AN ABSTRACT OF THE THESIS OF

Elizabeth Namagoba Kaweesa for the degree of Master of Science in Chemistry presented on October 2, 2018.

Title: Insights into Cytostatic and Cytotoxic Properties of the Natural Product Mensacarcin.

Abstract approved:

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Sandra Loesgen

Mensacarcin is a stereogenic complex polyketide with potent anti-tumor activity produced by a soil-dwelling *Streptomyces bottropensis*. The US National Cancer Institute (NCI) 60 human tumor cell line anticancer drug screen (NCI-60) reveals mensacarcin's cytostatic properties in almost all tested cell lines and distinct cytotoxic properties specifically in eight melanoma cell lines with an average IC_{50} value of 0.5-1 μM. We show that mensacarcin activates caspase-3/7-dependent apoptotic pathways and induces apoptosis in melanoma cells and not colon cancer carcinoma cells. Mensacarcin co-localizes in mitochondria and impairs mitochondrial function by either inhibiting mitochondria respiration directly or by causing general mitochondrial dysfunction. Combination of mensacarcin and the standard chemotherapeutic regime for BRAF-mutant melanoma, vemurafenib/zelboraf®, increases efficacy and cytotoxicity synergistically.

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Insights into Cytostatic and Cytotoxic Properties of the Natural Product Mensacarcin

by
Elizabeth Namagoba Kaweesa

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APPROVED:

Major Professor, representing Chemistry

Head of the Department of Chemistry

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Elizabeth Namagoba Kaweesa, Author

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CONTRIBUTION OF AUTHORS

I confirm that this work is my own and I have been involved in the conduct of this research, analysis of data, and preparation of this thesis. This thesis will cover data from a recently published article in the journal of biological chemistry as well as follow up studies. I conducted cell proliferation assays, subcellular fractionation, caspase activity assays, energetics study experiments, and combinatorial treatment studies.

Dr. Birte Plitzko conducted cell proliferation assays and mensacarcin mode of action studies as described in section 1.3 in Chapter one.

Prof. Sandra Loesgen was the major advisor on all projects and assisted with the design and writing of all chapters contained herein.

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CHAPTER 1

MENSACARCIN: Summary of previous study published in the *Journal of Biological Chemistry* 2018

This chapter has been published in JBC 2018 by Birte Plitzko, Elizabeth Kaweesa and Sandra Loesgen. EK contribution was: determining mensacarcin's apoptosis mechanism via cell proliferation assays, caspase activity assays, and western blot analysis to probe for apoptotic proteins.

Section 1.1 Introduction

Natural product drug discovery has led to the development and contribution of different drugs for various indications. In the early 19th century, drugs like quinine and morphine were isolated as pure, bioactive natural products from medicinal plants, previously only used in raw preparations. Major breakthroughs in medicine came with the discovery of early antibiotics like synthetic sulfa drugs and the fungal fermentation product penicillin, found by Alexander Fleming in 1929. The last has initiated the screening of microorganisms, mainly soil bacteria and fungi, to identify drug leads with antibacterial and anticancer activity (1). In the field of cancer research, 175 small molecules were discovered from 1981 to 2014 and approximately 50% were natural products or derived thereof (2). Currently, anticancer agents like doxorubicin and mitomycin also derived from natural sources are effectively used in the clinic as chemotherapeutics.

Screening of natural products for new drug leads remains to be a formidable task, but it continues to be the backbone of many innovations and contributions to medicine, biotechnology, and various multidisciplinary fields.

Mensacarcin is a highly oxidized and stereogenic complex molecule isolated from a soil dwelling bacterium, *Streptomyces bottropensis*. Mensacarcin was named after the location where the soil sample was obtained next to the University's cafeteria (German = mensa) in Göttingen, Germany. Mensacarcin's structure is closely related to another cytotoxic agent, cervicarcin which was isolated from a soil dwelling bacterium *Streptomyces ogaensis* in 1963 (3). The core structure of this polyketide consists of a three-membered ring system, it exhibits nine stereogenic centers, two epoxide groups. Epoxy ketones are electrophilic and reactive; therefore, strongly influence the pharmacodynamic properties of many bioactive natural products (4). The two epoxide moieties in mensacarcin seem to be key features and important for mensacarcin's cytotoxic activity (5). No total synthesis of mensacarcin has been published thus far but semi-synthetic modifications targeting the side chain epoxide revealed a correlation of cytotoxicity with the degree of oxidation in the side chain (6). In addition, Prof. Bechthold's group studied mensacarcin's biosynthesis which enabled heterologous expression of mensacarcin's biosynthetic gene cluster to yield mensacarcin and derivatives. Bechthold's group also explored the enzymology involved in the biosynthesis of mensacarcin, including a new mechanism of epoxide formation in polyketides via a luciferase-like monooxygenase (7).

Mensacarcin's cytotoxic activity was determined by the National Cancer Institute (NCI)-60 human tumor cell line screen in which it showed strong anti-proliferative effects against all tested cancer cell lines and low COMPARE correlations to known anti-cancer agents (7). Mensacarcin specifically showed strong cytotoxic effect in the melanoma cancer panel in the NCI in vitro cell assay and therefore, this research aims to mensacarcin's mode of action as a potential drug lead against melanoma.

Melanoma is a cancer of the neural crest-derived cells that provide pigmentation to skin and other tissues (8). According to the American Cancer Society, melanoma cases have been increasing for the last 30 years. In 2017, it is estimated that 87,100 new cases of melanoma will be diagnosed affecting mostly men United States with 9,730 related deaths (9). Current chemotherapy regimens offer very low success rates and no current treatments enhance patient survival once metastasis occurs (10, 11). Development of malign melanoma has been shown to be strongly associated with inactivation of the $p16^{INK4a}$/cyclin dependent kinases 4 and 6/retinoblastoma protein ($p16^{INK4a}$/CDK4,6/pRb) and $p14^{ARF}$/human double minute 2/p53 ($p14^{ARF}$/HMD2/p53) tumor suppressor pathways (12). These pathways help control the G_1 phase of the cell cycle and are most often inhibited via deletions or mutations in the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus on chromosome 9p21. Melanoma-associated oncogene activation most often targets Ras and its canonical effector pathways, RAF-MEK-ERK and PI3-Akt. Extensive studies about the RAF-MEK-ERK pathway led to the identification of mutations in the BRAF gene in about 67% of melanomas (13). Studies also show that 50% of melanomas have the BRAF-V600E mutation which is a hallmark for high risk melanoma associated with tumor drug resistance and low patient survival rates (14). As a result, BRAF has been used as a drug target leading to the development of inhibitors like vemurafenib and dabrafenib that are currently used as chemotherapeutics; unfortunately, with short term tumor repression, chemoresistance and patients relapse within six months. The limited amount of current treatments and their limitations, stress the need for new drug leads, targets and combinatorial therapies in order to treat BRAF-mutant melanoma (15).

Section 1.2 Summary of cytostatic and cytotoxic properties of mensacarcin

Mensacarcin's cytostatic and cytotoxic activity was investigated using different experiments to determine its apoptotic mechanism. Mensacarcin was tested in the NCI-60 cell screen and showed strong growth inhibition in all tested cell lines and relative selective cytotoxicity to melanoma cells. In all subsequent experiments, two BRAF-mutant melanoma cell lines SK-Mel 5 and SK-Mel 28 cells, were used as representatives from the NCI cell panel and a non-sensitive, non-BRAF mutant HCT-116 colon carcinoma cell line was used. Cell viability was monitored and quantitatively assessed via measuring reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) by viable cells to a purple formazan. Furthermore, mensacarcin cytotoxic effects were studied by using the lactate dehydrogenase (LDH) assay in which the effect of mensacarcin treatment on membrane integrity of cells and necrotic cell death was investigated (16). Both the MTT and LDH assay indicate that mensacarcin induced concentration- and time-dependent cell death in the two tested melanoma cell lines, concurrent with growth inhibition but only moderate LDH release. On the other hand, the HCT-116 colon carcinoma cells are strongly inhibited by mensacarcin in the cell viability assay but do not show signs of cell death in the LDH cytotoxicity assays (Fig. 1).

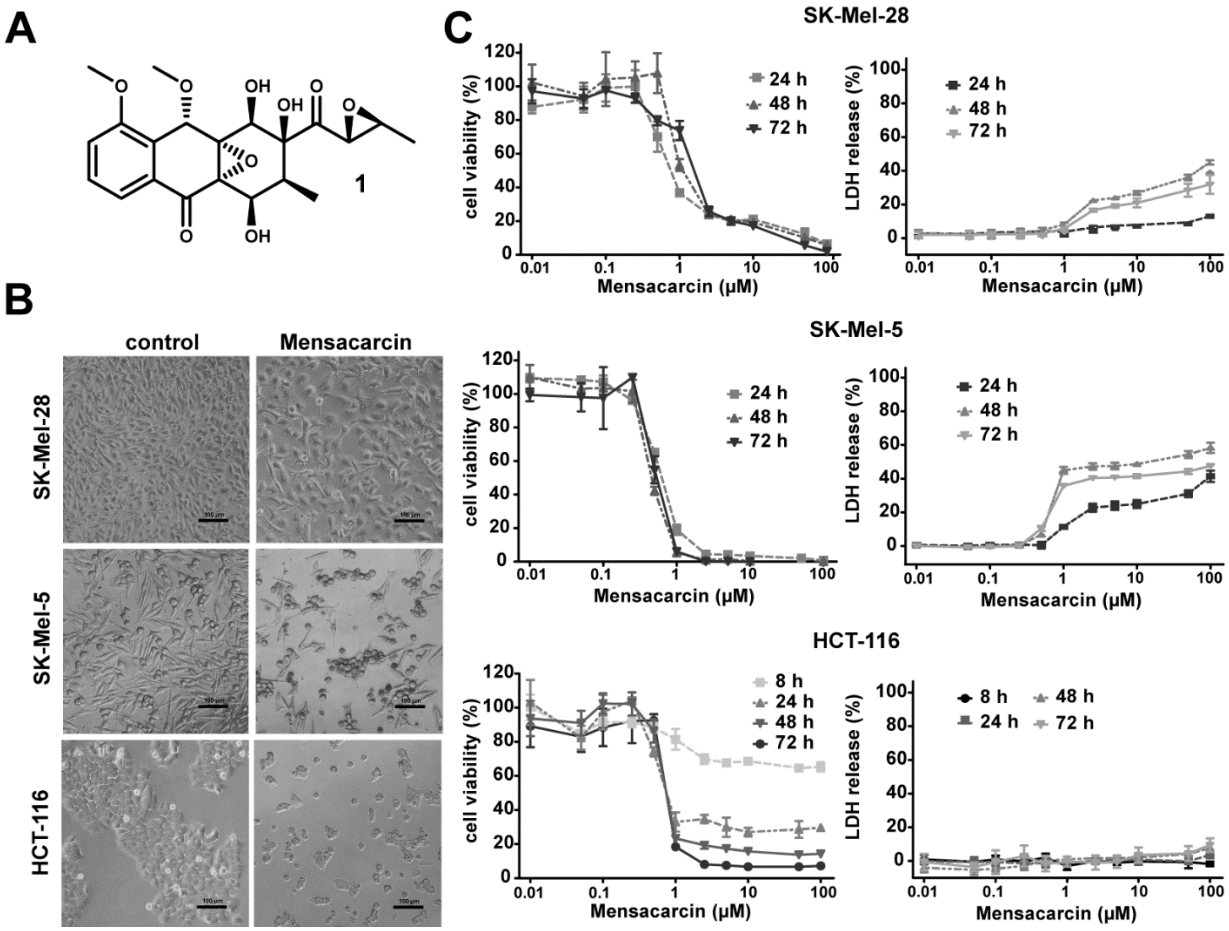


Figure 1. Evaluation of cytostatic and cytotoxic effects of mensacarcin in melanoma and colon cancer cells. (A) chemical structure of mensacarcin, (B) cell morphology with exposure to 1 μM mensacarcin examined by phase-contrast microscopy, (C) dose and time dependent growth inhibition and LDH release of mensacarcin in SK-Mel 28 and SK-Mel 5 melanoma cells and HCT-116 colon cancer cells.

Mensacarcin's ability to induce apoptosis and activate different apoptotic signaling pathways was studied due to its effective cytotoxicity in melanoma cells. Apoptosis events lead to the proteolytic activation of the caspase cascade, eventually resulting in the initiation of downstream apoptosis-executing processes. Melanoma cells were exposed to increasing mensacarcin concentrations for 6–48 h, and activation of caspase-3 and subsequent cleavage of PARP-1 was investigated by immunoblot analysis (17). Mensacarcin concentrations of 0.5–1 μM or greater induced the formation of 89-kDa PARP-1 fragments as well as caspase-3 activation in SK-Mel-28 and SK-Mel-5 beginning between 6 and 15 h after exposure. Cleavage of the pro-apoptotic proteins could not be observed in HCT-116 cells after 48 h of treatment.

Furthermore, to provide some insight into the potential mechanism of mensacarcin-induced cell death, cell-based assays were conducted to test the ability of mensacarcin to activate the downstream effector caspases of the major apoptotic signaling pathways: caspase-3 and caspase-7. Mensacarcin induced activation of caspase-3 and -7 in SK-Mel-28 and SK-Mel-5 cells over a time frame consistent with loss of cell viability (12 and 24 h) but did not induce caspase-dependent cell death in HCT-116 colon carcinoma cells (Fig. 2). In addition, mensacarcin was co-treated with Z-VAD_FMK, a pan caspase inhibitor (18). In all cases, mensacarcin's caspase activity was attenuated by pan-caspase inhibitor Z-VAD-FMK.

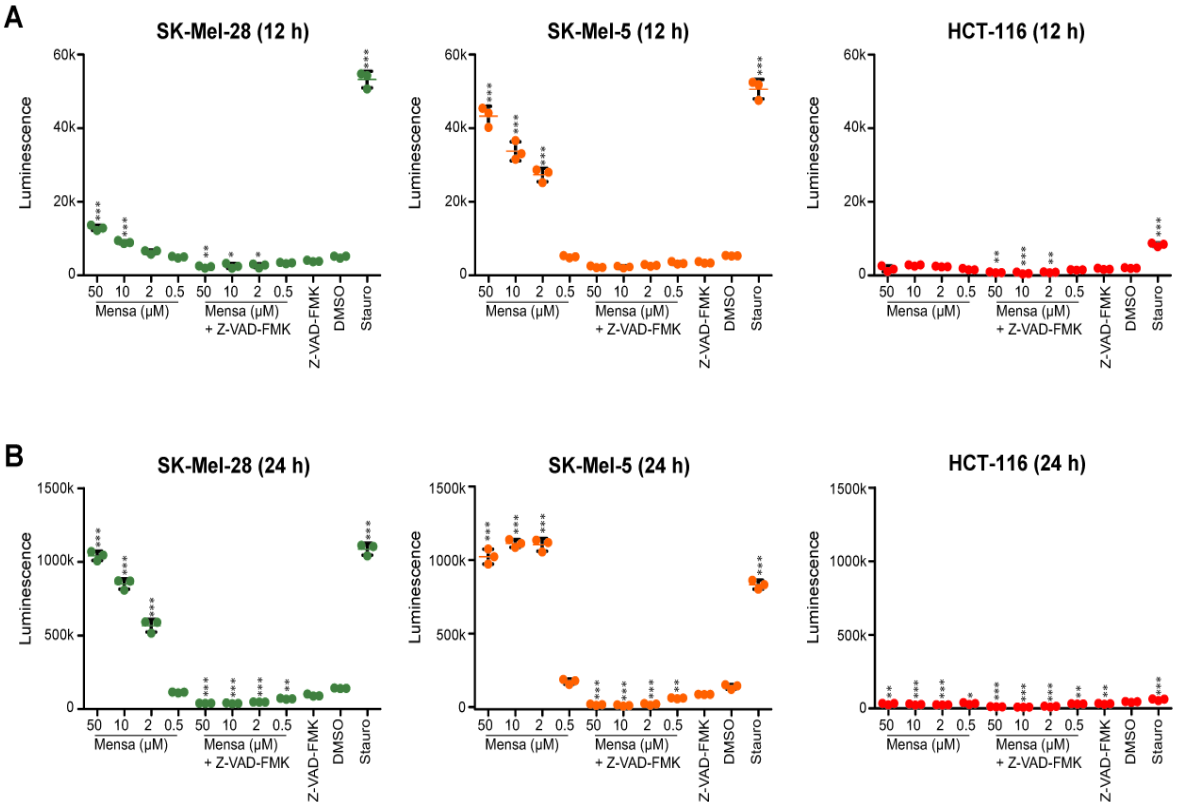


Figure 2. Mensacarcin induces activation of caspase-3 and -7 in melanoma but not colon cancer cells in a dose and time dependent manner (A) 12hrs and (B) 24hrs. Scatter plots represent mean ± S.D. of triplicates (n=3). *, $p < 0.1$; ***, $p < 0.001$ versus DMSO control.

To further confirm apoptosis and mitochondria-mediated programmed cell death, the release of the apoptogenic factor cytochrome c from the mitochondria into the cytosol was monitored (19). SK-Mel-5 cells were treated for 6 hours with 50 μM mensacarcin and 10 μM staurosporine, a known inducer of mitochondrial apoptosis. Cells were lysed and fractionated using differential centrifugation to obtain a cytosolic fraction for immunoblot analysis. The Western blot result analysis indicated that cytochrome c was released from the mitochondria into the cytosol for the mensacarcin and staurosporine treatment but not the negative control (Fig. 3).

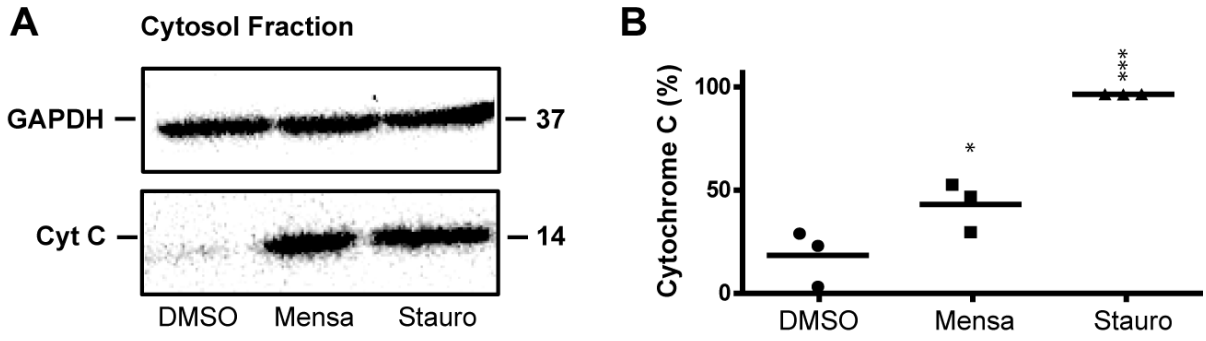


Figure 3. Mensacarcin treatment triggers cytochrome c release from mitochondria into the cytoplasm of melanoma cells. (A) cytosol fractions of SK-Mel-5 cells non-treated or treated with mensacarcin (50 μM) and staurosporine (10 μM) were analyzed for cytochrome C content via western blot analysis. (B) densitometric measurement of western blotting. Results are presented as mean ± S.D. of triplicates (n=3). *, $p < 0.1$; ***, $p < 0.001$ versus DMSO control.

Section 1.3 Summary of initial mensacarcin mode of action studies

Mensacarcin's cytostatic and cytotoxic activity as well as apoptotic mechanisms specifically against melanoma cancer cell lines motivated further study into its mechanism of action as a potential drug lead. Different experiments and assays both molecular and cellular based were used to investigate and provide insight into mensacarcin's mode of action. Mensacarcin's effect on the cell cycle distribution was investigated using flow cytometric analysis of propidium iodide (PI)-stained cells (20). Increasing mensacarcin concentrations resulted in a dose-dependent increase in apoptotic cells with hypodiploid DNA content for SK-Mel-28 and SK-Mel-5 cells (sub-G1 population) but produced only a minor increase of the sub-G1 population in HCT-116 cells. Phase distribution of the remaining cells did not change significantly in the presence of mensacarcin in melanoma cells, whereas in HCT-116 cells, a moderate S-/G2-phase arrest could be observed.

In addition, to determine mensacarcin's subcellular compartment target via fluorescence microscopy, a rhodamine labeled mensacarcin probe was synthesized. Rhodamine was linked to mensacarcin using tetramethylrhodamine-5-carbonyl azide (TMRCA) following established protocols (21). The rhodamine-mensacarcin conjugates, TMR-mensa also showed growth inhibition and cytotoxicity properties that were similar to unlabeled mensacarcin (Fig. 4). To study the subcellular distribution of TMR-mensa, SK-Mel-5 cells were incubated with the fluorescent probe for 20 min and co-stained with markers for the nucleus, endoplasmic reticulum, and mitochondria. Confocal fluorescence microscopy confirmed a quick uptake of TMR-mensa into the cells. Fluorescence imaging studies indicate that TMR-Mensa co-localizes with the

mitochondrial marker (Fig. 5). Therefore, mensacarcin induces mitochondrial toxicity in melanoma cells.

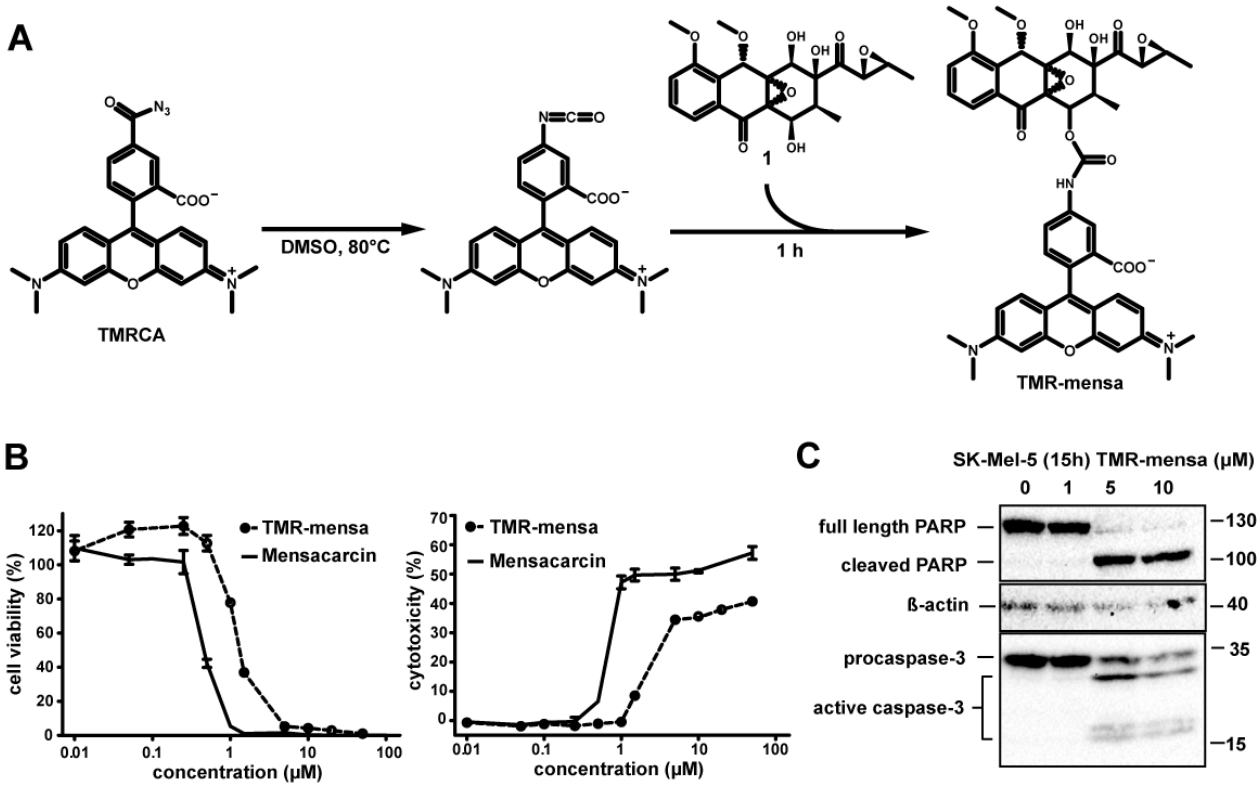


Figure 4. Preparation of fluorescently labeled mensacarcin. (A) Synthesis of rhodamine-mensacarcin conjugate. (B) Evaluation of rhodamine-mensacarcin conjugate cytotoxic and apoptotic activity. (C) Apoptotic cell death in SK-Mel-5 cells induced by TMR-Mensa evaluated by immunoblot analysis of caspase-3 activation and PARP-1 cleavage.

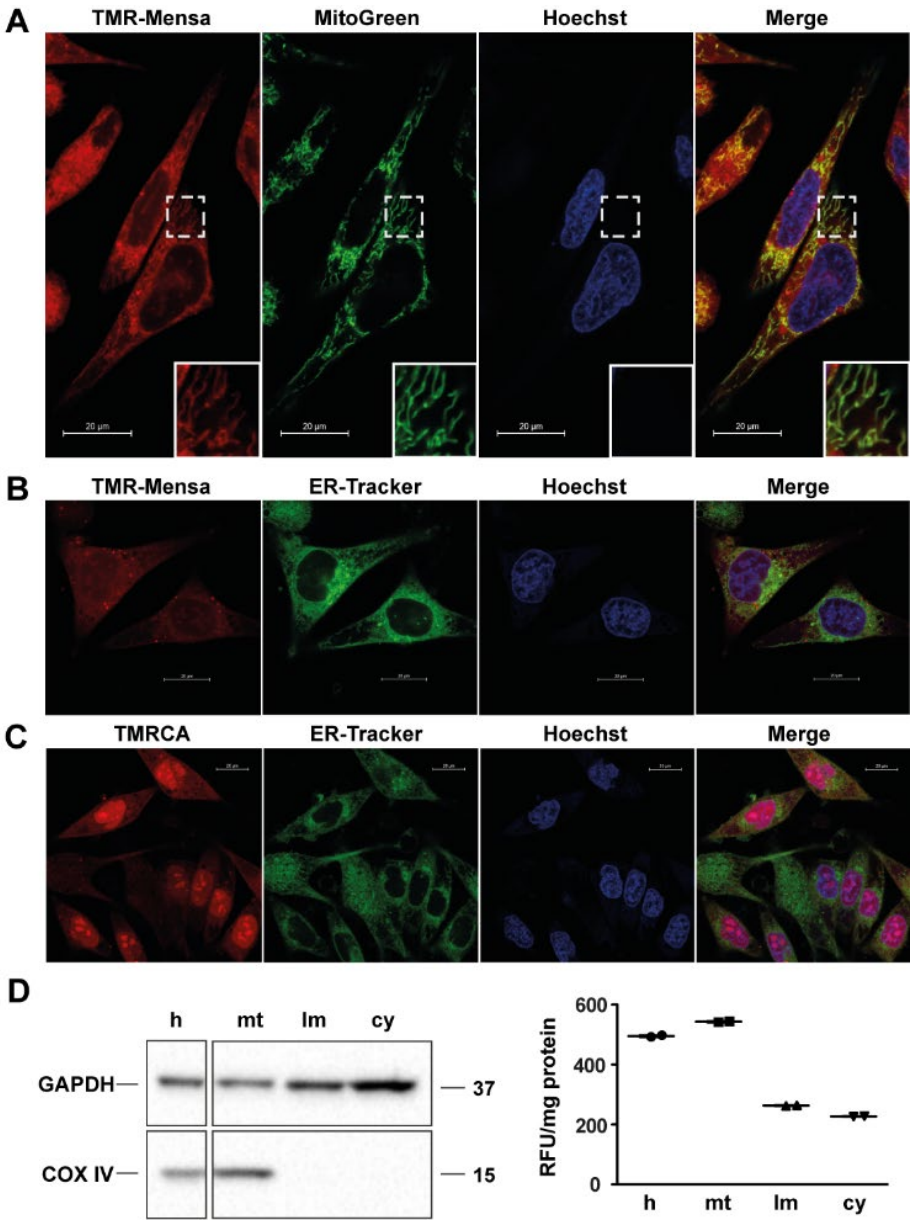


Figure 5. Cell localization studies in melanoma cells with a rhodamine-mensacarcin probe. (A) SK-Mel 5 cells were stained with TMR-Mensa, Hoechst 33342 (nucleus), and ER-tracker green (endoplasmic reticulum) or MitoGreen (mitochondria) for 20min. Images were captured by inverted confocal fluorescence microscopy and merged to examine co-localization. (B) TMR-Mensa does not colocalize with the ER-Tracker or Hoechst stain. (C) control cells were stained with uncoupled rhodamine dye which accumulates in the nuclei. (D) Cell subfractionations of TMR-mensacarcin treated SK-Mel-5 cells into homogenate*(h)*, mitochondrial fraction*(mt)*, light membrane fraction*(lm)*, and cytosolic fraction *(cy)*.

Section 1.4 Conclusion

Mensacarcin is a highly complex polyketide isolated from a soil bacterium, *Streptomyces bottropensis* with potent universal anti-proliferative effects in all tested cancer cell lines in the NCI-60 cell line panel but with selective cytotoxicity in melanoma cell lines. Mensacarcin induces apoptosis via the caspase dependent pathway and causes DNA damage as observed via the comet assay and nuclear alterations visualized with confocal microscopy. Fluorescently labeled mensacarcin with a rhodamine dye localized in the mitochondria of melanoma cells when exposed for 20 minutes. Metabolic flux analysis via the sea horse assay in melanoma cells reveals changes in oxidative consumption rate an indicator of mitochondria-perturbing mode of action. Mitochondria have emerged as a potential target for anticancer therapy (22). Mitochondria is the energy source of a cell and plays a pivotal role in cell death signaling; therefore, mitochondria can be a directed approach in cancer therapy.

In conclusion, mensacarcin strongly inhibits cell growth in human cancer cell lines but only induces apoptosis in some cell lines like melanoma cells. This data shows that mensacarcin localizes in the mitochondria and causes apoptosis via the caspase dependent pathways. This suggests that mensacarcin affects energy metabolism in melanoma cells and thereby a promising new compound as a potential anti-melanoma drug lead.

Chapter 2

Mensacarcin: Ongoing mode of action studies

This chapter covers experiments into the mode of action of mensacarcin. These experiments have been planned, conducted, and analyzed by EK and will be content of a future publication.

Section 2.1 Mensacarcin causes mitochondrial stress

Cell localization studies with a fluorescently labeled probe revealed that mensacarcin localizes in the mitochondria. Mensacarcin effects on mitochondrial function and cellular bioenergetics were measured using the seahorse extracellular flux analyzer. Energy metabolism studies of SK-Mel-28, SK-Mel-5, and HCT-116 cells before and after exposure to mensacarcin measured cellular oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) in real time as measures of mitochondrial respiration and glycolysis, respectively (23). The addition of mensacarcin induced a concentration-dependent reduction of basal OCR in both melanoma cell lines. With exposure to 50 μM mensacarcin, oxidative consumption decreased rapidly in the first 5 min of exposure and stabilized at 48% of the baseline in SK-Mel-28 and 74% in SK-Mel-5 after 60 min. The addition of the control, ATP-synthase inhibitor oligomycin (1 μM in well), which suppresses ATP production and reduces oxidative phosphorylation to the minimum necessary to balance proton leak, lowered OCR to approximately 30% of the baseline value. In contrast, mensacarcin had a minimal effect on oxygen consumption in HCT-116 cells, reducing the OCR only to 85% of control values at the highest dose tested (Fig. 6A). This is consistent with previous assays that showed that this colon carcinoma cell line is relatively insensitive to mensacarcin. For comparison, colon cancer cells were responsive to the ATP-synthase inhibitor oligomycin (1 μM in well), which lowered OCR to approximately 18% of the baseline.

In addition to measuring the OCR, the effects of mensacarcin and oligomycin on media acidification (ECAR) was measured. In agreement with numerous prior studies, oligomycin produced an increase in ECAR that represents a compensatory increase in glycolysis (24). Consistent with the previous assays OCR measurements, mensacarcin had no quantitative effects on ECAR in HCT-116 cells, however moderate effects on ECAR in the melanoma cells. In contrast to oligomycin, mensacarcin did not induce a compensatory increase in glycolysis. Low concentrations of mensacarcin (2 μM) yielded ECAR responses similar to control, whereas higher concentrations (50 μM) produced slight decreases in the acidification rate in melanoma cells. Overall, the metabolic flux analysis clearly revealed perturbation of the energy metabolism in the melanoma cell line, which prompted us to further explore the connection of energy supplying pathways and the mensacarcin's mode of action in this chapter.

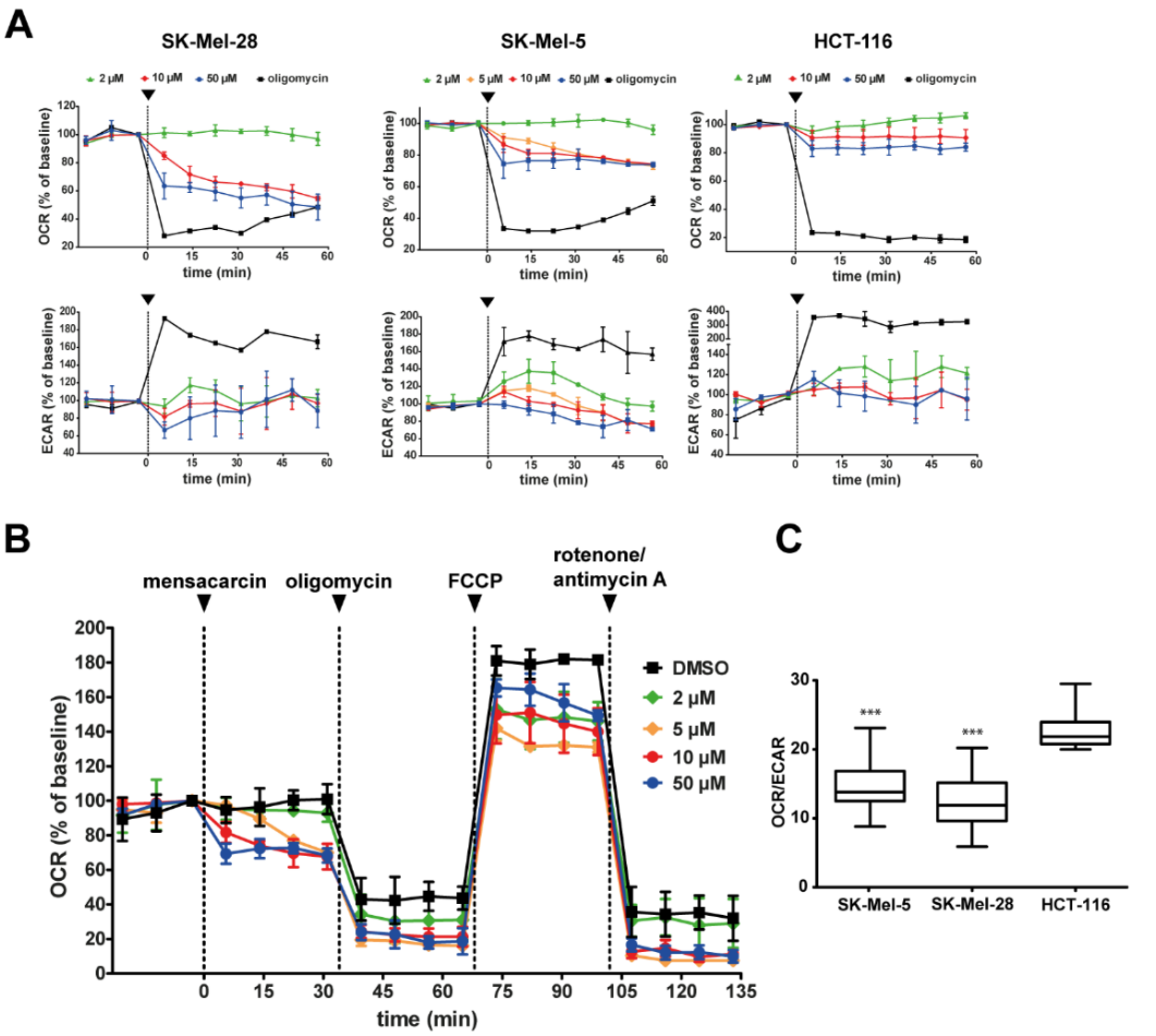


Figure 6. Mensacarcin impairs mitochondrial function in melanoma cells. (A) OCR and ECAR measurements using the Seahorse analyzer XF24. (B) OCR was measured after mensacarcin was injected in different concentrations followed by consecutive injections of controls. (C) basal energetics state measuring OCR/ECAR ratio of SK-Mel-28, SK-Mel-5, and HCT-116 cells (n=25). Error bars, S.D. ***, $p < 0.001$ versus DMSO control.

Section 2.2 Introduction into mitochondrial action and cell energetics

The mitochondrion is one of the major organelles of the cell, often called the powerhouse of the cell due to its function to synthesize ATP (25). Mitochondria consist of a double membrane; the inner and outer membranes that separates it from the cytosol. Different enzymes and processes occur in the different mitochondrial compartments to provide ATP as a n energy source. The citric acid cycle or the tricarboxylic acid cycle takes place in the mitochondrial matrix whereas the electron transport chain and ATP synthase reside in the inner membrane (26).

In addition to serving as the primary energy supplier, mitochondria have various functions in maintaining cellular homeostasis in different metabolic pathways. Mitochondria help regulate oxidative phosphorylation, the urea cycle, gluconeogenesis, thermogenesis, and calcium homeostasis among others (27). Mitochondria play a significant role in cell viability and cell death by regulating programmed cell death by generation of reactive oxygen species (ROS) which is the intrinsic pathway of apoptosis. ROS help regulate cell signaling processes in cytoprotection; however, in excess ROS lead to pathological processes (28). Different forms of apoptosis are initiated by the mitochondria via the formation of the mitochondrial permeability transition pore complex in response to oxidative stress or energy deficiency. Release of apoptotic factors from the mitochondria, disruption of ATP synthesis, and modification of cellular redox potential are a sign of apoptosis. Cytochrome c is an essential component in the respiratory chain but when released from the mitochondria to the cytosol, it binds to Apaf-1 to form an apoptosome which activates caspase dependent apoptosis leading to cell death. Also, another mitochondrial protein known as apoptosis inducing factor (AIF) initiates the caspase independent apoptotic pathway when it translocates into the nucleus, directly causing chromatin condensation and DNA fragmentation. (29). The different functions of the mitochondria determine cell life, cell death, and cell

functionality. Differences in energy metabolism between normal and cancerous cells were first observed by Otto Warburg in the 1920s and the phenomenon of aerobic glycolysis came to be known as the Warburg effect. The cancer cells high demand for growths requires energy, and their ability to generate ATP via oxidative phosphorylation is limited, forcing them to increase glycolysis. In addition, cancer cells are postulated more likely to produce ATP via glycolysis due to impaired mitochondrial respiratory capacity and low oxygen concentrations in tumors. The Warburg effect shows that cancer cells have upregulated glycolysis unlike normal cells; therefore this metabolic alteration can be exploited and the mitochondrial functions can be a potential drug targets (28). Mitochondrial DNA mutations were observed in cancer cells and it is important to note that 7 out of the 41 mitochondrial electron transport chain subunits are encoded by the mitochondrial genome and the rest by the nuclear genome (30). These changes in mitochondrial function may vary based on different types and stages of cancer as well as proliferation state. Cancer cells have reduced ATPase activity than normal cells including decreased electron transport chain complex activities. These differences in mitochondria and changes in ATP production by cancer cells provide a basis to target mitochondria for anticancer drug development (31).

Mitochondria targeting drugs

Several small and macromolecules have been discovered to act directly on the mitochondria and change its functions in cancer cells. These molecules target mitochondria using different mechanisms eventually leading to apoptosis. This group of molecules is referred to as “mitocans” an acronym for “mitochondrially targeted, apoptosis-inducing anticancer compounds” (32). Mitocans have been classified in different ways by several scientists depending on their structure or mechanism of action. These drugs have been classified into peptide and non-peptide

substances. The non-peptide mitocans include antioxidants that scavenge for reactive oxygen species in the cell as well as clinically approved drugs like paclitaxel and doxorubicin. Paclitaxel's mechanism of action is due to its ability to stabilize microtubules in the cell and doxorubicin main cytotoxic impact is attributed to its DNA intercalation (33). However, recent research studies show that the cytotoxic effects of both these widely used drugs are also associated with mitochondria dysfunction. These drugs induce the formation of the mitochondrial permeability transition pore complex (mPTPC) leading to apoptosis (27, 34). Examples of peptidic mitocans include, cyclosporine A, glutathione, gramicidin A among others and they all modulate mitochondria functions in different ways.

Furthermore, small molecules have been grouped based on their mechanism of action or target within the mitochondrion. First, compounds target the mitochondrial transmembrane potential for instance lipophilic cations like dequalinium can cross the double mitochondrial membranes and depolarizing the mitochondrial membrane which leads cell death and inhibition of tumor growth. Secondly, compounds target mitochondrial respiration for instance vitamin E analogues, resveratrol, and rotenone. These compounds are known to inhibit tumor growth and progression by inhibiting the different complexes in the mitochondrial respiratory chain/ electron transport chain (35). Dysfunction of the electron transport chain leads to leakage of electrons causing an increased production of reactive oxygen species (ROS) which lead to mitochondrial damage and subsequent activation of apoptosis. Thirdly, compounds target mitochondrial membrane permeability, for example Bcl-2 inhibitors. The mitochondrial permeability transition pore (MPTP) located between the inner and outer mitochondrial membranes is comprised of the adenine nucleotide transporter (ANT), voltage-dependent anion channel (VDAC), and cyclophilin D. These compounds cause the abnormal opening of the MPTP which leads to a decrease in

membrane potential and results in to the release of apoptotic factors from the mitochondria to the cytosol leading to apoptosis (36). Lastly, compounds like cisplatin and etoposide target mitochondrial DNA as well as other apoptotic signaling pathways. This leads to reduction in mitochondrial respiration and ATP production which leads to cell death (27, 32).

Lastly, some compounds target the cancer associated altered energy metabolism that is associated with mitochondrial dysfunction. Metabolic alterations are prevalent in cancer cells with the increase in glycolytic activity and enzyme activity and expression in glycolysis or tricarboxylic acid (TCA) cycle is altered in cancer cells. Glycolytic and TCA cycle inhibitors like 2-deoxyglucose, 3-bromopyruvate and dichloroacetate inhibit mitochondrial processes leading to apoptosis (29, 37).

Section 2.3 Studies to explore mensacarcin energetics effects

Previous experiments using fluorescence confocal imaging and the Sea horse flux analyzer indicate that mensacarcin localizes in the mitochondria and causes mitochondria dysfunction which leads to apoptosis in melanoma cells. This project sought to determine the mechanism of mensacarcin's activity in the mitochondria by focusing on how it can affect different energetic processes which lead to cell death. These experiments were explorations conducted to determine if mensacarcin cytotoxicity was enhanced by different carbohydrate concentrations, induction of reactive oxygen species, or by glycolytic inhibitors.

2.3.1 Reactive oxygen species study

Reactive oxygen species (ROS) are molecules and free radicals from molecular oxygen with superoxides being the main precursors. Superoxides are mainly located in cell membranes; in the mitochondria, they are located on the outer membrane, the matrix and both sides of the inner matrix. Formation of ROS and various byproducts in the mitochondria contribute to DNA damage and apoptosis in the cell (38). The cell has different enzymes and antioxidants that detoxify against ROS preventing cell damage. In this study, we sought to examine if mensacarcin activates ROS production in melanoma cells thereby inhibiting cell proliferation. The main objective of this study was to determine if mensacarcin cytotoxicity is because of ROS production. SK-Mel-5 cells were treated with mensacarcin in combination with vitamin E, an antioxidant which scavenges ROS (39). If mensacarcin causes cytotoxicity via ROS production, then expression of apoptotic proteins will be inhibited in cells treated with mensacarcin and vitamin E.

2.3.2 Carbohydrate study

According to the Warburg effect, cancer cells have upregulated glycolysis and produce ATP via aerobic glycolysis. Studying changes in mitochondrial processes and energy metabolism

in cancer cells provides insight on cell functionality and survival (40). Also, provides insight on possible molecular targets to inhibit cancer cell proliferation. The main objective of this study was to determine if mensacarcin cytotoxicity was dependent on glucose concentration.

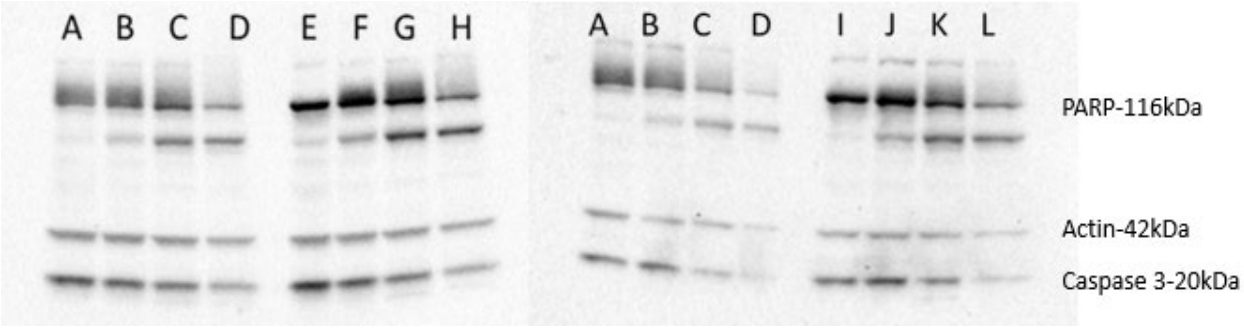
2.3.3 Glycolytic Inhibitor study

Wide spectrum of cancer cells rely on aerobic glycolysis for energy production and overall survival. This increased dependence on metabolic alterations and oncogenic signaling leads to cancer development. Targeting the glycolytic and related pathways in the mitochondria are potential strategies to inhibit cell proliferation and cancer cell metastasis (41). Therefore, glycolytic inhibitors are a potential new class of anti-cancer agents with a broad spectrum of therapeutic applications (42). Given mensacarcin's localization and potential activity in mitochondria, we sought to explore mensacarcin's potential effect in the glycolytic pathway and how that related to observed mitochondria dysfunction and apoptosis. In this study, SK-Mel-5 cells were treated with glycolytic and Krebs cycle inhibitors: 2-deoxyglucose (2-DG) and 3-bromopyruvic acid (3-BPA) in combination with mensacarcin. The goal of this study was to determine the cytotoxicity effects of mensacarcin and glycolytic inhibitors on melanoma cells in individual and combinatorial treatments. These experiments would provide insight into mensacarcin's mode of action via the different mitochondrial processes.

Section 2.4 Results

2.4.1 ROS study

In this study, induction of apoptotic proteins (PARP-1 and caspase-3) was analyzed after co-treatment of mensacarcin with the antioxidant vitamin E at different concentrations and compared to treatments with mensacarcin only. Co-treatment with vitamin E should be able to scavenge reactive oxygen species thereby inhibiting apoptosis. In the immunoblot analysis, no difference in the induction of pro-apoptotic proteins by co-treatment with vitamin E was observed (Fig. 7). The first results may indicate that mensacarcin cytotoxicity and mechanism of action may not rely on the generation of reactive oxygen species.



Lane	Mensa	Vit. E
A	0μM	0μM
B	0.5μM	0μM
C	1μM	0μM
D	2μM	0μM
I	0μM	50μM
J	0.5μM	50μM
K	1μM	50μM
L	2μM	50μM

Lane	Mensa	Vit. E
A	0μM	0μM
B	0.5μM	0μM
C	1μM	0μM
D	2μM	0μM
I	0μM	100μM
J	0.5μM	100μM
K	1μM	100μM
L	2μM	100μM

Figure 7. Western blot analysis probing apoptotic proteins in cellular protein obtained from melanoma cells treated with mensacarcin and vitamin E at different concentrations

2.4.2 Carbohydrate study

Oncogenic signals in tumor cells contribute to metabolic reprogramming and aerobic glycolysis of cancer which affects cellular metabolism. Multiple studies indicate that metabolic alterations increase tumor growth; therefore, inhibiting glycolysis has resulted in reduced tumor growth (43). Previous metabolic flux experiments revealed that mensacarcin is lowering the oxygen consumption rate in melanoma cells, therefore potentially targeting oxidative phosphorylation. Melanoma cells cultured in media with low glucose concentration (5.5mM), would adopt their energy metabolism and rely more on oxidative phosphorylation. The treatment with mensacarcin should have stronger effects if ATP productions via oxidative phosphorylation is increased and the target. Whereas, at high glucose concentrations (25mM), melanoma cells would rely on glycolysis more, leading to a decrease in mensacarcin cytotoxicity. Galactose was chosen as a non-glycolytic active sugar replacement. A recent study on BAY 87-2243, an inhibitor of complex I of the electron transport chain, showed that the treatment of melanoma cells cultured in high glucose concentrations decrease its cytotoxicity due to enhanced ATP production by compensatory glycolysis (44).

Our results show that cell growth inhibition and cytotoxicity was not affected by treatment of mensacarcin in cells cultured in media with either low or high glucose concentrations. In this experiment, mensacarcin cytotoxicity remained consistent across all treatments without significant changes (Fig. 8). These results need to be repeated with optimized media conditions, the slow growing melanoma cells used in the study showed responses to high and low glucose concentrations in previous studies (43).

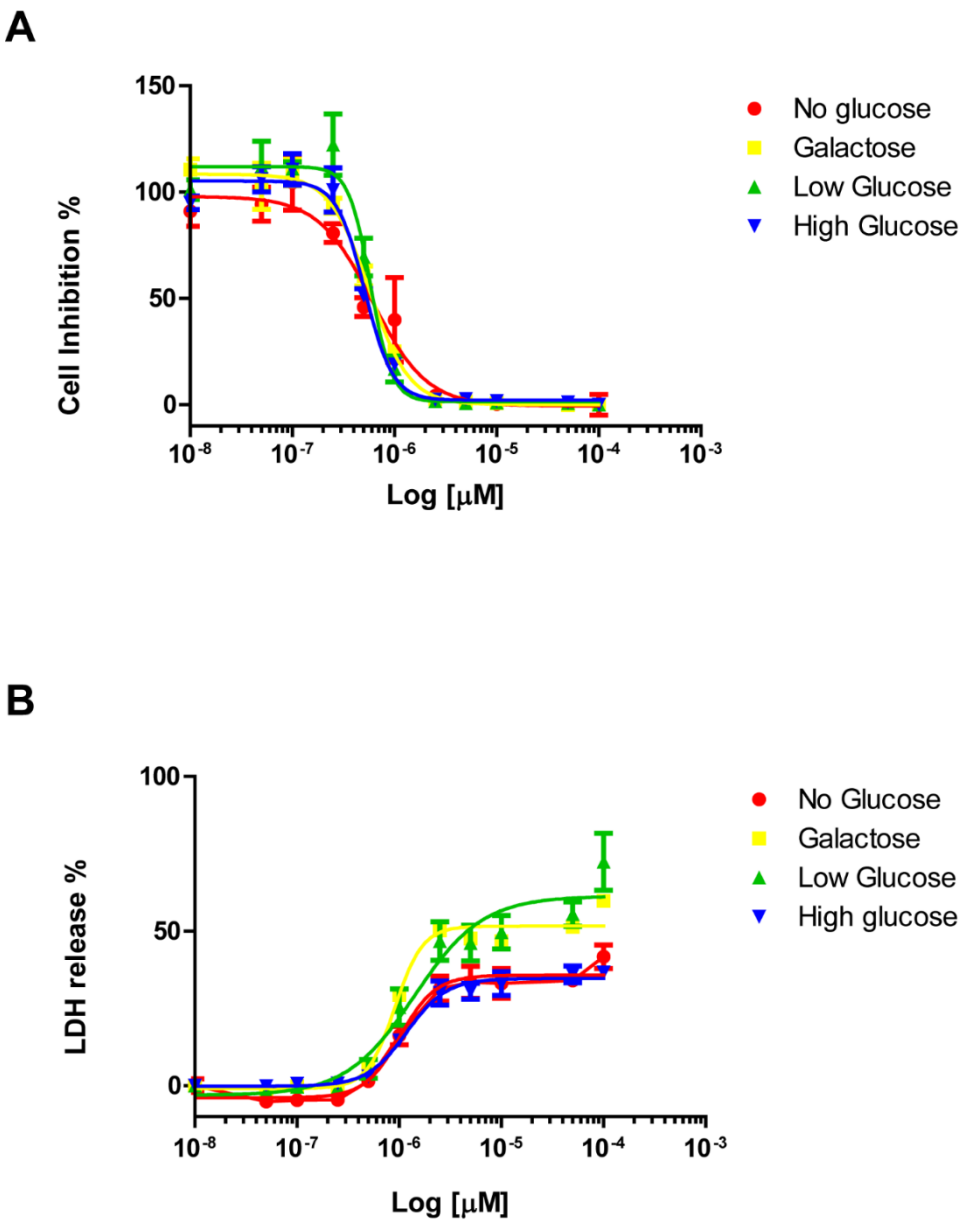


Figure 8. IC_{50} curves of mensacarcin treatment on SK-Mel-5 cells cultured in media with no glucose, galactose (5.5mM), low glucose (5.5mM), high glucose (25mM). Cell viability was determined by an MTT assay (A) and LDH assay (B).

2.4.3 Glycolytic inhibitor study

Cancer cells produce energy by glycolysis via lactic acid fermentation known as the Warburg effect. Over the last several years, experiments have been conducted to induce cytotoxicity by inhibiting glucose metabolism with glycolytic inhibitors

In this study, an increase in cell growth inhibition was observed with the combinatorial treatment of mensacarcin and glycolytic inhibitors: 2-deoxyglucose (2DG) and 3-bromopyruvic acid (3-BPA) in comparison to mensacarcin treatment alone (Fig. 9a and b). All treatments were done in triplicates and 1% (v/v) DMSO was used as a negative control.

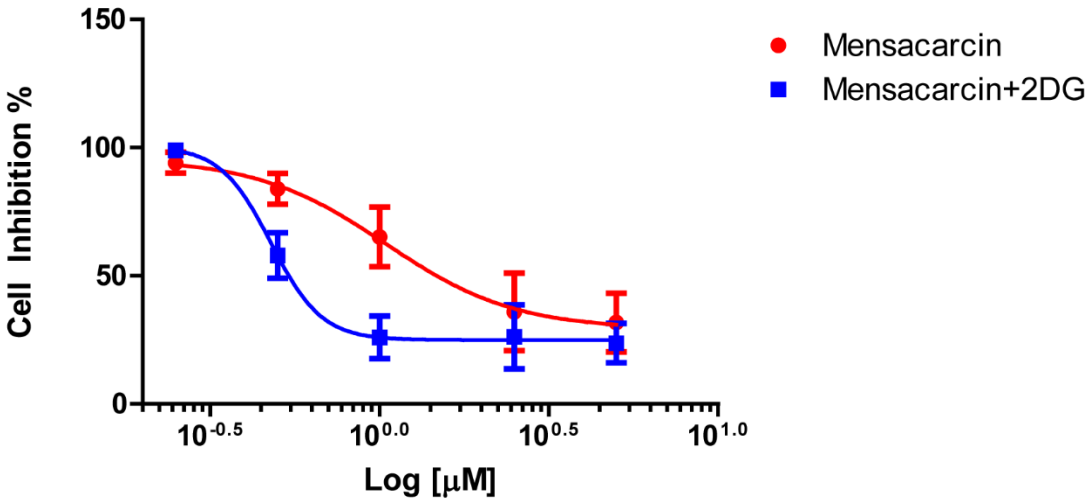


Figure 9a. Dose dependent growth inhibition of SK-Mel-5 cells treated with mensacarcin (5-0.25µM) and mensacarcin plus 2mM 2-deoxyglucose for 24 hrs.

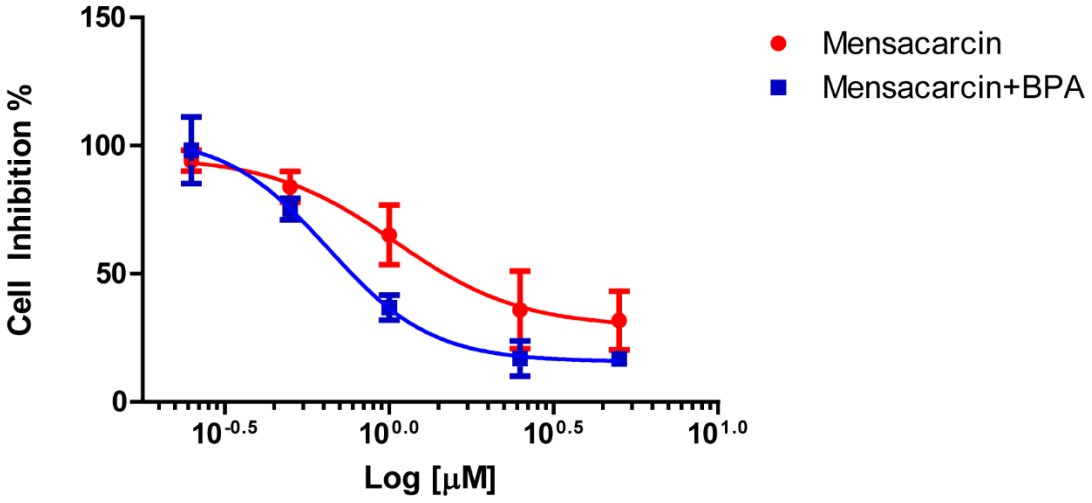


Figure 9b. Dose dependent growth inhibition of SK-Mel-5 cells treated with mensacarcin (5-0.25µM) and mensacarcin plus 10µM 3-bromopyruvic for 24 hrs.

Section 2.5 Conclusion

A synthesized mensacarcin-rhodamine probe localizes in the mitochondria as observed by confocal fluorescence microscopy. We hypothesize that mensacarcin also localized to the mitochondria and induces apoptosis in melanoma cells via mitochondria dysfunction. Energy metabolism experiments with mensacarcin using the sea horse flux analyzer indicate reduction of oxygen consumption rate, a measure of mitochondria function. Different energetics studies were conducted to determine mensacarcin's mechanism of action in the mitochondria. In the reactive oxygen species (ROS) study, no changes in mensacarcin cell toxicity were observed when mensacarcin was co-treated with vitamin E, an antioxidant (Fig. 7), indicating that mensacarcin does not cause apoptosis by generating ROS.

Future studies to determine mensacarcin's ability to generate ROS are planned via a colorimetric assay which measures glutathione levels in melanoma cell extracts treated with mensacarcin. Glutathione is an antioxidant present in healthy cells and reduction of glutathione levels indicates oxidative stress (45). Also, in an initial experiment, mensacarcin effects on cell proliferation was not changed if the media contained different glucose concentration (Fig. 8); however, a synergistic increase in cell growth inhibition was observed when mensacarcin was co-treated with glycolytic inhibitors, 2-deoxyglucose and 3-bromopyruvic acid than in individual treatments (Fig. 9). Future experiments will explore mensacarcin's mechanism of action via cell energetics and particularly glycolysis inhibition causing apoptosis.

CHAPTER 3: Mensacarcin's application against melanoma

This chapter covers experiments into the application of mensacarcin as a potential melanoma treatment. These experiments have been planned, conducted, and analyzed by EK and will be content of a publication.

Section 3.1 Introduction into combinatorial cancer chemotherapies

Chemotherapy has been the conventional treatment for any type of cancer including surgery and radiotherapy. These methods have gained success over the years but do not completely lead to full recovery or eradication of the tumors. In addition, chemo and radio therapy is very toxic towards normal cells which leads to lowered immune response and patient susceptibility to more infections. On the other hand, surgery is effective but to only localized tumors. Targeted therapies and immunotherapy studies have been conducted in preclinical studies to develop new cancer treatment strategies in which the host immune system can be armed and activated to control any disease. The tumor micro-environment is complex with different proteins, receptors, and pathways involved that it requires a combination of treatments affecting different targets which can help control tumor growth and eliminate the cancer (46).

Chemotherapy and immunotherapy combinatorial treatments can eradicate the cancer by enhancing the immune system via increasing antigen cross-presentation, activation of dendritic cells, inhibition of immunosuppressive cells, homeostatic proliferation, and upregulation of molecules among others. Ipilimumab is an FDA approved antibody that is specific for cytotoxic T lymphocyte-associated antigen 4. This treatment is given to patients with advanced melanoma and it acts by blocking inhibitory signals for T activated cells thereby increasing T cell responses which

contribute to tumor destruction (47). A taxane therapy involving taxol was found to increase T-cell precursors and not deplete them. In addition, the first clinical trial to show that docetaxel can be administered with immunotherapy was done with metastatic androgen-independent prostate cancer patients. l. This combinatorial treatment improved patients' response to docetaxel without inhibiting specific T-cell responses. Another clinical study done with small cell lung cancer patients show that chemotherapy response was closely associated with induction of immunological response via antibody drug conjugates. These experimental studies show that chemotherapy synergize with immunotherapy to enhance more anti-tumor immunity (48).

Combinatorial treatment in melanoma can be between anti-cancer drugs with different modes of action or that target different metabolic pathways as well as combination of chemotherapy and immunotherapy.

Cell signaling pathways in melanoma

The pathways that are hyperactivated in melanoma include the MAPK pathway, BRAF, MEK, and PI3K among others. Overstimulation of the MAPK induces RAS to interact with the RAF kinase family which leads to phosphorylation and activation of MEK 1/2 and ERK 1/2 (49). This leads to activation of multiple downstream effector proteins that are involved in cell survival and cell cycle progression. On the other hand, activation of the PI3K pathway recruits downstream effector proteins like mTOR, ribosomal protein S6 kinase 1 and eukaryotic translation initiation factor 4E binding protein 1 (50). Both pathways act independently and activate different downstream effector proteins; however, they also have the capability to cross-interact via positive and negative feedback interactions. For instance, hyperactivation of MAPK pathway can induce RAS and PI3K pathways or the inhibition of the PI3K pathway can increase phosphorylation of MAPK pathway molecules such as ERK. In addition, this cross interaction activates different

downstream proteins like c-myc transcription factors thereby causing melanoma progression. Because of the interaction of the different pathways, treatment and therapy with a single agent can yield partial positive results and acquired resistance to the drug. Combinatorial treatment with different drugs or inhibitors that simultaneously target the different pathways can inhibit progression of melanoma in patients (51).

Section 3.2 Combinatorial treatment in melanoma

Melanoma is the leading cause of skin cancer deaths worldwide. Over the last years, more insight into genetic alterations have led to more understanding of how melanoma progresses. The key cell signaling pathways that are involved in melanoma progression are an indication of a molecularly complex disease. Some of these pathways include the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/AKT, Wnt and c-jun-NH_2-kinase (43). Further research studies identified the BRAF mutation prevalent in 66% of malignant melanomas with 80% of the cases with a single substitution in the kinase domain (V599E). This led to the development of the first fragment-based drug, vemurafenib, the first oral BRAF-mutant inhibitor drug approved by the FDA in 2011 (52).

Vemurafenib had an 81% response rate in patients and showed an increased efficacy in patients compared to patients treated with dacarbazine. Subsequently, another BRAF inhibitor, dabrafenib and a mitogen-activated protein/extracellular signal-regulated kinase (MEK 1/2) inhibitor trametinib were developed and showed impressive clinical results. However, most patients relapsed quickly and developed chemo-resistance within 6.7 month in the case of vemurafenib and within 5.5 months in the cases for dabrafenib and trametinib. Several molecular processes lead to melanoma onset and progression which therefore requires new combinatorial treatments or co-targeting strategies (53).

Clinical trials or treatment of melanoma have incorporated combinatorial treatment and therapy in which several different inhibitors are used to target different pathways that are affected in melanoma. Combinatorial treatment can be incorporated by simultaneously targeting proteins or substrates in the same pathway and this is referred to as vertical co-targeting. It can also be done by targeting molecules in different pathways causing a dual blockade of both signaling pathways and that is referred to as parallel co-targeting.

Vertical co-targeting has been done in the MAPK and PI3K/Akt/mTOR pathways. Hyperactivation of both these pathways has been associated with the development and progression of melanoma. As a result, vertical co-targeting of small molecules that inhibit mTOR kinase, MAPK and PI3K activity have been studied. For instance, a synergistic genotype and dose-dependent anti-proliferative effect in melanoma cells was observed by combining rapamycin, an mTOR inhibitor and PI3K inhibitors (BKM-120, LY294002 and ZSTK474) leading to cell cycle arrest and increase in apoptosis in melanoma cells (54).

The MAPK pathway has been blocked by several inhibitors that target the downstream RAS/RAF/MEK/ERK signaling pathways. Combination of different MEK and BRAF inhibitors has led to a decrease in tumor growth, cell proliferation and viability and as a result an increase in apoptosis. Combination of trametinib and dabrafenib diminished expression of pro-proliferative molecules cyclin D1, downregulated genes involved in cell proliferation and survival, reduced skin lesion formation and upregulation of molecules involved in apoptosis. Cell Inhibition of melanoma cells was also observed with the combinatorial treatment of vemurafenib and MEK inhibitors. Increase in the cleavage of caspase-3 and PARP-1 as well as reduction in cyclin D was an indicator of increased apoptosis (55). Furthermore, synergetic effects of lonafarnib and sorafenib, a multi-kinase/RAF inhibitor inhibited melanoma cell growth and invasive tumor growth in a skin

culture. Lastly, co-treatment of metastatic melanoma with cobimetinib and vemurafenib reduced tumor volume and growth by inhibiting expression of CRAF, pMEK and RAS proteins as well as a metabolic enzyme hexokinase II (56).

On the other hand, parallel co-targeting of MAPK and PI3K pathways have been used to counteract melanoma resistance mechanisms to targeted therapies. Different co-targeting approaches in melanoma are currently being incorporated due to the different pathway convergences, cross-activation as well as inhibition. For instance, selumetinib a MEK inhibitor when combined with different PI3K/mTOR inhibitors apoptosis and tumor regression is observed. Also, the treatment of cobimetinib and trametinib in combination with compounds that target different pathways such as PI3K, Akt and MTOR pathways result into reduced cell viability, improved apoptosis and reduced tumor growth both in vivo and in vitro (57).

Section 3.3 Vemurafenib and mensacarcin combinatorial treatment

Combinatorial treatment unlike single treatment is improving the efficacy of anticancer therapies and is providing more insight into clinical therapy treatments. Vemurafenib was developed as a specific inhibitor to the BRAF gene which is the most frequent mutation in 60-70% of malignant melanomas. This drug exhibits potent tumor reduction responses in most melanoma patients prolonging the median survival in relation to chemotherapy. However, different resistance mechanisms in multiple pathways have been discovered to contribute to vemurafenib resistance in patients between 5-7 months. Changes and amplification in the mutant BRAF gene have led to MAPK pathway dependent resistance mechanisms, including development of secondary mutations in the NRAS and MEK pathways. In order to overcome these resistance mechanism, combinatorial treatment of vemurafenib with other BRAF or MEK inhibitors has been incorporated in different clinical trials and has prolonged the median survival rate and in melanoma patients (51, 53).

Given the observed selective mensacarcin cytotoxicity in melanoma cell lines and unique mode of action in cellular bioenergetics in the mitochondria, we were interested in the efficacy of combinatorial treatment with a current chemotherapeutic. Combinatorial treatment in different melanoma cell lines has previously been done with inhibitors in inter-related kinase pathways most of which are synthetically derived. Combinatorial treatment with vemurafenib a synthetic BRAF inhibitor and mensacarcin, a naturally derived small molecule will give more insight on drug resistance mechanisms and how to develop targeted therapies to treat melanoma and other cancer types. In addition, combinatorial treatment between the different compounds will study the efficacy of combinatorial vs single drug treatment in the development of drug-targeted therapies. Individual and combinatorial treatment was also conducted in vemurafenib resistance cell lines to determine mensacarcin cytotoxic activity in cells that have developed resistance. In this study, cell viability assays were conducted in a concentration and time dependent manner.

Section 3.4 Results

Vemurafenib combination treatments with MEK inhibitor, cobimetinib or with PI3K/Akt/mTOR inhibitors resulted in increased apoptosis and tumor regression Also, a decrease in the expression of 2-deoxy-2[fluorine-18]-D-glucose (FDG), a malignancy biomarker was observed in the combinatorial treatment (44, 49).

In this combinatorial study, increase in cell growth inhibition was observed when cells were treated with mensacarcin in combination with vemurafenib than individual treatments. Also, enhanced synergistic effects are observed with the combinatorial treatment of vemurafenib and mensacarcin than individual treatments in parental SK-Mel-5 and SK-Mel-28 cell lines (Fig.10, 11). In both cell lines, the IC_{50} values of the combinatorial treatments are lower than the individual treatments, synergism was assessed via Compusyn software (58). Whereas mensacarcin acts fast

within minutes, vemurafenib is a slow acting chemotherapeutic, showing effects after 72hrs, hence two different treatment times to evaluate combinatorial vs. individual treatment were chosen.

Co-treatment of melanoma cells with vemurafenib and the natural compound mensacarcin increased cell growth inhibition synergistically than individual treatments. These results possibly indicate that mensacarcin is not a BRAF inhibitor and possibly targets another pathway, thereby, in combination, increasing cell cytotoxicity. Future studies will be conducted in vivo with a hypothesis that tumor regression and decrease in vemurafenib resistance when co-treated with mensacarcin. In collaboration with Prof. Amanda Lund (OHSU), tumored mice will be treated with mensacarcin and mensacarcin/vemurafenib combinations to assess in vivo efficacy.

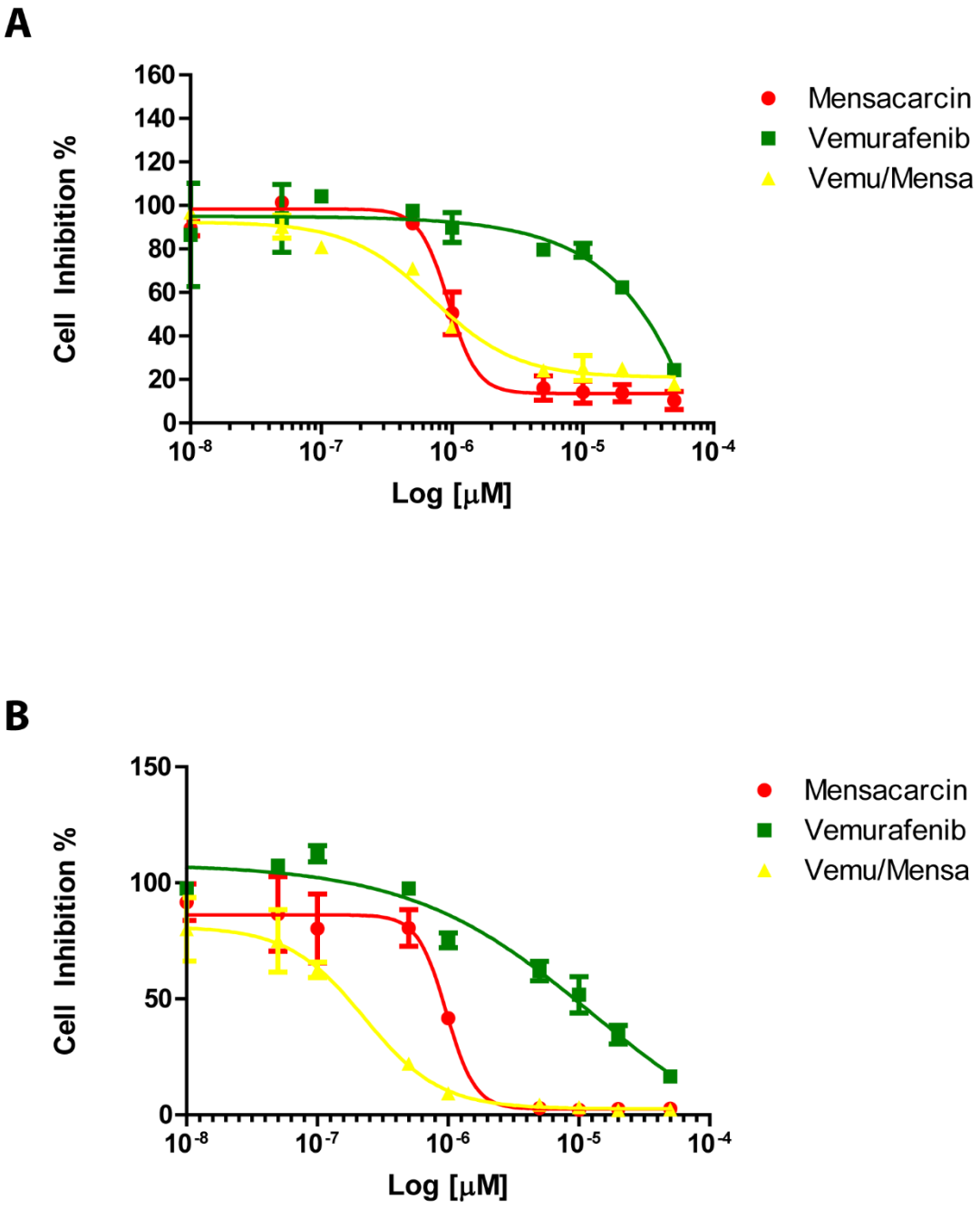


Figure 10. Dose and time dependent growth inhibition of SK-Mel-5 cells treated with mensacarcin and vemurafenib individually and in combination for 24 hrs (A) and 72 hrs (B).

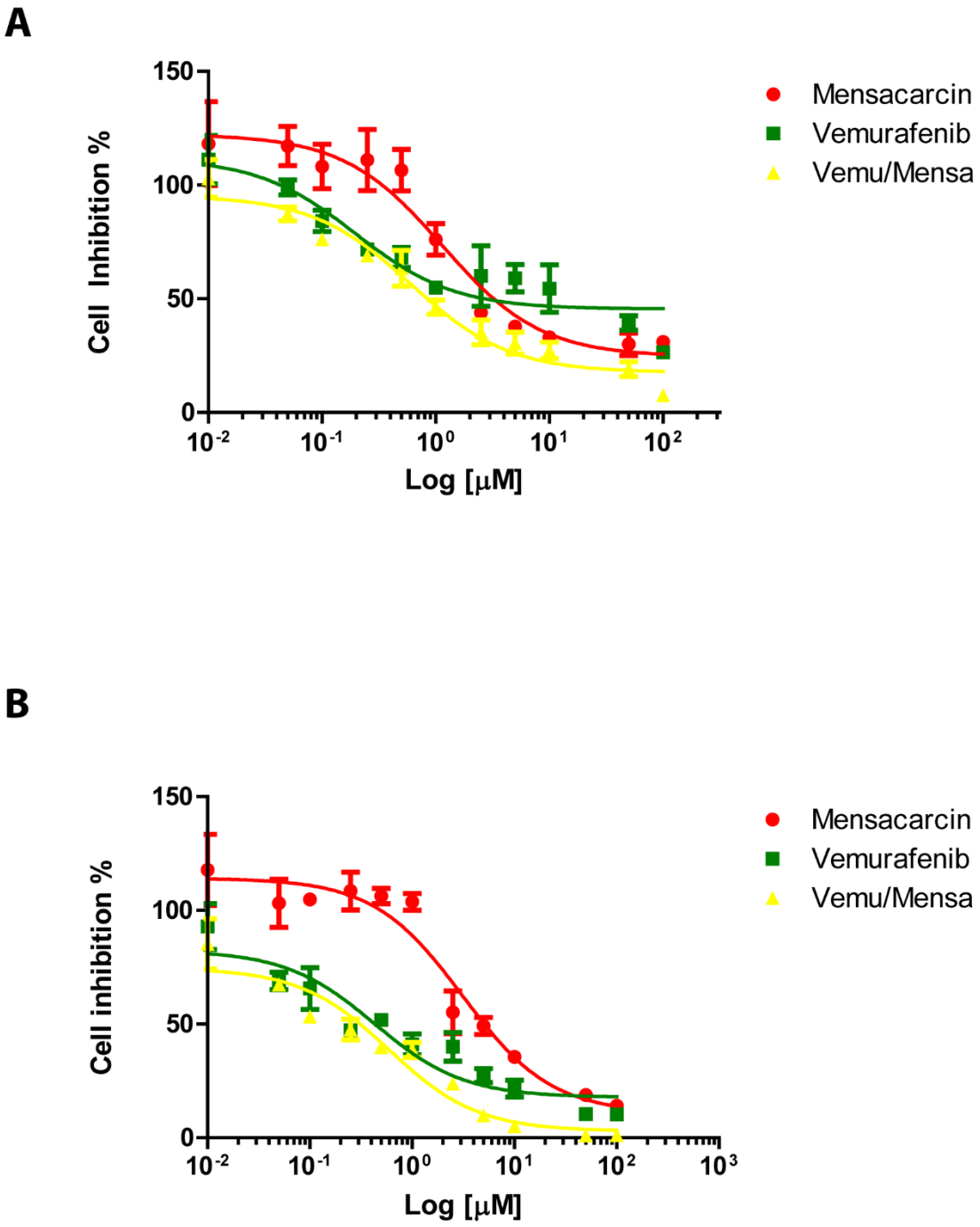


Figure 11. Dose and time dependent growth inhibition of SK-Mel-28 cells treated with mensacarcin and vemurafenib individually and in combination for 24hrs (A) and 72hrs (B).

Chapter 4: CONCLUSION

Mensacarcin is a stereogenic complex polyketide isolated from *Streptomyces bottropensis* near a cafeteria at the University of Gottingen in Germany. This molecule inhibits cell growth across all cell lines tested in the NCI-60 cell panel but is only selectively cytotoxic to melanoma cell lines. Mensacarcin's cytotoxicity is induced by the caspase-dependent apoptotic pathway as observed by experiments and assays that measure caspase-3 and -7 activity as well as the release of cytochrome c. Apoptosis was also evident by DNA and nuclear damage in the comet assay and confocal microscopy respectively. Rhodamine labeled mensacarcin localized in the mitochondria which was indicative of mensacarcin's possible site of action. In addition, Sea horse flux analyzer experiments with mensacarcin treated cells show a decrease in oxygen consumption rate which is indicative of mitochondria dysfunction.

Mensacarcin energetics study show no evidence of apoptosis by the generation of ROS. Also, mensacarcin treatment of cells cultured in different glucose concentrations shows no difference in cell growth inhibition thereby, does not provide any insight if the mitochondria dysfunction caused by mensacarcin stems from glycolysis or oxidative phosphorylation pathways. However, cell growth inhibition was observed with the combinatorial treatment of mensacarcin with two glycolytic inhibitors: 2-deoxyglucose and 3-bromopyruvic acid. Statistical enhancement of cytotoxicity was observed in the case of 2-deoxyglucose and dichloroacetate combination treatments, but not 3-bromopyruvic acid. Lastly, combinatorial treatment of mensacarcin with the currently, clinically used drug vemurafenib showed increased cytotoxicity and synergistic effects in melanoma cell lines.

Given our current studies on mensacarcin's cytostatic and cytotoxic activity, further experiments incorporating pulldown assays and proteomics will focus on determining

mensacarcin's mechanism of action and target in BRAF-mutant melanoma cells as well as non BRAF-mutant cell lines like colon carcinoma cell line HCT-116. In addition, synthesis of mensacarcin derivatives will explore the different structure activity relationships of mensacarcin and the changes in biological activity. This can also lead to the development of new and compounds with unique cytotoxic effects that will be further be studied as potential anti-cancer drug leads.

CHAPTER 5: EXPERIMENTAL PROCEDURES

Reagents and cell lines

Two parental melanoma cell lines with the BRAF mutation were used: SK-Mel-5 and SK-Mel-28. SK-Mel-5 cells were obtained from the NCI cell line repository (Frederick, MD). SK-Mel 28 cells were a kind gift from Dr. Nupur Pande (Oregon Health and Science University, Portland, OR). Minimum essential medium, DMEM, trypsin/EDTA (0.25%/2.21 mM), and penicillin/streptomycin solution were obtained from Corning Life Sciences (Corning, NY). FBS was obtained from Atlanta Biologicals (Flowery Branch, GA). Anti-PARP-1 (catalog no. 9542) and anti-caspase-3 (catalog no. 9662) antibodies were obtained from Cell Signaling Technologies (Danvers, MA). Mensacarcin was received from Bioviotica (Göttingen, Germany), and its purity and stability in stock solutions were tested by LC-MS analysis before usage.

Cell Culture

SK-Mel-5 and SK-Mel-28 cells were grown in minimum essential medium supplemented with 10% (v/v) FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). The cell lines were maintained in a humidified chamber at 37 °C with 5% CO2. The passage number for cells used in experiments never exceeded 15 passages. All cell lines were tested mycoplasma- negative by real-time PCR (Myco Solutions mycoplasma detection kit, Akron Biotech, Boca Raton, FL).

Cell proliferation assays

Cell viability was determined by measuring the reduction of the tetrazolium salt MTT by metabolically active cells (49). Cells were plated into 96-well plates at a density of 60,0000 cells/ml and maintained overnight before treatment was started with the addition compounds to each well. After the designated time, MTT (5 mg/ml in PBS) was added to each well at a final concentration of 0.5 mg/ml. The plates were incubated for 2 h at 37 °C. The medium was removed,

cells were lysed, and the purple formazan product was solubilized by the addition of 50 µl of DMSO. Absorbance was measured at 550 nm. Metabolic activity of vehicle-treated cells (0.5% DMSO) unless otherwise stated) was defined as 100% cell growth.

MTT specifics for ROS study: SK-Mel 5 cells were seeded into 12-well plates. Different concentrations of mensacarcin were used to treat the cells (0.5, 1 and 2 µM). In addition, different vitamin E concentrations (50 and 100 µM) were used in co-treatment of the cells with mensacarcin in the respective treatment plates for 24 hrs.

MTT specifics for carbohydrate study: SK-Mel 5 cells were cultured in flasks with different glucose and galactose concentrations: no glucose, 5.5 mM glucose, 5.5 mM galactose and 25 mM glucose. Cells were treated with different mensacarcin concentrations (0.01-100 µM) for 24hrs.

MTT specifics for glycolytic inhibitor study: SK-Mel-5 cells were treated with different mensacarcin concentrations (0.01-100 µM) in combination with 2-DG (2 mM), 3-BPA (10 µM) and DCA (20 mM) for 24 hrs.

MTT specifics for combinatorial treatment: SK-Mel-5 and SK-Mel-28 cells were treated with mensacarcin only, vemurafenib only, and in combination at a constant ratio for 24 and 72hrs at different concentrations (0.01-100 µM).

Cell cytotoxicity assay

Cell cytotoxicity was determined by measuring the extent of lactate dehydrogenase (LDH) release of cells. Cell were plated into 96-well plates and maintained overnight before treatment was started with the addition of mensacarcin. After the designated time, LDH activity of the cell supernatant was measured. Briefly, 50 µl of supernatant of each well was transferred to a new well plate, and 50 µl of iodonitrotetrazolium lactate mix was added (100 µl of iodonitrotetrazolium solution (25 mg/ml in DMSO), 100 µl of phenazine methosulfate solution (7.75 mg/ml in PBS),

2.3 ml of NAD^+ (2.8 mg/ml in PBS), and 2.5 ml of lithium lactate solution (150mM lithium lactate in 50 mM Tris, pH 8.5). Well plates were incubated for 15 min at room temperature in the dark, and the reaction was stopped by the addition of 50 µl of 1 M acetic acid. Absorbance was measured at 490 nm. LDH activity of lysed vehicle-treated cells (0.5% DMSO unless otherwise stated) was defined as 100% cell cytotoxicity.

Western blot Analysis

Immunoblot analysis of PARP-1 cleavage and caspase-3 activation followed previous described procedures.

Data Analysis

IC_{50} curves and values for the different treatments were generated using Graph Pad Prism to determine the interactions between respective treatments.

Statistical Analysis

Statistical analyses were done using GraphPad Prism version 5.0. The significance of observed differences was evaluated by one-way analysis of variance followed by Bonferroni multiple-comparison post hoc test. In all cases, $p < 0.05$ was significant. Drug combination index (CI) was obtained using Compusyn software. CI>1, CI=1, CI<1 indicate antagonism, additive effect, and synergism respectively.

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