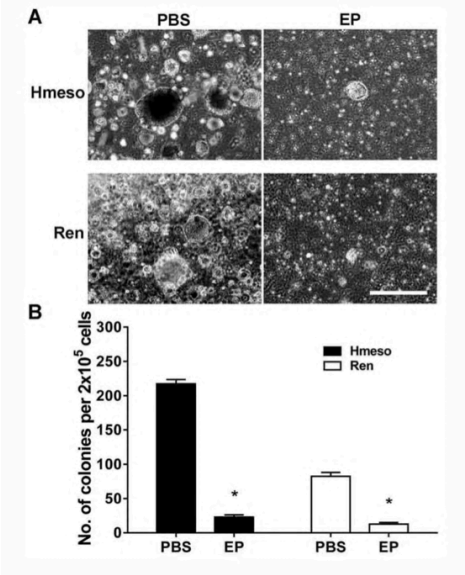


Figure 4. EP disrupts the malignant phenotype of MM cells. A and B. Anchorage-independent cell growth (REN and HMESO 4 x 10^3 cells) was determined by the soft agar assay. After 23 days of culture, the number and size of the colonies formed in the EP-treated and vehicle (PBS) control were evaluated. MM cells treated with EP (0.5 mM) formed smaller (A) and fewer (B) colonies in soft agar compared to vehicle (PBS) control. Original magnification, x40. Figure shows representative images from 2 experiments done in duplicate. Colonies larger than 0.1 mm in diameter were counted using ImageJ software. *P<0.05; EP versus vehicle (PBS) control. Error bars represent SEM. Scale bar=0.5mm.

3.4. Ethyl Pyruvate interferes with the activation of NF-κB in malignant mesothelioma cells.

Previously we have shown that HMGB1 autocrine stimulation enhances the transcription of multiple genes controlled by the activation of NF-κB and downstream genes in MM cells[8]. Regulators of NF-κB, such as IκBα, are NF-κB dependent, generating auto-regulatory feedback loops[69]. Therefore, we tested in REN cells if EP interferes with HMGB1-dependent NF-κB activation by measuring IκBα mRNA expression by quantitative real time PCR.

In REN cells treated with recombinant HMGB1, EP significantly reduced IκBα mRNA expression to levels comparable to control (Fig. 5A). We also assayed NF-κB nuclear translocation in REN and PHI cells by Western blotting.

We found that EP significantly reduced TNF-α-induced NF-κB nuclear translocation in both cells types (Fig. 5B).

These results indicate EP is able to inhibit NF-κB nuclear translocation and its transcriptional activity induced by HMGB1 stimulation in MM cells.

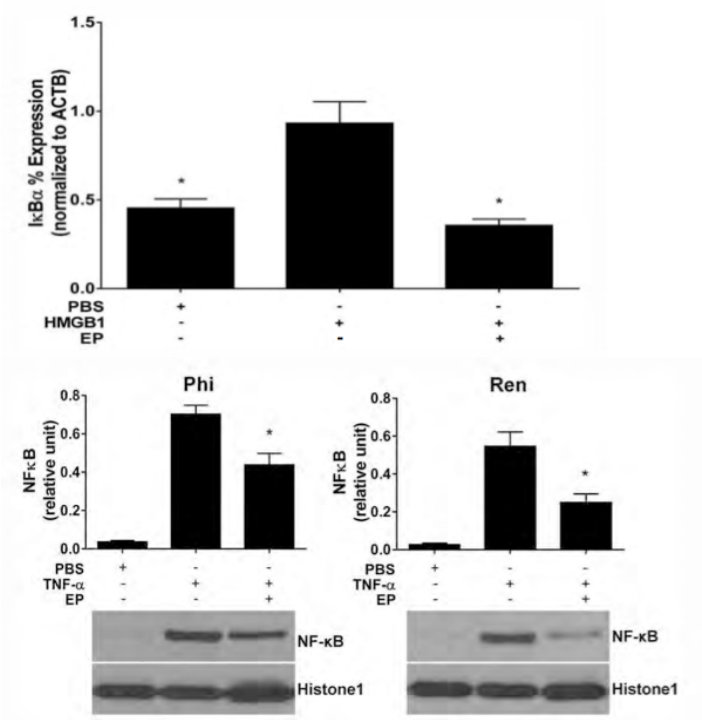


Figure 5. EP suppresses HMGB1-induced NF-κB activation. A. Quantitative real time PCR assay. REN cells (5 x 10^6) were pretreated with or without EP (2.5 mM) for 24 h then stimulated by purified recombinant HMGB1 (100 ng/ml) for 2 h. The cells were then harvested and mRNA extracted and used with IκBα-specific primers to determine the levels of IκBα mRNA. Treatment with EP decreased HMGB1-induced IκBα transcript levels, which suggests an inhibitory effect of EP on NF-κB activation. IκBα mRNA expression was normalized to β-actin (ACTB). Experiments were done in triplicate and performed twice. Error bars represent SEM. *P<0.05; HMGB1+EP versus HMGB1. B. Cells were stimulated with TNF-α (1 ng/ml) for 30 minutes and then treated for 48 h with EP (2.5 mM). Experiments were done in triplicate and performed twice. Error bars represent SEM. *P<0.05; TNF-α+EP versus TNF-α.

3.5. Withaferin A induces apoptosis and the passive release of HMGB1.

WA has previously been shown to reduce tumor growth in malignant mesothelioma *in vitro* and *in vivo* making it an attractive potential chemotherapeutic for MM [29]. It has also been found to inhibit NF-κB activation

with HMGB1-stimulated HUVEC cells[27]. To investigate the effects of WA on MM cells, we first treated MILL, REN, and PHI with a range of WA treatment (0-40μM) for 72 hours. After a 72-hour treatment with WA, all three MM cell lines displayed a decrease in cell viability in a dose-dependent manner (Fig. 6A). The decrease in cell viability did not greatly differ between cell lines with high HMGB1 release (REN and PHI) and low HMGB1 release (MILL), indicating that in our experimental setting, a correlation between HMGB1 release and WA effect on MM survival was not found.

To determine the mechanisms through which WA caused a decrease in cell viability, we measured cytotoxicity for 72 hours using LDH assay. After a 72-hour WA treatment, MILL and REN exhibited a dose-dependent increase in cytotoxicity, which indicates that WA may induce necrosis and/or apoptosis in MM cells (Fig. 6B).

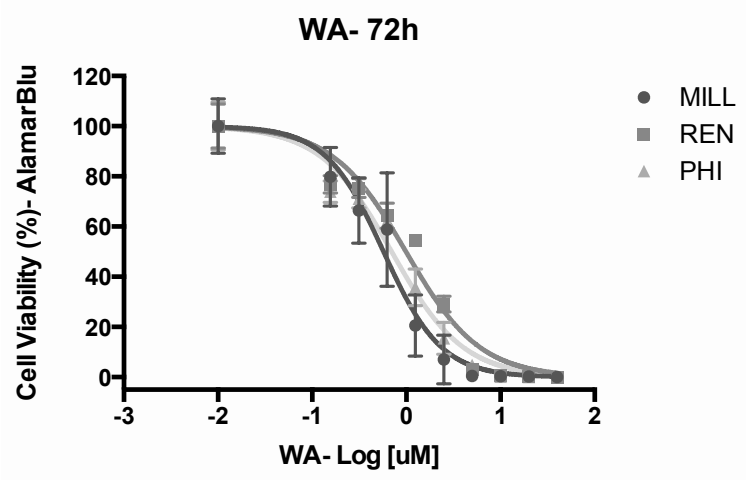
To determine whether the cell death and cytotoxicity that we observed at 72 hours resulted from necrosis or late-stage apoptosis of MM cells, we treated MILL and REN with WA for 6 hours and measured apoptosis, viability, and cytotoxicity using the ApoTox-Glo Triplex Assay. In both MM cell lines, MILL and REN displayed a dose-dependent increase in caspase 3/7 activation (Fig. 6C). MILL and REN also exhibited a significant decrease in viability that correlates with the increase in caspase 3/7 activation (Fig. 6D). Cytotoxicity in MILL increased, but was not significantly higher than control. REN displayed no increase in cytotoxicity compared to control. Comparable results were obtained in PHI cells (data not shown).

To further investigate the role that WA plays in decreasing cell viability and its relationship with HMGB1, we measured the release of HMGB1 secretion in MM cells after a 24-hour WA treatment. After treatment with WA for 24 hours, all three MM cell lines showed an increase in HMGB1 release, with MILL showing a decrease at 10 μM. This decrease could be due to WA inhibiting HMGB1 or significant cell death, responsible for reduced HMGB1 levels detected. PHI

exhibited a significantly high release of HMGB1 at 5 and 10 μM WA compared to MILL and REN and to its control (Fig. 6E).

These results reveal that WA is able to decrease cell viability and increase cytotoxicity in a dose-dependent manner. These effects are likely to be due to WA inducing apoptosis in a dose-dependent manner. The high levels of extracellular HMGB1 observed in MM treated with WA, may be likely result from passive release of HMGB1 due to the high cytotoxicity seen in the later time points.

A.



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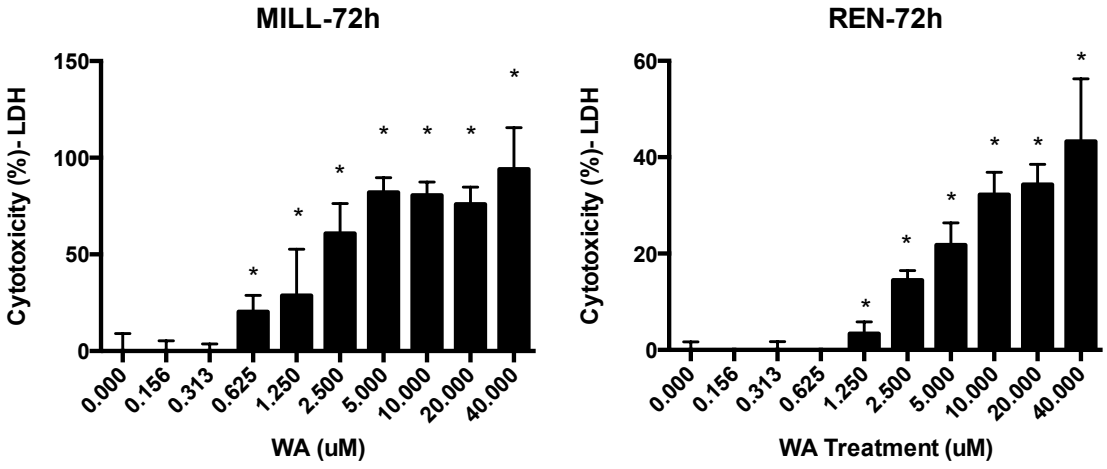
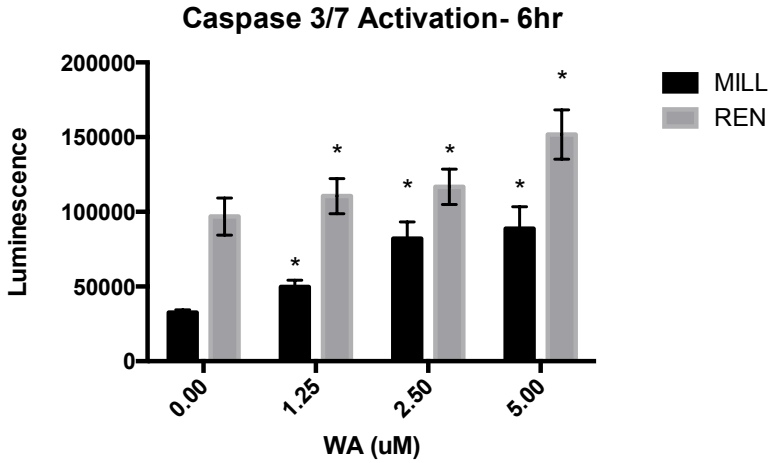


C.


Caspase 3/7 Activation- 6hr
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WA (uM)
MILL
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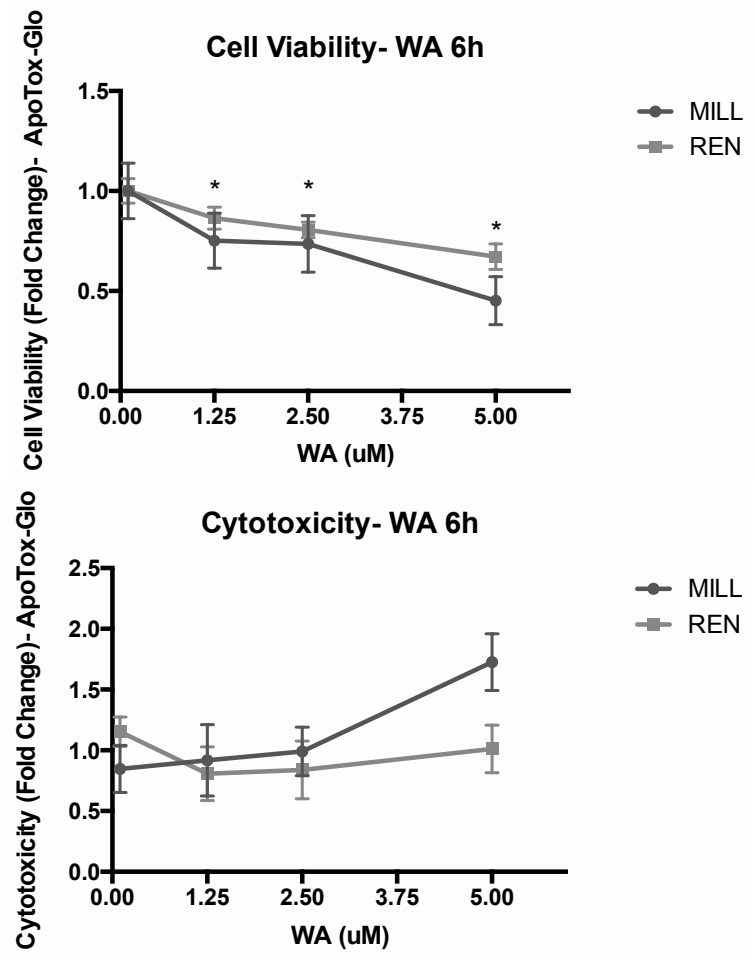
*

D.


Cell Viability- WA 6h
Cell Viability (Fold Change)- ApoTox-Glo
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WA (uM)
MILL
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*
Cytotoxicity- WA 6h
Cytotoxicity (Fold Change)- ApoTox-Glo
2.5
2.0
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WA (uM)
MILL
REN

E.

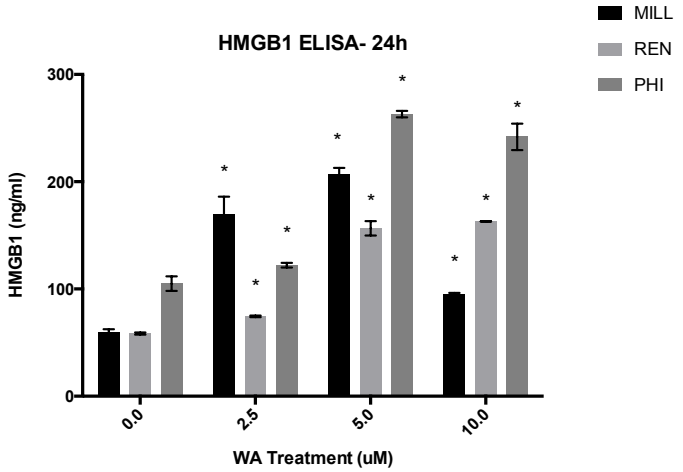


Figure 6. WA induces apoptosis and the passive release of HMGB1. A. Cell viability was determined by the AlamarBlu Assay. MM cells were plated in 1% FBS DMEM, then treated with vehicle (0.1% DMSO) or indicated doses of WA. IC50 for MILL, REN, and PHI was 572.5 nM, 990.1 nM, and 698.4 nM, respectively. Error bars indicate SD. Data represents one of three experiments performed where the mean of 6 replicates for each concentration was calculated. B. Cytotoxicity was determined by LDH assay. MM cells, seeded in 1% FBS DMEM, were treated with either vehicle (0.1% DMSO) or indicated doses of WA. Error bars indicate SD. Data represents one of three independent experiments performed with six replicates. *P<0.05; WA versus control. C. Caspase 3/7 activation was measured by the ApoTox-Glo Triplex Assay. Increase in apoptosis is indicated as an increase in luminescence. Error bars indicate SD. Data represents one of three independent experiments performed with quadruplicates. *P<0.05; WA versus control. D. Viability and cytotoxicity was measured by ApoTox-Glo Triplex Assay fluorescence using the fluorogenic, cell-permeant peptide substrates GF-AFC and bis-AAF-R110, respectively. Data represents the fold change compared to untreated cells. Error bars indicate SD. Data represent one of three independent experiments performed in quadruplicates. *P<0.05; WA versus control. E. The release of HMGB1 in MM cells was determined by HMGB1 ELISA Kit as described in Material and Methods. Culture media for each cell line were collected and concentrated (50x) under identical conditions. MM cell lines were treated with indicated doses of WA for 24h. Error bars indicate SD. Data represents one of three independent experiments performed in duplicates. *P<0.05; WA versus control.

3.6. Withaferin A at low concentrations inhibits NF-κB nuclear translocation in TNF-α stimulated MM cells.

It has been shown previously that TNF-α treatment of mesothelioma cell induces NF-κB nuclear translocation after 30 minutes of treatment [4]. To evaluate

if the NF-κB pathway could be a possible mechanism to induce apoptosis by WA, we incubated the MM cell lines MILL and PHI with low to high concentrations of WA for 24 hours (0-10μM), followed by a 30-minute TNF-α treatment. Densitometry analysis by Image Studio (LI-COR Biosciences) revealed that for all three MM cell lines, all concentrations of WA, starting at 0.5μM, reduced NF-κB nuclear translocation upon TNF-α exposure in a dose-dependent manner. NF-κB was also found in the cytoplasmic fraction of the cell, but did not differ in a dose-dependent manner (Fig. 7). Similar results were obtained in REN cells at 24 and 48 hours and in MILL and PHI cells at 48 hours (data not shown).

These results indicate that WA may be effective in inhibiting NF-κB pathway in mesothelioma.

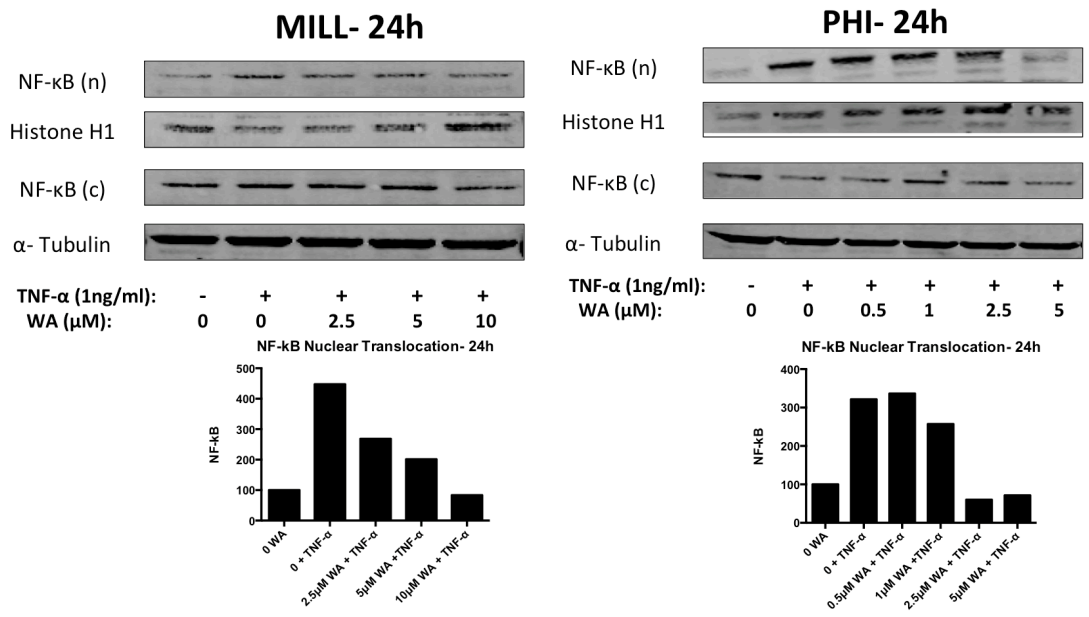


Figure 7. WA at low concentrations inhibits NF-κB nuclear translocation in TNF-α stimulated MM cells. MM cells were treated with vehicle (0.8% DMSO; 0 WA) or with the indicated concentrations of WA for 24h. Levels of nuclear and cytoplasmic NF-κB, Histone H1, and α-tubulin were determined by Western blotting. The percentage of NF-κB nuclear translocation was determined by Image Studio Software (Odyssey). Nuclear NF-κB was first normalized to Histone H1 and then normalized to control (0 WA).

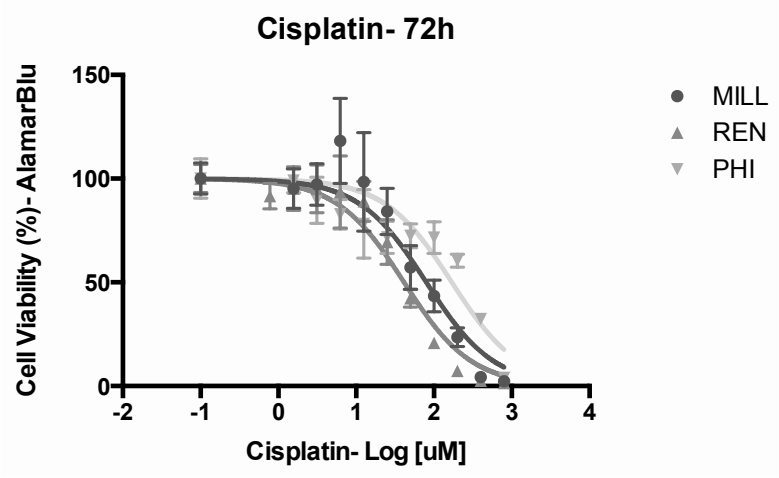
3.7. Ethyl Pyruvate and Cisplatin, a common MM chemotherapeutic, decreases MM cell survival.

Cisplatin alone and in combination with pemetrexed has commonly been used as a chemotherapeutic treatment to mesothelioma[24]. We tested MM cell viability upon cisplatin treatment for 72 hours in MILL, REN, and PHI cell lines. In all cell lines, cisplatin decreased cell viability in the high micromolar range, with REN appearing as the most sensitive to this treatment (IC50: 41.32 μM), although the difference was not significant when compared to MILL and PHI, (IC50 79.85μM and 172.9μM, respectively), (Fig. 8A).

In our previous experiments we found that EP significantly decreased survival of MM cells. Here we sought to investigate whether combining EP with the standard MM chemotherapeutic, cisplatin, may be therapeutically beneficial in MM. We tested survival of MIL REN and PHI cell lines treated with various concentrations of ethyl pyruvate in the presence or absence of cisplatin. In MILL cells treated for 48 hours, the combination of 5 and 10mM EP to either 10 or 40μM cisplatin significantly decrease survival compared to corresponding concentrations of EP or cisplatin alone (Fig. 8B). Similar results were obtained in REN and PHI cells (data not shown).

These results indicate that the addition of EP to cisplatin treatment may reduce the concentration of cisplatin required to achieve the same or stronger effect on MM survival, compared to cisplatin treatment alone.

A.



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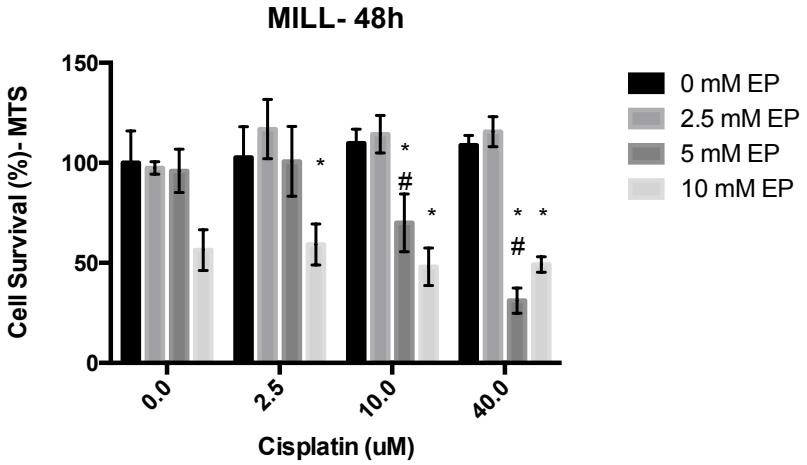

Figure 8. EP and Cisplatin, a common MM chemotherapeutic, decreases MM cell survival. A. Cell viability was determined by AlamarBlu assay. Cells were treated with vehicle (0.4% DMSO in 1% FBS DMEM) or indicated doses of cisplatin for 72h. IC50 for MILL, REN, and PHI were 79.85 μM, 41.32 μM, and 172.9 μM, respectively. Error bars indicate SD. Data indicates the mean of 6 replicates. B. Cell survival was determined by MTS assay. Cells were treated with indicated doses of EP for 24h. After 24h, cells were treated with a combination of cisplatin and EP for 48h. Vehicle for EP treatment was 1%FBS DMEM. Vehicle for cisplatin treatment was 0.5% DMSO in 1% FBS DMEM. Error bars indicate SD Data indicates the mean of 4 replicate. *P<0.05 Cisplatin+EP versus Cisplatin; #P<0.05 Cisplatin+EP versus EP alone.

3.8. Withaferin A and cisplatin decreases MM cell viability.

In an ovarian cancer *in vitro* model, treatment with cisplatin plus WA was shown to induce cell death in a time- and dose-dependent manner[30]. To determine the effect of cisplatin in combination with WA in MM, we treated MILL, REN, and PHI with low amount of WA and cisplatin concentrations around the average IC50 determined previously for 72 hours (Fig. 6A and 8A). An additive effect was observed in MILL cells, with the cisplatin in combination with WA at every concentration of the two drugs, compared to single treatments with the respective concentrations (Fig. 9). Similar results were obtained with REN (data not shown).

These results indicate WA as an effective anti-cancer agent that can be used in combination with cisplatin with low IC50 in all three MM cell lines.

Furthermore, our data indicates that WA may additively lower the IC50 of cisplatin,

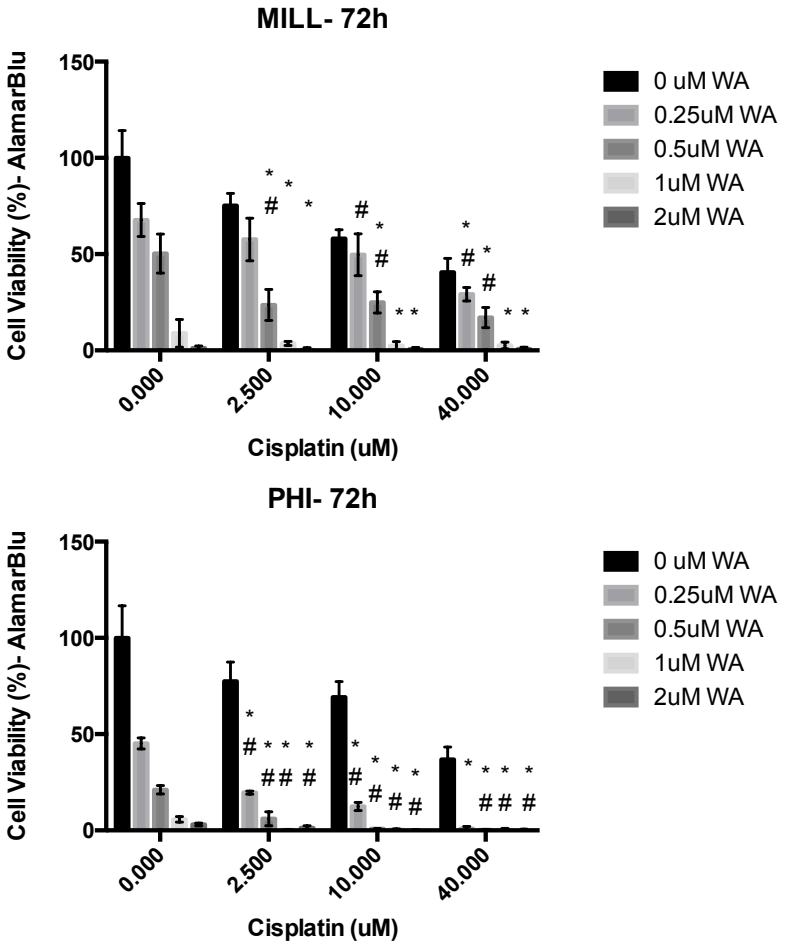

Figure 9. WA and cisplatin decreases cell viability in MM cells. Cell Viability was determined by AlamarBlu assay. Cells were treated with vehicle (0.2% DMSO, 1% FBS DMEM) or indicated doses of WA and cisplatin, in combination or alone for 72 hours. Data indicates the mean of 4 replicates. . *P<0.05 Cisplatin+WA versus cisplatin alone; #P<0.05 Cisplatin+WA versus WA alone.

CHAPTER 4. Discussion

MM is an aggressive cancer with dismal prognosis and poor therapeutic options. MM is renowned for its resistance to conventional therapies and the patients' median survival is 1 year from diagnosis[2]. The high mortality rate for MM, underscores the need for novel targeted therapies for this deadly disease. Currently, there is no effective standard therapeutic care for MM. Chemotherapy remains the standard of care for MM and is based on the combination of Cisplatin and Pemetrexed, which only extends the survival time by about 11 weeks[24]. Nevertheless, the major concerns of this treatment are its short duration of response, rapid relapse, and acute toxicity.

In this study, we sought to investigate the potential use of the HMGB1 inhibitor, EP, for the treatment of MM, determine its mechanism of action, and investigate if EP treatment could enhance chemosensitivity of MM to cisplatin. We have previously demonstrated the critical role of HMGB1 in the initiation and progression of MM[8]. Moreover, HMGB1 was shown to promote chemo-resistance through the upregulation of autophagy[13]. This evidence strongly suggests that HMGB1 may be an attractive molecular target for the treatment of MM alone and/or in combination with standard chemotherapy. Our *in vitro* data shows that EP was able to reduce survival and migration of MM cells (Figs. 1, 2, and 3), via suppression of HMGB1-induced NF-κB activation (Fig. 5). Importantly, EP effectively disrupted the MM malignant phenotype as measured in an *in vitro* soft agar colony formation assay (Fig. 4). We are currently testing the efficacy of EP *in vivo* using a mouse xenograft model of human MM. Although preliminary, our results indicate that EP was able to reduce tumor growth in mice treated with EP, 100 mg/kg/day, administered intraperitoneally, three times a week, for 10 weeks. Overall, our findings indicate for the first time that EP alone can be a novel therapeutic tool for the treatment of MM tumors. Furthermore, we hypothesized that by inhibiting HMGB1, EP may also sensitize MM cells to chemotherapeutics. To test our hypothesis we pretreated MM cell lines with different concentrations of EP for 24 hours, before addition of cisplatin

at concentrations below IC50 in MM cells. Our results showed a significant decrease in survival of MM cells treated with cisplatin after pre-treatment with EP, when compared to survival of MM cells treated with the corresponding concentrations of EP or cisplatin alone (Fig. 8B). Further studies will be needed to dissect the exact molecular interactions that allow for an increased sensitivity to cisplatin in the presence of EP.

WA is a novel natural product, which is currently being investigated as a potential therapeutic in different tumor types[70, 71]. WA is an attractive candidate for the treatment of MM, as previous reports indicate that WA may at least in part function through inhibition HMGB1 signaling. WA was found to inhibit HMGB1-mediated transendothelial migration of human leukocytes upon LPS-stimulation and suppress HMGB1-induced production of IL-6, TNF-α, and NF-κB in human endothelial cells[27]. In cancer cells, WA has been shown to decrease cell proliferation and migration, and to induce apoptosis in different preclinical tumor models[26, 29, 65]. However the relationship between HMGB1 and WA has not been clearly elucidated. In our *in vitro* model we found that WA significantly reduced MM survival at concentrations in the low micromolar to nanomolar range (Fig. 6A). At late time points of treatment, WA induced dose-dependent cytotoxicity in all MM cell lines tested (Fig. 6B). This may result from a process of necrosis or from a late stage of apoptosis. After a 6-hour treatment, WA exerted a decrease in MM survival, coupled with an induction of apoptosis, as demonstrated by the activation of caspase 3/7, while no significant cytotoxicity was detected at this time point (Figs. 6C and 6D). These results indicate that WA may induce apoptosis, however we cannot exclude that WA also acts via other mechanisms. We then, investigated the molecular signaling affected by WA and we found that WA inhibited the translocation of NF-κB to the nucleus in a dose dependent manner (Fig. 7). According to previously published data on WA's mechanism of action, WA may inhibit the NF-κB pathway directly or via inhibition of HMGB1[27]. To begin to address this question, we measured HMGB1 release by ELISA upon WA treatment in MM cells. From this experiment, we observed an increased

release of HMGB1 (Fig. 6E). This is likely due to passive release of HMGB1 resulting from the overall high percentage of cell death induced by WA at that time point. Measurement of HMGB1 levels at earlier time points will be performed to further investigate whether at least part of the effects of NF-κB inhibition exerted by WA are mediated by HMGB1. Overall, our preliminary studies on WA, which showed to be effective at low concentrations in an *in vitro* setting, indicates that WA alone may represent a novel natural therapeutic product for the treatment of MM. Further experiments will be performed to evaluate anti-tumor activity of WA *in vivo* using a mouse xenograft model. Finally, our data shows that WA effectively increased the sensitivity of MM cells to cisplatin, at all tested concentrations, which strongly suggests the potential application of adding WA to the current standard of care for MM (Fig. 9).

Overall, this study has illuminated the beneficial effects of WA and EP as stand alone treatments as well as their potential to reduce chemoresistance and increase sensitivity to cisplatin for the treatment of MM.

CHAPTER 5. Materials and Methods

5.1. Cell Cultures

Malignant mesothelioma cell lines MILL and PHI were established from surgically resected human malignant mesothelioma specimens. REN cells were provided by Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA), whereas all other cell lines used in this study were provided by Dr. Harvey I. Pass (New York University).

5.2. Reagents and Materials

Withaferin A was obtained from Chromadex (Irvine, CA, USA). EP was obtained from Sigma-Aldrich (St. Louis, MO, USA). EP and Human recombinant TNF-α were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cisplatin was obtained from Selleckchem (Houston, TX, USA). Mouse monoclonal anti-NF-κB (F-6), mouse monoclonal anti-alpha tubulin (4G1), and mouse monoclonal anti-histone H1 (AE-4) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-histone H3 was obtained from Abcam (Cambridge, MA, USA).

5.3. Nuclear Extract Preparation

Cell pellets were resuspended in buffer A (10 mM Hepes, pH 7.9/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM DTT/0.5 mM PMSF) and placed on ice for 15 minutes, and 25 µl of 10% Nonidet P-40 was added to each tube followed by vortexing for 10 seconds. The samples were then centrifuged for 1 min at 13,200 rpm at 4°C. The supernatants (cytoplasmic fraction) were then transferred to a pre-chilled 1.5 ml microcentrifuge tube. The remaining pellets were washed with 500µl of cold PBS and then centrifuged for 1 min at 13,200 rpm at 4°C. The supernatants were discarded. The nuclear pellets were suspended in 30 µl buffer C (20 mM Hepes, pH 7.9/400 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM DTT/1 mM PMSF) and then put on ice for 30 min. The pellets

was resuspended every 30 min for up to 4 hours. After 4 hours, tubes were vortexed for 10 seconds then centrifuged for 10 minutes at 13,200 rpm at 4°C. The supernatants (nuclear fraction) were then transferred to a pre-chilled 1.5 ml microcentrifuge tube. The protein concentration was determined with the Bradford assay. The primary antibody mouse monoclonal anti-NF-κB was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies: mouse monoclonal αTubulin and mouse monoclonal anti-histone H1 (Santa Cruz Biotechnology) were used as loading controls for the cytoplasmic and nuclear fractions, respectively.

5.4. Cell Survival, Cytotoxicity, and Viability Assays

AlamarBlu (AbD Serotec) and CellTiter 96 Aqueous Cell Proliferation Assay-MTS (Promega) were used to evaluate cell viability. The lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche) was used to evaluate cytotoxicity. Malignant mesothelioma cells (2×10^3 cells per well) were incubated for 72 hours in DMEM with 1% FBS containing either Withaferin A or Cisplatin. Malignant mesothelioma cells (1×10^4 cells per well) were incubated for 24 hours in the HMGB1 antagonist EP alone. For combination studies, MM cells (2×10^3 cells per well) were incubated for 24 and 48 hours with the HMGB1 antagonist EP together with Withaferin A or Cisplatin for 24 and 48 hours. Doses are as indicated. All assays were run using the BioRad Model 680 Microplate Reader and Perkin Elmer Wallac Victor 3 1420 Multilabel Counter.

5.5. HMGB1 ELISA

The human HMGB1 ELISA Kit (IBL International) was used to measure the levels of HMGB1 in conditioned media of malignant mesothelioma cell lines. Samples were tested in duplicate. To detect extracellular HMGB1 released by malignant mesothelioma cells lines, 2×10^6 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 1% FBS for 24h. Cells were plated in DMEM with 1% FBS, then treated with vehicle (0.8% DMSO) or WA treatment

(2.5, 5, or 10 μM). The culture media was then collected and concentrated by ultrafiltration using Amicon Ultra Centrifugal Filters (Millipore), and 10ul aliquots were assayed in duplicate to the ELISA. All culture media were collected under identical conditions.

5.6. ApoTox-Glo Triplex Assay

The ApoTox-Glo triplex assay determines the cell viability, cytotoxicity, and caspase activation events within a single well (96-well plate, Corning). 5 x 10^3 cells per well and cultured for 24 hours. Viability, cytotoxicity, and caspase 3/7 activation was measured after a 6-hour treatment of vehicle (indicated as 0; 0.5% DMSO, 1% FBS DMEM) or WA treatment (1.25, 2.5, or 5 μM). Part one of the assay simultaneously measures cell viability and cytotoxicity by the markers glycylphenylalanyl-aminofluorocoumarin (GF-AFC) and bis-alanylalanyl-phenylalanyl- rhodamine 110 (bis-AAF-R110). Live cell proteases produce AFC and dead cell proteases produce R110. These products are determined by a different excitation and emission spectra. Caspase 3/7 activity was measured by an increase of luminescence. The fluorescence signal to determine cell viability was recorded with $400_{Ex}/505_{Em}$ filters, and the fluorescence signal monitoring the cell cytotoxicity was recorded using $485_{Ex}/520_{Em}$ filters. After measurement of cell viability and cytotoxicity, 100 ml of the Caspase-Glo 3/7 assay was added to all wells, and the luminescence signal from the caspase- 3/7 activation was measured after 30 min of incubation. Assays were run using a Perkin Elmer EnVision Multimode Plate Reader.

5.7. Statistical Analysis

All statistical analyses in this project were performed on mean values using Prism (GraphPad Software). The significance of differences between groups was determined using a paired Student's *t-test* after assessing that distribution was normal and the variances were equal in all compared groups or

an unpaired Student's *t-test* between only two groups of interest. Statistical significance was denoted by *P* values smaller than 0.05.

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