Investigation of Cell Death in Response to the Marine Natural Product Mandelalide A

by
Karina Destine

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in BioHealth Sciences
(Honors Scholar)

Presented May 31, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Karina Destine for the degree of Honors Baccalaureate of Science in BioHealth Sciences presented on May 31, 2016. Title: Investigation of Cell Death in Response to Marine Natural Product Mandelalide A .

Abstract approved:

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Jane Ishmael, Ph.D.

The structural and chemical diversity of natural compounds represents a vast amount of potential in terms of new drug discovery. This project examines the cytotoxic potential of the marine natural product mandelalide A and seeks to understand whether or not exposure to this compound induces programmed cell death in cancer cells grown in culture. Human HeLa cervical cancer cells were examined for changes in morphology as well as expression of caspase-3 and poly(ADP-ribose) polymerase (PARP1), classic biomarkers of cell death signaling by apoptosis, when exposed to mandelalide A. It was found that mandelalide A caused time and concentration-dependent changes in HeLa cell morphology; cells treated with mandelalide A appeared rounded and eventually detached from the cell culture plates whereas vehicle-treated cells showed normal morphology and growth characteristics. Western blot analysis of HeLa cell lysates, grown in the presence and absence of mandelalide A, revealed cleaved forms of caspase-3 and PARP1 in mandelalide A treated cells, indicating apoptosis has occurred. Taken together these findings provide the first evidence that mandelalide A can induce time and concentration-dependent changes in HeLa cells that are consistent with apoptotic cell death.

Key Words: natural products, cell death, apoptosis, mandelalide A

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

Karina Destine, Author

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INTRODUCTION

Natural products hold a vast amount of potential when it comes to drug discovery. The structural and chemical diversity exhibited by these compounds have led to numerous discoveries, so many that claims have arisen stating that a new golden age of natural product drug discovery is currently emerging (Shen, 2015). In search of new natural compounds, attention has been directed towards the sea because while marine environments comprise about 71% of the earth's surface, it remains relatively unexplored with only 5% of the deep sea explored and only 0.01% of the deep sea floor sampled (Newman & Cragg, 2015). More specifically, interest has been targeted towards locating compounds with cytotoxic properties since marine environments have historically been ideal places to look for cytotoxic compounds with high potencies. This logic makes sense because if these compounds did not have a high potency, compounds would be rendered useless when exposed to water as they would immediately become diluted to ineffective amounts (Haefner, 2013).

Natural products with cytotoxic properties are of relevance because they have the potential to reveal new mechanisms of action and, after understanding how they work, could ultimately inspire the development of new drugs to treat human cancers. Modern chemotherapy is based on the use of drugs that either inhibit the growth or kill cancer cells. The discovery of new compounds with cytotoxic properties is important as human cancer cells have the ability to evade or resist cell death. This may be an inherent property of the cancer cell or can be acquired during treatment leading to drug resistance and disease progression.

According to the Nomenclature Committee on Cell Death (NCCD), cell death is acknowledged when three criteria have been met: (1) a cell must lose the ability to maintain the barrier function of its cytoplasm, (2) the cell breaks apart causing the formation of apoptotic bodies, and (3) the cell must be engulfed by a phagocytic cell assuming a phagocytic cell is located in a close proximity to the dead or dying cell in question (Galluzzi et al., 2015). In addition, when discussing cell death, one must be very precise as the occurrence of cell death can be classified into two categories. The first category is accidental cell death (ACD) which is death caused by various incidents such as the cell's exposure to a harsh environment like a high pH (Galluzzi et al., 2015). The main point is that with ACD, there is no way to control or inhibit cell death from occurring. The second category is regulated cell death (RCD) and this can be defined as death following initiation of some sort of mechanism by either a pharmacological or genetic intervention. This kind of death has evolved as a protective mechanism to help maintain cellular homeostasis (Galluzzi et al., 2015). As opposed to ACD, RCD can be controlled by the cell, and is identified as a subgroup of RCD known as programmed cell death (PCD) (Galluzzi et al., 2015).

One way to induce PCD is through a pathway known as type I cell death or apoptosis. Apoptosis is determined morphologically by the presence of cytoplasmic shrinkage, chromatin condensation originating at either the nuclear membrane (marginalization) or the whole nucleus (pyknosis), breakdown of the nucleus into fragments (karyorrhesis), or a compromised cell membrane in which the cell is unable to hold its plasma membrane together, creating a leaky membrane that it spills out its contents (blebbing) (Galluzzi et al., 2015).

Another way to study apoptosis is through the use of biochemical markers. Apoptosis is a highly conserved form of PCD and multiple biomarkers have now been identified in this well-studied pathway. The intracellular protein caspase-3 is a reliable biomarker of apoptosis as it is a crucial effector to both intracellular and extracellular activation of apoptosis in cells (Figure 1). Intracellularly, an intrinsic pathway is initiated from the mitochondria reacting to B-cell CLL/lymphoma 2 associated X protein (BAX) or BH3 interacting domain death agonist (BID) which are members of the Bcl-2 family involved in mitochondrial outer membrane permeabilization (Galluzzi et al., 2015). Downstream from here, the mitochondria will activate cytochrome c to bind to apoptotic peptidase-activating factor 1 (APAF1) and procaspase-9 to create a death inducing signaling complex (DISC) apoptosome to activate caspase-9, which in turn activates caspase-3 (Jin and El-Deiry, 2005). Another intrinsic pathway can be started in response to endoplasmic reticulum (ER) induced stress which will then induce ER bound caspase-12 to signal and activate caspase-3 (Hitomi et al., 2004). Extracellularly, an extrinsic pathway can be generated with the binding of Fas, a ligand in the tumor necrosis factor (TNF) family, to a Fas receptor on the cell. Downstream, this will cause the formation of a Fas-associated protein with death domain **(**FADD) which will bind to caspase-8 to form a DISC that will activate caspase-3 (Li et al., 1988). Ultimately, activation of caspase-3 will affect a biomarker known as poly(ADP-ribose) polymerase-1 (PARP1) which plays a critical role in deoxyribonucleic acid (DNA) repair. Once caspase-3 is activated, it will cleave PARP1 and eliminate the cell's ability to repair DNA breaks.

An inability to hold DNA together causes instability in the cell, enabling apoptosis to occur.

Emphasis has been placed on a marine product called mandelalide A (Figure 2) because when exposed to human lung cancer and mouse neuroblastoma cells, it exhibited low nanomolar cytotoxicity (Sikorska et al., 2012). This compound is the first of a series of related natural product structures discovered by Dr. Kerry McPhail from a new species of *Lissoclinum* ascidian found in Algoa Bay, South Africa (Sikorska et al., 2012). More recent studies, with other mandelalides in the series (B and E), confirmed the ability of the mandelalides to reduce the viability of laboratory cultured human cancer cells such as: NCI-H460 lung, HeLa cervical, U87-MG glioblastoma, and HT116 colon (Nazari et al., 2016). Mandelalide B is a close analogue of mandelalide A and induced a concentration-dependent cytotoxic response in all four cell lines tested (Nazari et al., 2016). From mandelalide B a ranked order of vulnerability was noticed with HeLa being the most vulnerable, followed in order by NCI-H460, HCT116, then U87-MG (Nazari et al., 2016).

Although the mandelalides appear to reduce the viability of human cancer cells, more studies are needed in order to determine if exposure to mandelalide A just stops cells from growing or causes cells to die. This thesis will investigate mandelalide A-induced changes in cell morphology and potential changes in the expression of caspase-3 and PARP1, as biomarkers of cell death signaling in human cancer cells. I will use HeLa, the most vulnerable human cancer cell line of those tested to date (Nazari et al., 2016), to test the hypothesis that mandelalide A induces time and concentration-dependent programmed cell death.

MATERIALS AND METHODS

All experiments were repeated at least three times on independent cultures.

Reagents:

Mandelalide A was a kind gift from Dr. Amos Smith. For all studies, mandelalide A was reconstituted in 100% dimethyl sulfoxide (DMSO). Final concentrations of DMSO never exceeded 0.1 % in culture medium.

Analysis of Cell Morphology:

Prior to treatment, HeLa cell lines were seeded in 6 cm^2 cell culture dishes at a density of 6,000 cells per well and allowed to settle to the bottom of the cell culture dishes overnight. All cell culture dishes were left to incubate at 37°C with 5% CO_2 in a medium containing Eagle's minimum essential medium (MEM), 10% fetal bovine serum (FBS), and 1% penicillin streptomycin solution.

For the first independent culture, 3 cell culture dishes were treated. Each cell culture dish received 3 µl of one of three different treatments: vehicle-control 0.1% DMSO, 1 nM of mandelalide A, or 3 nM of mandelalide A. Cell culture dishes were left to incubate for 12 hours before morphological analysis.

For the second independent culture, one cell culture dish was left as an untreated control while 3 cell culture dishes were treated with similar conditions to the first independent culture. Each cell culture dishes received 3 µl of a separate treatments containing either vehicle-control 0.1% DMSO, 1 nM of mandelalide A, or 3 nM of mandelalide A. Cell culture dishes were left to incubate for 12 hours before morphological analysis.

Analysis of cell morphology was conducted with a microscope at 10X magnification and an image was captured for each cell culture plate. Cells from each cell culture plate were examined for both morphology and growth characteristics. Observed results were compared between mandelalide A treated cells and the controls of untreated (when present) and 0.1% DMSO to account for any differences.

Cell Harvesting:

HeLa cell lines were seeded in 6 cm^2 cell culture dishes at a density of 6,000 cells per well and allowed to settle to the bottom of the cell culture dishes overnight. On the day of the experiments, each cell culture dish with the exception of an untreated control, received 3 µl of their respective treatment which varied in increasing concentrations of 1 nM to 100 nM of mandelalide A or vehicle-control 0.1 % DMSO. Cell culture dishes were then left to incubate at 37°C with 5% CO_2 in a medium containing MEM, 10% FBS, and 1% penicillin streptomycin solution at various time exposures up to 48 hours.

After incubation, cells from each cell culture dish were harvested. The cell culture dishes were placed on ice and 1 mL of medium was collected and transferred into a 1.5 mL microcentrifuge tube twice. Consequently, the cell culture dishes were washed with 3 mL of phosphate buffered saline (PBS) (Appendix) and aspirated. Next, 70 µl of lysis buffer (Appendix) was applied to the plates. Cell lysates were then scraped off the plate, collected into a 1.5 mL microcentrifuge tube to be set aside for later. The 1.5 mL microcentrifuge tubes containing the 1 mL of media were then centrifuged at 2500 rpm for 5 minutes. After being centrifuged, the media was

aspirated from each 1.5 mL microcentrifuge tube with the pellet kept and 20 µl of lysis buffer from the 1.5 mL microcentrifuge tubes set aside that corresponded to the applied treatment was added to break up each pellet. This was then transferred back into the 1.5 mL microcentrifuge tubes set aside and centrifuged at 13,000 rpm for 20 minutes. Once completed, the supernatant was kept with the pellets removed.

Normalization of Cell Lysates:

The cell lysate samples were normalized using a standard bicinchoninic acid assay (BCA) in a 96 well plate. The first lane contained increasing concentrations of 0 to 7 µl of bovine serum albumin (BSA) at a concentration of 2 mg/mL with each well filled with ddH_2O to a total volume of 20 µl and was used as a standard to compare the cell lysate samples to. The samples were placed in the two lanes that followed consecutively after the first lane in a 1:10 dilution with 18 µl of ddH_2O to 2 µl of cell lysate sample. BCA protein assay reagents A (3 ml) and B (60 µl) from Pierce BCA Protein Assay Kit were mixed together thoroughly and 80 µl of the combined reagents were added to each well. The 96 well plate was incubated at 37°C with 5% CO_2 for 30 minutes. After incubation, the 96 well plate was read at an absorbance setting of 562 nM. The absorbance readings were used to create running lysates of diluted samples of either 20 µg/ 20 µl or 40 µg/20 µl in lysis buffer and 4X Laemmli buffer (Appendix).

Western Blot Analysis:

Running lysate samples were prepared for western blot by initially being warmed up in a water bath a little above body temperature (37° C) and then centrifuged for 15 seconds. 20 µl of the running lysate as well as a 5 µl of Thermo Scientific PageRuler Plus Prestained Protein Ladder (10 to 250 kDa) were loaded into either a 10% or 12.5% acrylamide gel (Appendix) with a 4.5% stacking gel (Appendix). Acrylamide gels immersed in 1X Tris-Glycine SDS Western Blotting Running Buffer (Running Buffer) diluted from a 10X Running Buffer (Appendix) were run at 100 V for 15 minutes and then at 160 V for at least 60 minutes until the bands in the DNA ladder had distinctly separated.

For protein transfer, Polyvinylidene fluoride (PVDF) membranes were run at 95 V for 1 hour and 30 minutes in an Electro-Transfer Buffer (Appendix). Strips were blocked in 5% dry milk in Tris Buffered Saline and Tween 20 (TBST) (Appendix) for 45 minutes. The strips were subsequently bathed in 2 baths of TBST for 5 minutes each and incubated with primary stock antibodies from Cell Signaling Technology in specified dilutions (anti-PARP1 in 1:1000 dilution, anti-caspase-3 in 1:1000 dilution, anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) in 1:2000 dilution, and anti-tubulin in 1:1000 dilution) with 5% BSA in TBST overnight.

Once incubated, the strips were washed in 2 baths of TBST for 5 minutes each, bathed in the secondary antibody (anti-rabbit in a 1:2000 dilution with 5% dry milk in TBST), and 3 baths in TBST for 5 minutes each. Strips were then observed via enhanced chemiluminescence (ECL) by applying 0.6 mL of ECL reagents (Solution A Luminol Enhancer Solution and Solution B Peroxide Solution) each from

a Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate kit. Images were captured on a Thermo Scientific myECL Imager.

If necessary, membranes were stripped and reprobed by washing the PVDF membrane 4 times for 5 minutes each in a TBST bath. PVDF membranes were then incubated and washed in the dark for 30 minutes bath in stripping buffer (Appendix). Finally PVDF membranes were washed in a TBST bath 6 times for 5 minutes each. After this step, primary antibodies can be added to the PVDF membranes and western blotting technique can proceed as normal.

RESULTS

Morphological Changes in Response to Mandelalide A

Independent cultures of human HeLa cervical cancer cells were left to incubate for 12 hours and with one of three conditions: DMSO (0.1%), mandelalide A (1 nM), or mandelalide A (3 nM). Under examination with a microscope at 10X mandelalide A treated HeLa cells were compared to the vehicle-control DMSO treated HeLa cells which adhered as expected and showed normal morphology (Figure 3). In the conditions of 1 nM and 3 nM of mandelalide A, a majority of treated HeLa cells adhered to the plate and showed normal morphology, however some of the treated HeLa cells lifted off the plate, aggregated together, and appeared round in shape. Treated HeLa cells that lift off the plate also appear smaller in size when compared to HeLa cells that have not lifted off the plate such as those seen in HeLa cells incubated in the condition of vehicle-control DMSO.

More independent cultures of HeLa cells were then left to incubate for a longer period of 72 hours in one of four conditions: untreated, DMSO (0.1%), or mandelalide A at a higher concentration of either 30 nM or 100 nM (Figure 4). Upon examination with a microscope at 10X, in the conditions of 30 nM and 100 nM mandelalide A, HeLa cells had detached from the plates, aggregated together, and appeared round in shape. The untreated control HeLa cells and HeLa cells with the vehicle-control DMSO continued to adhere to the plates and showed normal morphology. In addition, the treated HeLa cells that lift off the plate also appeared much smaller in size when compared to cells that have not lifted off the plate such as those seen in HeLa cells incubated in the condition of the untreated control or the

vehicle-control DMSO. It should also be noted that due to this longer incubation period, at 72 hours, there were dead HeLa cells in both of the untreated and vehicle-control DMSO conditions. However, more death occurred in the plates with treated HeLa cells as opposed to the controlled conditions.

Analysis of Caspase-3 and PARP1 Cleavage in HeLa Cell Lysates

Classic biomarkers, caspase-3 and PARP1, were detected in HeLa cell lysates via western blot analysis (Figure 5). It should also be noted that due to the specific antibody used, caspase-3 was expressed in its cleaved form, whereas anti-PARP1 recognized both cleaved and full-length protein. Western blots were run with independent cultures of treated human HeLa cervical cancer cells.

The first independent culture was run in a western blot with the loading control GAPDH used for comparison against PARP1 and the cleaved form of caspase-3 in HeLa cells receiving either a 12, 24, or 30 hour treatment in one of four conditions: untreated, vehicle-control DMSO (0.1%) , mandelalide A (1 nM), and mandelalide A (3 nM) (Figure 5A). From this western blot, four trends are noted. First, as time and concentration of mandelalide A increased, two bands, corresponding to the full length and cleaved forms of PARP, were detected starting in HeLa cells harvested after 24 hours that received treatment of 1 nM of mandelalide A. Another trend noted is with HeLa cells harvested after 12, 24, and 30 hours that received a treatment of mandelalide A at 3 nM, the bands of PARP1 expression were either lighter or thinner than PARP1 bands expressed by the controls (untreated and DMSO) and HeLa cells treated with 1 nM of mandelalide. In addition, as time and

concentration of mandelalide A increase, expression of the cleaved form of caspase-3 appeared only in HeLa cells harvested after 24 hours that received a treatment of mandelalide A at 1 nM and 3 nM and in HeLa cells harvested after 30 hours that received a treatment of mandelalide A at 3 nM. Lastly, the bands of the cleaved form of caspase-3 that are expressed appear darker as the time the HeLa cells were harvested and the concentration of mandelalide A the HeLa cells were exposed to increased.

Lysates from the other independent culture were analyzed by western blot with two loading controls, GAPDH and tubulin, which used for comparison against PARP1 and cleaved caspase-3. In these studies HeLa cells were treated for 12, 24, or 30 hour with: DMSO (0.1%), mandelalide A (1 nM), or mandelalide (3 nM) (Figure 5B). Trends observed are consistent with the ones noted in the other western blots. Two bands (corresponding to full length and the cleaved form of PARP1) were observed in HeLa cell lysates harvested after 12 hours that received a treatment of mandelalide A at 3 nM, in HeLa cells harvested after 24 hours that received a treatment of mandelalide A at 1 nM and 3 nM, and in HeLa cells harvested after 30 hours that received a treatment of mandelalide A at 1 nM and 3 nM. When treated HeLa cells that exhibited PARP1 expression were compared to the PARP1 expression of the vehicle-control DMSO, the bands of PARP1 expression were lighter or thinner in all mandelalide A treated HeLa cells. In addition, as time and concentration of mandelalide A increased, expression of the cleaved form caspase-3 only appeared in HeLa cells harvested after 24 hours that received a treatment of mandelalide A at 3 nM and HeLa cells harvested after 30 hours that received a treatment of mandelalide

A at 1nM and 3 nM. The bands of the cleaved form of caspase-3 that are expressed also appear darker as the time the cells were harvested and the concentration of mandelalide A the cells were exposed to increased.

DISCUSSION

The results of studies designed to assess morphological changes in response to mandelalide A are consistent with the hypothesis that cells undergo cell death as a consequence of treatment. The round shape of mandelalide A-treated cells is indicative of one of the first criteria outlined by NCCD, and this abnormal shape may be the result of the cell's inability to maintain barrier function of its cytoplasm. Rounded shape can also occur as a result of cytoplasmic shrinkage and potentially explains why HeLa cells that have lifted off the plate appear smaller in size than cells that continue to adhere to plates such as in the untreated control and vehicle control. This is important as cytoplasmic shrinkage is a morphological determinant used to identify when apoptosis is occurring in a cell (Galluzzi et al., 2015).

The results of the morphological changes in response to mandelalide A also eliminate the possibility that the cytotoxic property of marine natural product only stops cells from growing as opposed to causing cells to die. If mandelalide A stopped cells from growing, mandelalide A-treated cells would have been less abundant on the culture plates but would have been expected to shown normal morphology similar to the plates with the untreated control and the vehicle-control DMSO when viewed under the microscope. Together, the results strongly suggest that exposure to mandelalide A caused the death of the HeLa cells.

Evidence of cell death in response to mandelalide A was also achieved through analysis of caspase-3 and PARP1 expression. The full length form of PARP1 was expressed in all conditions of HeLa cells, and this makes sense as PARP1 is involved in DNA repair, something all cells need. Yet, the cleaved form of PARP1

was strongly expressed only when the cleaved form of caspase-3 was expressed as well. These results support what is known about the apoptosis cell death signaling pathway since cleavage of caspase-3 means that caspase-3 was activated and so downstream, cleavage of PARP1 occurs, ultimately allowing the cell to undergo apoptosis. This pattern of expression only occurred in HeLa cells treated with mandelalide A which provides more conclusive evidence that mandelalide A causes apoptotic cell death. In addition, the trends noted with cleaved form of caspase-3 showing darker bands of expression and the full length form of PARP1 showing lighter bands of expression over increased time and treatment to a higher concentration of mandelalide A support the hypothesis of mandelalide A inducing time and concentration-dependent programmed cell death in cancer cells.

Comparison of Mandelalide A to Ecteinascidin-743

It is worth comparing mandelalide A to other marine natural products to emphasize the potential good mandelalide A could do once its mechanism of action is better understood and able to harnessed in order to developed into a new drug to treat human cancers. Drugs that target apoptotic pathways are of great importance because if utilized correctly, these drugs can be used to inhibit the ability of a human cancer cell to evade or resist death.

Success has been seen in a marine natural product known as ecteinascidin-743 (ET-743). Originally isolated from a Caribbean tunicate *Ecteinascidia turbinate,* it became the first marine derived anti-tumor drug on the market and is sold commercially as Trabectedin (Vollmar and Von Schwarzenberg, 2010). ET-743 shows different anti-proliferative properties depending on concentration. At 1-10

ng/ml, it can negatively affect transcription, but at a higher concentration of 10-100 ng/ml apoptosis can be induced intrinsically through activation of mitochondrial cytochrome c release, c-Jun NH_2-terminal kinase, and caspase-3 (Vollmar and Von Schwarzenberg, 2010). As both mandelalide A and ET-743 share the ability to induce apoptosis by caspase-3 activation at low concentrations, once the full mechanism of action is discovered for mandelalide A, it could be developed into a successful cancer treating drug such as ET-743.

Future Studies

To create more morphological evidence of cell death being achieved, HeLa cells could be incubated for longer periods of time and introduced to phagocytic cells to see if the two other criteria for cell death from the NCCD could be observed (formation of apoptotic bodies and engulfment by phagocytosis cells). Other morphological determinants of apoptosis could be examined for as well should incubation time increase.

In regards to examining biomarker expression, while cleavage of caspase-3 and PARP1 have been identified indicating the activation of cell signaling for apoptosis, more specifics on the mechanism of action for mandelalide A still need to be determined. Future studies could be targeted towards determining if the pathway is trigged intrinsically in the mitochondria or the ER, or extrinsically by receptor binding of a ligand in the tumor necrosis factor family such as Fas.

Lastly, while useful, apoptosis assays have limitations that need to be taken into consideration as well. It is important to note that only one human cancer cell line, HeLa, was examined in this project. This is a potential weakness as different cancer

cell lines do not behave in the same way when exposed to identical situations (Sundquist et al., 2006). This being said, other human cell lines such as the other three (NCI-H460, HCT116, then U87-MG) noted for their vulnerability in the study by Nazari et al. should be tested to see if these cell lines behave in similar ways to HeLa cell lines. Should mandelalide A induce other cell death signaling aside from just apoptosis, this would only help elevate mandelalide A to a status of higher priority to focus on developing as a drug to treat cancer. Another future study to consider is testing mandelalide A on normal human cell lines in order to see if there is a difference in response between normal human cells versus cancerous human cells.

Concluding Remarks

Both pieces of evidence from the trends observed by morphological and biomarker expression of caspase-3 and PARP1 in HeLa cells support the hypothesis that mandelalide A induces time and concentration-dependent programmed cell death through apoptosis. The ability of mandelalide A to induce apoptosis makes it a marine natural product worthy of further investigation.

If with further research mandelalide A is discovered to ill-suited for drug development, it is still a natural product of value as mandelalide A can be used to further exemplifies the structural and chemical diversity of natural products and encourage others to tap into the undiscovered potential organic compounds offered by the sea.

FIGURES

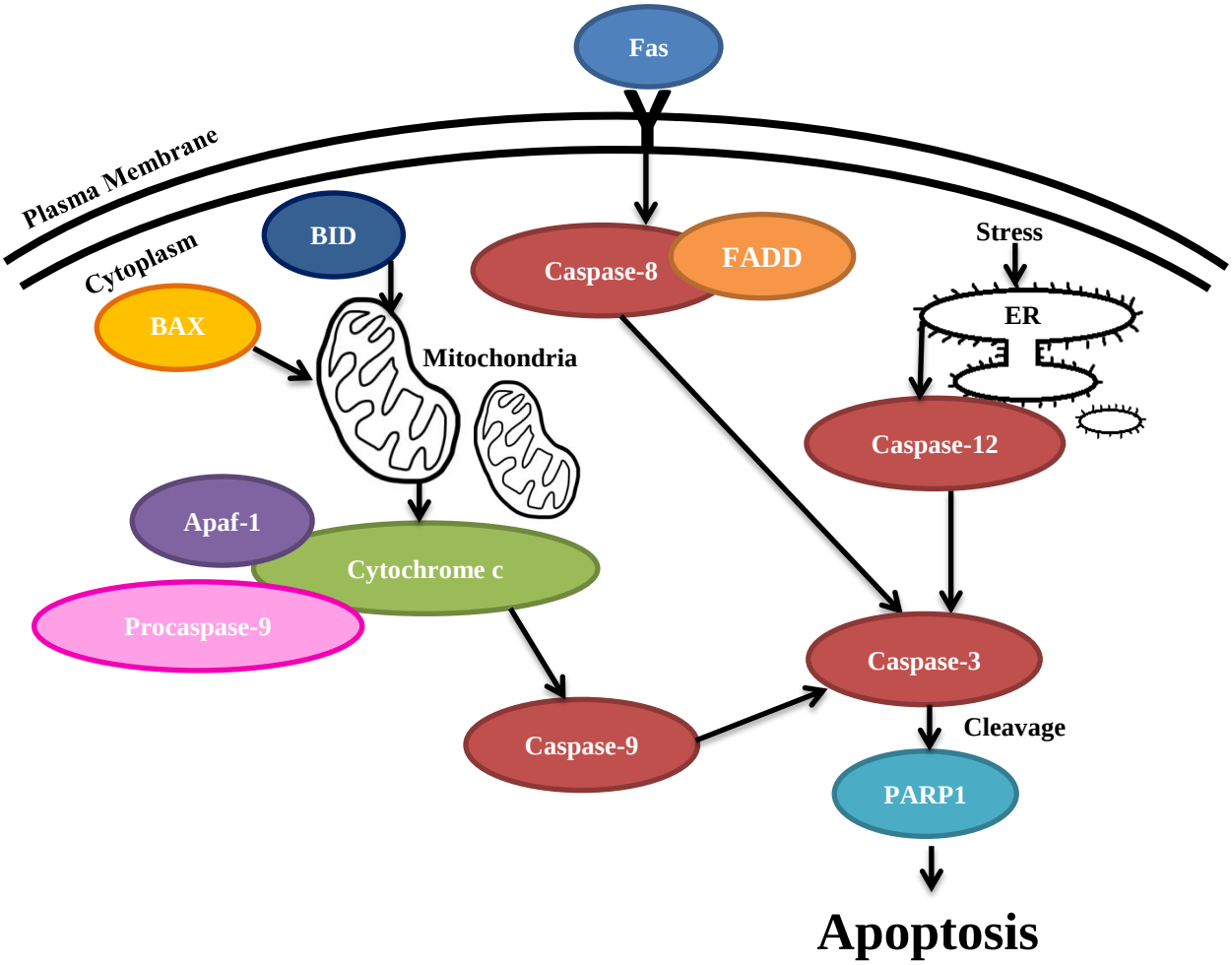
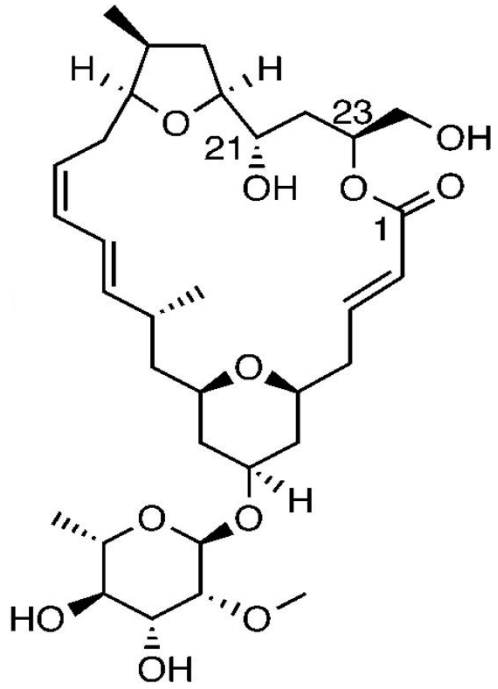


Figure 1: Apoptosis Signaling Pathway

Both intracellular and extracellular pathways can activate caspase-3 to cleave PARP1 to allow for apoptosis to occur.



Mandelalide A

Figure 2: Structure of Mandelalide A

Mandelalide A is the first in a series of natural products discovered by Dr. Kerry McPhail. It was isolated from a recently discovered species of *Lissoclinum* ascidian from Algoa Bay, South Africa. Structure was obtained from *Mandelalides A-D, Cytotoxic Macrolides from a New Lissoclinum Species of South African Tunicate* (Sikorska et al., 2012).

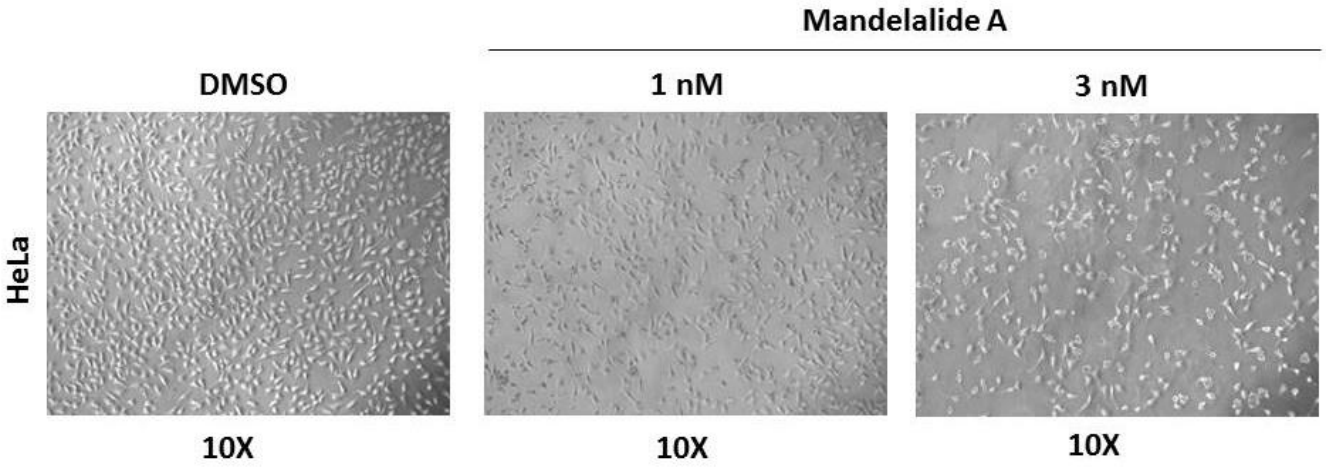


Figure 3: Morphological Changes in Response to Mandelalide A After 12 Hours

Examination of HeLa, human cervical cancer cells, after a 12 hour exposure to mandelalide A (1 nM or 3 nM) via a microscope at 10X. In response to a lower concentration of mandelalide A and exposure time, the same result as treated cells detach from the plates whereas untreated and vehicle-treated control cells adhere as expected.

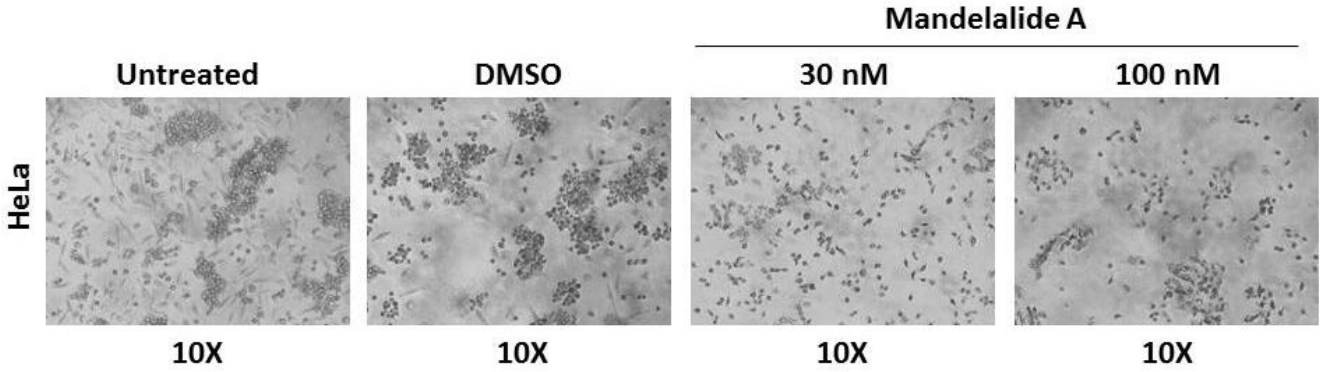


Figure 4: Morphological Changes in Response of Mandelalide A after 72 Hours

Examination of HeLa, human cervical cancer cells, after a 72 hour exposure to mandelalide A (30 nM or 100 nM) via a microscope at 10X. In response to exposure to mandelalide A, results consistent with Figure 3 occur as treated cells detach from the plates whereas untreated and vehicle-treated control cells adhere as expected.

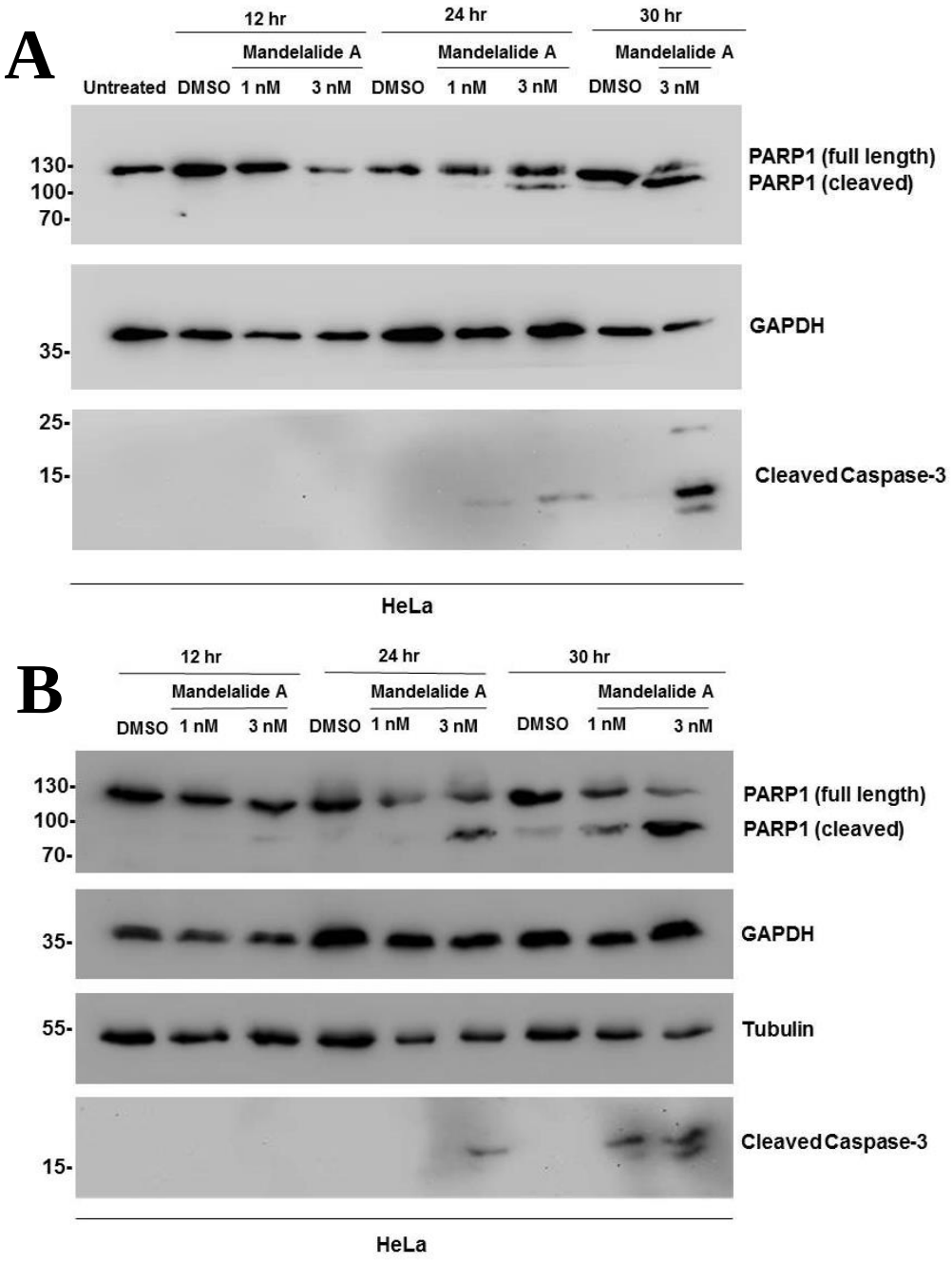


Figure 5: Caspase-3 and PARP1 Cleavage in HeLa Cells

A) Western blot with loading control, GAPDH, and classic biomarkers, PARP1 and caspase-3 (in the cleaved form), in HeLa cells. HeLa cells were incubated for either 12, 24, or 30 hours and received one of four treatments: vehicle-control DMSO, mandelalide A at 1 nM, or mandelalide A at 3 nM.

B) Western blot with loading controls, GAPDH and tubulin, and classic biomarkers, PARP1 and caspase-3, (in the cleaved form) in HeLa cells. HeLa cells were incubated for either 12, 24, or 30 hours and received one of four treatments: vehicle-control DMSO, mandelalide A at 1 nM, or mandelalide A at 3 nM.

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APPENDIX

1X Phosphate Buffered Saline (PBS)

NaCl	8 g
KCl	0.2 g
$Na_2HPO_4 \cdot 7H_2O$	1.44 g
KH_2PO_4	0.24 g
HCl	Adjust pH of solution to 7.4
diH_2O	Bring volume to 1 L

Lysis Buffer

Tris-HCL 1 M pH 7.5	0.5 mL
EGTA 0.5 M	0.02 mL
EDTA 0.5 M	0.02 mL
Triton X-100 10%	1 mL
Sucrose	0.92 g
Na_3VO_4 100 mM (*fresh)	0.1 mL
NaF 1M (**fresh)	0.5 mL
Na Pyrophosphate	0.022 g
Benzamidine***	0.02 mL
PMSF****	0.02 mL
diH_2O	Bring volume to 10 mL

* Na_3VO_4: 0.018g in 1 mL diH_2O
**NaF: 0.042g in 1 mL diH_2O
***Benzamidine: 1mM in 10mL
****PMSF: 100mM in 10 mL

4X Laemmli Buffer for 200 mL

Tris	0.8 M
SDS (w/v)	8%
EDTA	5 mM
Glycerol	40%
DTT	0.2 M
Bromophenol Blue	4 mg

Running Acrylamide Mini-Gels with 10% Lower Gel for 10 mL

10% Lower Gel Stock	2.5 mL
30% Acrylamide	3.3 mL
ddH_2O	4.2 mL
TEMED	5.0 µl
10% APS	50 µl

Running Acrylamide Mini-Gels with 12.5% Lower Gel for 10 mL

12.5% Lower Gel Stock	2.5 mL
30% Acrylamide	4.2 mL
ddH_2O	3.3 mL
TEMED	5.0 µl
10% APS	50 µl

4.5% Stacking Gel for 5 mL

10% Lower Gel Stock	1.25 mL
30% Acrylamide	0.75 mL
ddH_2O	3.0 mL
TEMED	5.0 µl
10% APS	25 µl

10X Tris-Glycine SDS Western Blotting Running Buffer (Running Buffer)

Glycine	144 g
TRIS	30.3 g
SDS	10 g
diH_2O	Bring to volume of 1 L

Electro-Transfer Buffer

TRIS	6.06 g
Glycine	28.8 g
Methanol	100 mL
diH_2O	Bring to volume of 1 L

Tris Buffered Saline and Tween 20 (TBST)

NaCL	8 g
KCL	0.2 g
Tris	3 g
HCl	Adjust pH of solution to 7.4
Tween 20	0.5mL
diH_2O	Bring to volume of 1 L

Stripping Buffer

TRIS	0.76 g
SDS	2.00 g
β-mercaptoethanol	700 µl

diH_2O	Bring to volume of 100 mL
HCl	Adjust pH of solution to 6.8
Heat	50-60°C