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The Effects of Marine Natural Products on the Microenvironment of Pancreatic Cancer

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ABSTRACT

Pancreatic cancer is the 4th leading cause of cancer deaths in the United States, with that number increasing each year. New drugs need to be developed to treat this disease. One source of new drugs is marine natural products. Two compounds—HB-131 and HB-018—were tested for potential anti-cancer properties. Pancreatic cancer cells were exposed to these compounds for six hours and the conditioned media from this treatment was used to further our understanding of the effects of these compounds. A western blot was used to determine matrix metalloproteinase (MMP) expression, while a zymogram was used to determine MMP activity. A sandwich enzyme-linked immunosorbent assay (ELISA) array was used to determine the effects of these compounds in 15 different cytokines. HB-131 did not inhibit any of the cytokines tested, which suggests that its inhibition of CCL-2 is specific and not caused by inhibiting protein synthesis. In addition to inhibiting degranulation, HB-018 appeared to downregulate the inflammatory cytokine IL-23, while upregulating the anti-inflammatory cytokine IL-10. HB-131 and HB-018 lowered MMP-2 activity, which might translate to lower metastatic activity. From these findings, HB-131 and HB-018 are one step closer to making it to clinical trials.

INTRODUCTION

Pancreatic cancer is an aggressive disease with a five year survival rate of 8%, making it the fourth leading cause of cancer deaths in the United States. (American Cancer Society, 2018). Pancreatic cancer killed 31,270 diagnosed patients in 2004, and that number has increased to an estimated 44,330 for 2018. (American Cancer Society 2018; Freelove & Walling, 2006). Most pancreatic cancers are pancreatic ductal adenocarcinomas (PDAC), with nonspecific early symptoms that cause the cancer to be more advanced at diagnosis (Freelove & Walling, 2006). A

higher incidence of PDAC is found in patients who have chronic pancreatitis, because inflammation, if not controlled, can lead to cancer development (McKay et al., 2008; Coussens, 2010).

The tumor microenvironment is composed of tumor cells, non-tumor cells, extracellular matrix, cytokines, growth factors, and exosomes that regulate autocrine, paracrine and endocrine communication (Ansari, 2018). Inflammation within this environment can lead to cancer initiation, and/or promote cancer growth, tissue invasion, and metastasis (McKay et al., 2008). Tumors promote local and systemic inflammation by hijacking the immune response to create an environment that fosters tumor growth and progression (Farrow et al., 2008; McKay et al., 2008).

Part of this immune response includes mast cells, which are granulocytic immune cells known for their activity in wound healing and allergic reactions (Rigoni et al., 2015). Inflammation around tumors causes infiltration of mast cells that then facilitate cancer growth, especially in PDAC (Theoharides, 2008). Strouch et al. (2010) found that mast cell infiltration was significantly increased in pancreatic cancer, and increased infiltrating mast cells correlated with higher grade tumors and worst survival.

C-C Motif Chemokine Ligand 2 (CCL-2) is one of the chemokines responsible for the recruitment of mast cells into the tumor microenvironment (Theoharides, 2008). Some tumor cells can regulate chemokine expression to recruit inflammatory cells, as well as, use chemokines to promote tumor growth and progression (Coussens & Werb, 2002). CCL-2 is a target for treatment to halt the migration of mast cells to the tumor microenvironment.

Other mast cell mediators that can be considered pro-tumor are matrix metalloproteinases (MMPs). MMPs are proteolytic enzymes that regulate cancer-cell growth, apoptosis, tumor

angiogenesis, and immune surveillance by degradation of proteins (Egeblad & Werb, 2002). MMPs are produced by different cells in response to inflammatory signaling. While in healthy tissue they support wound healing, in cancer their production favors neoplastic spread and metastasis. The efficacy of MMP inhibitors may be mediated through anti-inflammatory actions (Coussens & Werb, 2002).

Current treatments for pancreatic cancer include: surgery, radiation therapy, chemotherapy, and targeted drugs (American Cancer Society, 2018). Natural products from terrestrial plants and microbes have been a source of drug molecules, but the development of marine natural products is on the rise (Molinski, 2009). At Harbor Branch Oceanographic Institute, 2 compounds isolated from marine sponges— HB-131 and HB-018—have been identified to have anti-inflammatory properties (Guzmán, 2018, personal communication). These compounds were tested on PANC-1, an epithelioid cell line started from a human pancreatic carcinoma of ductal cell origin (Lieber et al., 1975). Soucek et al. showed that mast cells played an essential role in pancreatic cancer initiation and progression in pancreatic cancers with myc mutations (Soucek 2007). This observation has since been confirmed by many groups in all forms of pancreatic cancer. The purpose of this project is to further investigate the anti-inflammatory effects that HB-131 and HB-018 have against pancreatic cancer.

MATERIALS & METHODS

Compounds. HB-131 and HB-018 were obtained from the Harbor Branch Oceanographic Institute Pure Compound Collection. Stock HB-131 was at a concentration of 1 mg/mL in DMSO and stock HB-018 was at a concentration of 1 mg/mL in methanol. Curcumin (Calbiochem #239802 Lot 000095943) was at a concentration of 1 mg/mL in ethanol. DMSO

was purchased from Sigma-Aldrich, St. Louis, MO. Methanol used in the experiments was purchased from Fisher Scientific, Fair Lawn, NJ. Ethanol was purchased from Pharmco Products Inc., Brookfield, CT. For the experiments, the compounds were diluted in media before addition to the cells. HB-131 and control DMSO were used at a 5 μg/mL concentration, HB-018 and control methanol at 4 μg/mL and curcumin and control 1% DMSO in ethanol at 16 μg/mL. Concentrations were determined using the dose required to see 50% inhibition (IC_{50}) of CCL-2 secretion by PANC-1 cells for HB-131 and of mast cell degranulation for HB-018.

Cell Culture. The PANC-1 cells were ordered through Sigma-Aldrich from the European Collection of Authenticated Cell Cultures (ECACC) in February 2015. Cells were maintained in complete RPMI media (described below) at 37°C under 5% CO_2 at a density of $1x10^6$ cells/mL. Cells were maintained for a maximum of 20 passages when a new aliquot was thawed. The doubling time was 52 hours. The cells were split on Mondays and Thursdays, with the experiments taking place on Tuesdays and Fridays.

Complete RPMI Media. A 500 mL bottle of RPMI 1640 medium was supplemented with 10% fetal bovine serum (FBS), 0.11 mg/mL sodium pyruvate, 4.5 g/L D-glucose, 18 mM HEPES Buffer, 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, 2 mM L-glutamine and 50 μg/mL gentamicin. The solution was then filter sterilized using a 2.0 μm bottle-top filter or 500 mL bottle filter unit. The media was kept refrigerated and warmed to room temperature before use.

Splitting Cells. Cell confluency was checked under an Olympus inverted microscope at 10x magnification. Media was aspirated off before the cells were washed with 1 mL of pre-warmed (37°C) trypsin. The wash was removed before 3 mL of pre-warmed trypsin was added and the cells were incubated for 8 minutes at 37°C. The cells and trypsin were then placed in a

15 mL tube and 3 mL of room temperature media was added. To count the cells, 25 µL of cells and 25 µL of Trypan blue were mixed and placed in a hemocytometer. The top left corner and bottom right corner were chosen to count, without including cells on the borders. Live and dead cells were counted, viability calculated, and the number of cells per milliliter was determined. Depending on the cell count, about 2 mL of cells was added back to the flask along with 22 mL of fresh, room temperature media.

Treatment. Compounds were mixed in media at room temperature. Old media was aspirated from wells before 200 µL of treatment was added to desired wells. The plate was incubated for 6 hours at 37°C. Media was removed and saved for testing and now referred to as conditioned media. Zymogram duplicates and western duplicates were placed in tubes with 9 volumes of ethanol and left in the freezer until testing. ELISA duplicates were placed in a new plate, wrapped in parafilm and aluminum foil, and placed in the refrigerator until testing. After the media was removed, a viability assay was conducted on the remaining cells.

Viability Assay. A lysis buffer was prepared by diluting stock lysis buffer (Promega P/N E1500) 1:5 with distilled water. The media was removed by aspiration and 50 µL of lysis buffer was added to each well. The cells were then incubated at room temperature with shaking for 2 minutes. After mixing with the pipette, 20 µL of each lysate was transferred to a new, black 96-well plate. Next, 20 µL of 1.5uM ethidium bromide was added to each well. The plate was allowed to equilibrate for 30 min at room temperature (RT) away from light. The fluorescence was read at an excitation of 340 and an emission of 590 with a plate reader (NOVOstar, BMG Labtech Inc., Durham, NC). The raw data was analyzed in Microsoft excel to find the averages and standard deviations. Possible outliers were found using a Grubbs' test.

Western Blot. Protein from the conditioned media was used for confirmation westerns loading 5 µg protein per lane. Protein was run in a pre-cast denaturing 15% SDS-PAGE gel (Bio-Rad, Hercules CA), which was then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules CA), and blocked with 5% non-fat milk in Tris-buffered saline containing Tween-20 (TBST) buffer for 1 hr at RT. After repeated washing, the membrane was incubated with primary antibody overnight at 4˚C, repeatedly washed and incubated for 1 hr at RT with horseradish peroxidase conjugated secondary antibody. Detection of proteins was done with chemiluminescence (Amersham Biosciences, Piscataway, NJ), followed by imaging with the ChemiDoc MP System and densitometry analysis using Image Lab 4.1 software (Bio-Rad, Hercules, CA). Primary antibody used was matrix metalloproteinase 2 (cat # 4022S; Cell Signaling Technologies, Beverly, MA). Primary antibody used was matrix metalloproteinase 9 (cat # 13667S; Cell Signaling Technologies, Beverly, MA). Primary antibody for loading control used was Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (cat# 2118L; Cell Signaling Technologies, Beverly, MA). For confirmation experiments for the zymograms, 18 µg protein per lane was used. Peroxidase-conjugated anti-rabbit IgG secondary antibody was obtained from Jackson ImmunoResearch (West Grove, PA). All antibodies were used at manufacturer's recommended dilutions.

Zymogram Gel Casting. A 4 mg/mL solution of gelatin (Sigma G6144 Type A: From Porcine Skin 75-100 Bloom) was made along with 10 % resolving acrylamide gel and 4% stacking gel without adding the ammonium persulfate (APS) or N, N, N', N', tertramethylethylenediamine (TEMED). The resolving and stacking gel mixtures were degassed for approximately 1 hour using a vacuum with sonication. During this time the gel apparatus was assembled. Grease or other lubricant was used between the spacers and the glass and along the

bottom to create a seal. Tape was also applied to the bottom and sides carefully folding the corners to prevent leaking. Once the TEMED and APS were added to the resolving gel mixture, it set for at least 1 hour with water overlaying the solution. APS and TEMED were added to the stacking gel solution and it was poured on top of the solid resolving gel after water was removed. A comb was placed in the stacking gel and it set for at least 1 hour. Gels were stored at 4°C in plastic bags with combs inserted until use.

Zymography. Tubes were removed from the freezer and centrifuged. After discarding the supernatant, the pellet was washed and re-suspended in 40 µL of double distilled water. Samples were subjected to electrophoresis in precast bisacrylamide gel containing gelatin. Gels were incubated in renaturing solution (2.5% triton X) for 30 min at RT, in developing solution (50mM Tris, 200mM NaCl, 5mM $CaCl_2$, 0.02% Brij-35) for 30 min at RT, in stain solution (40% methanol, 10% acetic acid, 0.5% coomasie blue) for 1hr at RT and in de-stain solution (40% methanol, 10% acetic acid) for 30-60 mins until clear bands appeared on a blue background. Gels were imaged using a ChemiDoc MP system (Bio-Rad, Hercules, CA). The image was inverted to get better quantitation. Densitometry was quantitated using Image Lab 4.1 software (Bio-Rad, Hercules, CA).

ELISA. An 8-point calibration curve was prepared using the calibrator and sample diluent from the Quansys Biosciences Q-Plex Array: Human Cytokine High Sensitivity (15-plex) kit (REF 112449HU, LOT HCHK171023). The samples were diluted 1:3 with sample diluent before 50 µL of the samples and the calibrator were added to the Q-Plex™ Array 96-well plate. The plate was covered with a provided plate seal and left to shake on a Lab-Line Instruments, Inc (Melrose Park, ILL) Tilter Plate Shaker on speed 6 (500 RPM) for 3 hours at RT. The plate was washed with 300 µL of 1X wash buffer (kit) 3 times and flicked off the plate before 50 µL of

detection mix (kit) was added to each well and left to shake for 90 minutes at RT at 500 RPM. The plate was washed 3 times with 1X wash buffer and flicked off before 50 µL of Streptavidin-HRP 1X (kit) was added, the plate returned to the plate shaker, and left for 15 minutes at RT at 500 RPM. The plate was washed 6 times with 1X wash buffer and 50 µL of mixture of substrates (3 mL Substrate A : 3 mL Substrate B) was added to each well. The plate was imaged within 15 minutes of substrate addition using a BioRad ChemiDoc MP. The image was analyzed using Q-View™ Software.

RESULTS

Viability Assay. Every time media was collected, a viability assay was conducted to check cytotoxicity. Overall, no changes in viability were seen in any of the experiments performed. PANC-1 cell line, passage 7, sample 1 (PANC-1 P7 S1) showed a larger value for curcumin, which was attributed to its strong red color, so 4 washes of phosphate buffered saline (PBS) were performed before lysis for all other samples. A representative graph of viability results for all samples can be seen in Figure 1.

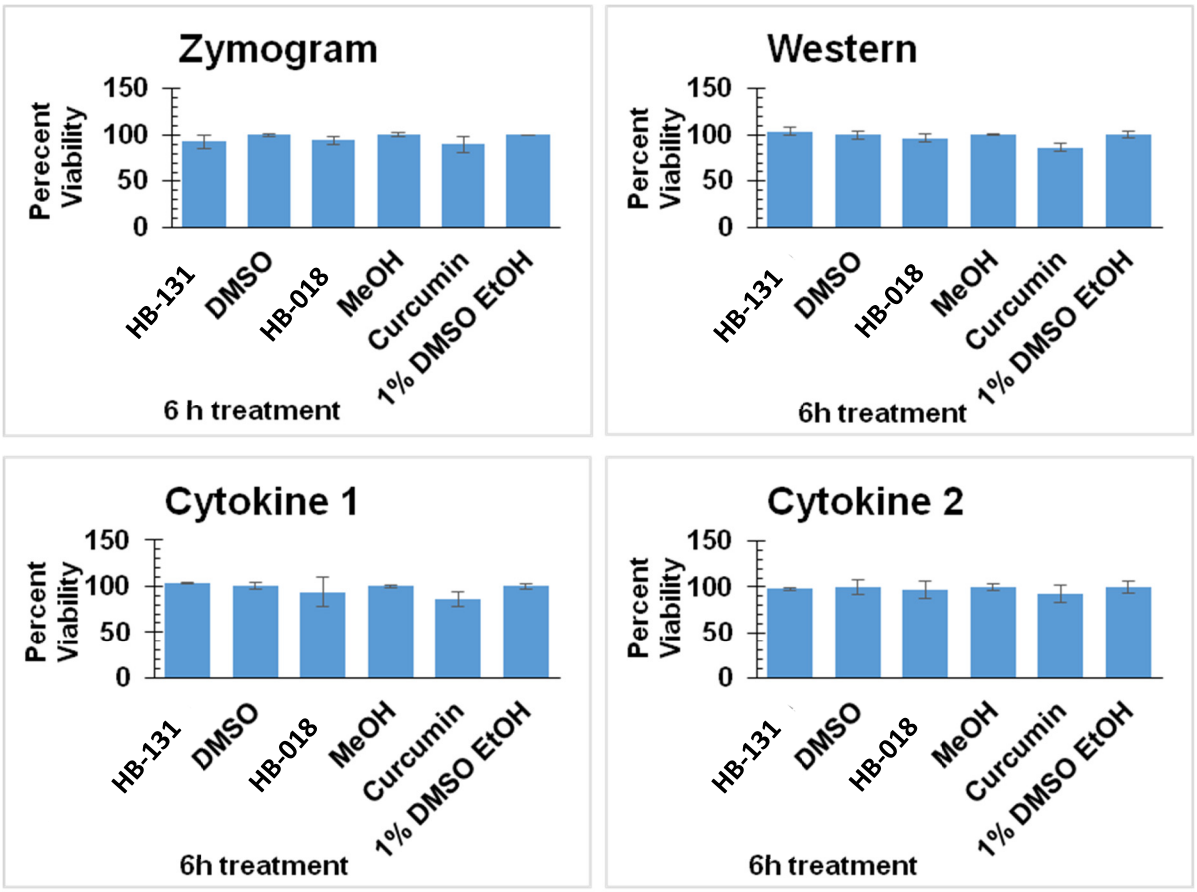

Figure 1. Viability assay results of PANC-1 P8 S2.

Western Blot. For PANC-1 P7 S1, the western blot procedures were followed as described above. Anti-bodies for MMP-9 and GAPDH were applied and no bands were detected. The gel used was expired, so it was assumed that the gel was the issue for the lack of MMP-9 detection; GAPDH cannot be detected in conditioned media. For PANC-1 P8 S2, a new box of gels were opened. The conditioned media was resuspended in 30 µL of water and split into 2 tubes. The samples did not sink into the lanes, so the gel was discarded. This was likely the result of too much ethanol left in the sample. The tubes of resuspended PANC-1 P8 S2 that did not receive 6X sample buffer were respun and suspended in 15 µL of water and the protocol was continued. The tape was not removed from the gel prior to electrophoresis, so when no bands were observed again, it was assumed that was the issue. More of leftover PANC-1 P8 S2 was

later tested. The samples were left in the freezer suspended in 15 µL of water, 500 µL was added before spinning at 15,000 RCF for 15 minutes at 4°C. HB-018 did not sink as well as the other samples, so it did not show up as well on the gel. The bands were very faint for all samples and appeared white. For PANC-1 P9 S3, the samples were split into two 2 mL eppendorf tubes prior to any spinning and the protocol was continued. The samples still rose out of the lanes when loading, so 500 µL of water was added to the other set of the sample (other 2 mL eppendorf). These tubes were spun for 15 minutes at 15,000 RCF at 4°C and suspended in 15 µL of water before the protocol was continued. Two sets of bands for MMP-2 appeared at molecular weight 64 and molecular weight 72 (Figure 2). These molecular weights correspond to the sizes of the protein. For PANC-1 P10 S4, an extra water wash was added to the protocol to accommodate for the lack of drying that caused the samples to rise during loading. It was later determined that this wash caused most of the pellet to redissolve in the water, which caused most of the sample to be lost while decanting. PANC-1 P10 S4 was not tested. PANC-1 P15 S5 was washed with a larger amount of ethanol and allowed to dry for ~20 minutes before being suspended in 15 µL of water. The samples were thick and hard to pipette before loading, so they were transferred to a smaller tube and 15 µL of water was added along with 3 µL of sample buffer. No bands appeared for PANC-1 P15 S5, but the large blotch suspected to be albumin that had appeared on all Ponceau S staining was visible. PANC-1 P16 S6 was initially resuspended in 30 µL of water to prevent the issues faced in PANC-1 P15 S5. The same blotches occurred, but also streaked further down the gel as well and appeared white. The only usable western blot data was from PANC-1 P9 S3 (Figure 2).

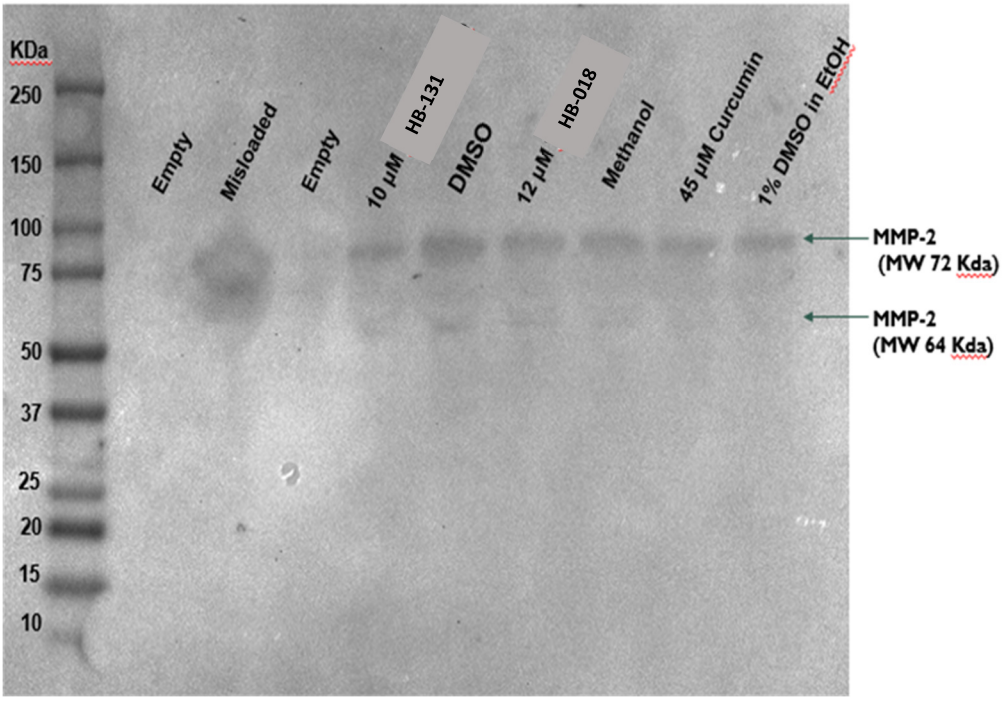

Figure 2. Western blot of PANC-1 P9 S3. Primary antibody: MMP-2 1:1000 Secondary antibody: Donkey- Anti-Rabbit IgG 1:10,000. Exposure 300 seconds.

Zymography. Since only 3 gels were cast successfully, only 3 sets of samples were tested. PANC-1 P9 S3 showed white bands around 72 kDa indicating that MMP-2 activity was seen. HB-131 had a 26% decrease compared to its control DMSO. HB-018 had a 36% decrease in activity compared to methanol, and curcumin had a 42% decrease compared to 1% DMSO in ethanol (Figure 3). PANC-1 P10 S4 showed the same white bands at 72 kDa with a 1% decrease in HB-131, 145% increase in HB-018, and 51% decrease in curcumin. PANC-1 P15 S5 showed a 24% decrease in HB-131, 35% decrease in HB-018, and 80% decrease in curcumin at the 72 kDa bands. The 3 samples were averaged together, but the HB-018 value was skewed due to the outlier of PANC-1 P10 S4. PANC-1 P9 S3 and PANC-1 P15 S5 were averaged (± standard deviation) to give a 25 ± 1.7 % decrease in HB-131, 35 ± 0.7 % decrease in HB-018, and 61 ± 27.3 % decrease in curcumin (Figure 4).

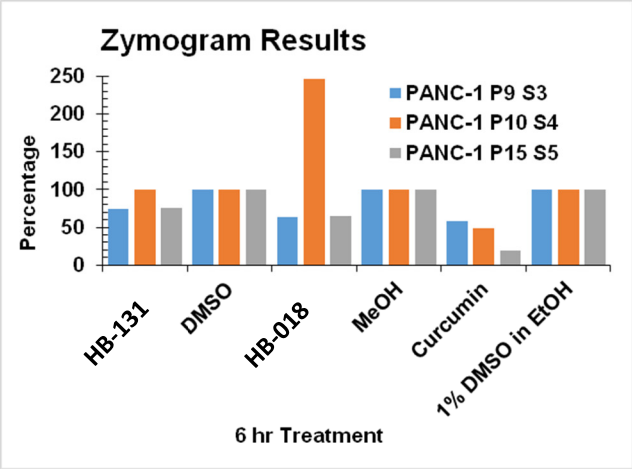

Figure 3. Zymogram results from 3 experiments.

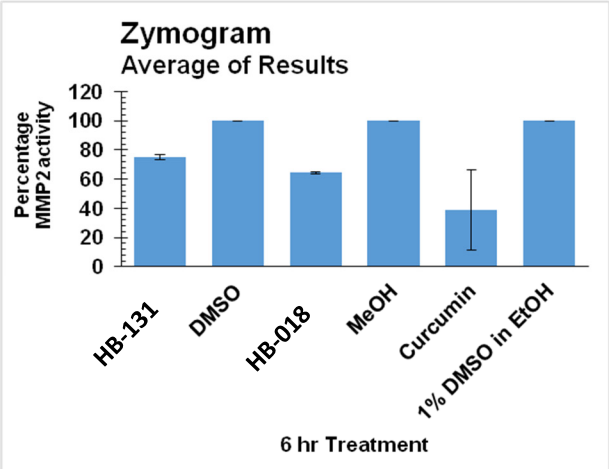

Figure 4. Average of PANC-1 P9 S3 and PANC-1 P15 S5 zymograph results.

ELISA. The ELISA array was conducted following manufacturer's instruction described in the methods section. This plate allows to test 15 cytokines within one well (Figure 5a). Conditioned media from three PANC-1 experiments were chosen to be tested in this assay. The rest of the plate were filled with a calibration curve and with samples obtained from three experiments using AsPC-1 pancreatic cancer cells conducted by Ms. Tara Pitts. Multiple exposures were captured using the BioRad ChemiDoc MP (Hercules, CA) and the one judged to be the best was inverted and saved as a TIFF file for analysis. The image obtained at 295.4 seconds exposure (Figure5b) was analyzed using Q-view software (Quansys Biosciences, Logan UT).

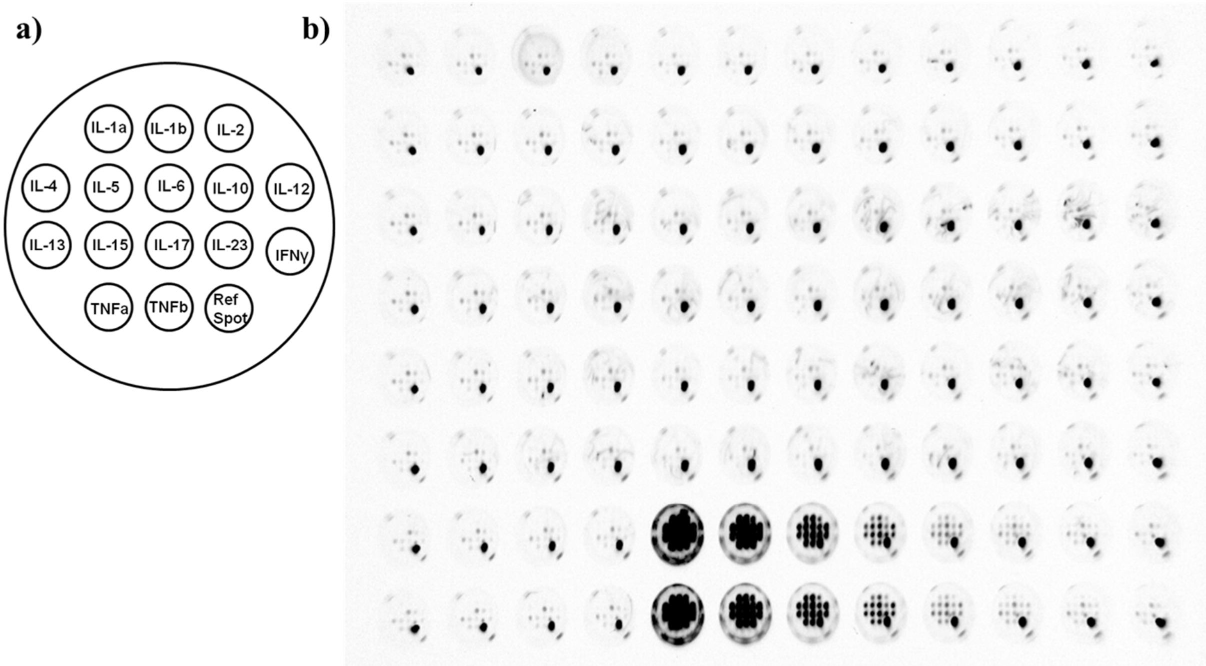

Figure 5. a) Key of cytokines and locations for ELISA array plate provided by Quansys Biosciences. **b)** Image of cytokine array at 295.4 seconds used for analysis.

Cell Line:	PANC-1	AsPC-1									
1	2	3	4	5	6	7	8	9	10	11	12
DMSO	DMSO	5 µg/mL HB-131	5 µg/mL HB-131	MeOH	MeOH	4 µg/mL HB-018	4 µg/mL HB-018	1 % DMSO in EtOH	1 % DMSO in EtOH	16 µg/mL curcumin	16 µg/mL curcumin
DMSO	DMSO	5 µg/mL HB-131	5 µg/mL HB-131	MeOH	MeOH	4 µg/mL HB-018	4 µg/mL HB-018	1 % DMSO in EtOH	1 % DMSO in EtOH	16 µg/mL curcumin	16 µg/mL curcumin
DMSO	DMSO	5 µg/mL HB-131	5 µg/mL HB-131	MeOH	MeOH	4 µg/mL HB-018	4 µg/mL HB-018	1 % DMSO in EtOH	1 % DMSO in EtOH	16 µg/mL curcumin	16 µg/mL curcumin
DMSO	DMSO	5 µg/mL discodermide	5 µg/mL HB-131	MeOH	MeOH	4 µg/mL HB-018	4 µg/mL HB-018	5.6 µg/mL bromotopsentin	6.3 µg/mL discorhabdin A	2.3 µg/mL isobatzelline C	0.9 µg/mL secobatzelline A
DMSO	DMSO	5 µg/mL HB-131	5 µg/mL HB-131	MeOH	MeOH	4 µg/mL HB-018	4 µg/mL HB-018	5.6 µg/mL bromotopsentin	6.3 µg/mL discorhabdin A	2.3 µg/mL isobatzelline C	0.9 µg/mL secobatzelline A
DMSO	DMSO	5 µg/mL HB-131	5 µg/mL HB-131	MeOH	MeOH	4 µg/mL HB-018	4 µg/mL HB-018	5.6 µg/mL bromotopsentin	6.3 µg/mL discorhabdin A	2.3 µg/mL isobatzelline C	0.9 µg/mL secobatzelline A
1 % DMSO in EtOH	1 % DMSO in EtOH	1 % DMSO in EtOH	NT	calibrator	calibrator 1:3 dilution	calibrator 1:9 dilution	calibrator 1:27 dilution	calibrator 1:81 dilution	calibrator 1:243 dilution	calibrator 1:729 dilution	sample diluent
11 µg/mL curcumin	11 µg/mL curcumin	11 µg/mL curcumin	NT	calibrator	calibrator 1:3 dilution	calibrator 1:9 dilution	calibrator 1:27 dilution	calibrator 1:81 dilution	calibrator 1:243 dilution	calibrator 1:729 dilution	sample diluent

Figure 6. Plate scheme used for ELISA courtesy of Dr. Esther Guzmán.

A full 96-well plate was used, but this project will focus on the first 3 rows of data (Figure 6).

Each row represents a separate experiment.

The image was analyzed providing a resulting concentration for each antibody within each well. The average of the treatments were averaged and their standard deviation were calculated. In some cases, the results were beyond the limits of the assay and no results were obtained for a particular cytokine. The results for the PANC-1 experiments are summarized in a graph shown in Figure 7 (separate document). The most unexpected result was that curcumin increased the expression of IL-23 by 279.3 pg/mL. HB-131 did not show significant differences from DMSO, its solvent control, for any cytokine. In addition, for most cytokines detected for HB-131 and DMSO the concentration was below 50 pg/mL. PANC-1 cells treated with HB-018 had about 16.4 ± 0.7 pg/mL IL-23 while cells treated with methanol had a concentration of IL-23 of 57.4 ± 1.0 pg/mL. This means HB-018 inhibited IL-23 secretion about 3.5 fold compared to methanol. HB-018 also promoted IL-10 with a 31.2 pg/mL difference between itself and methanol.

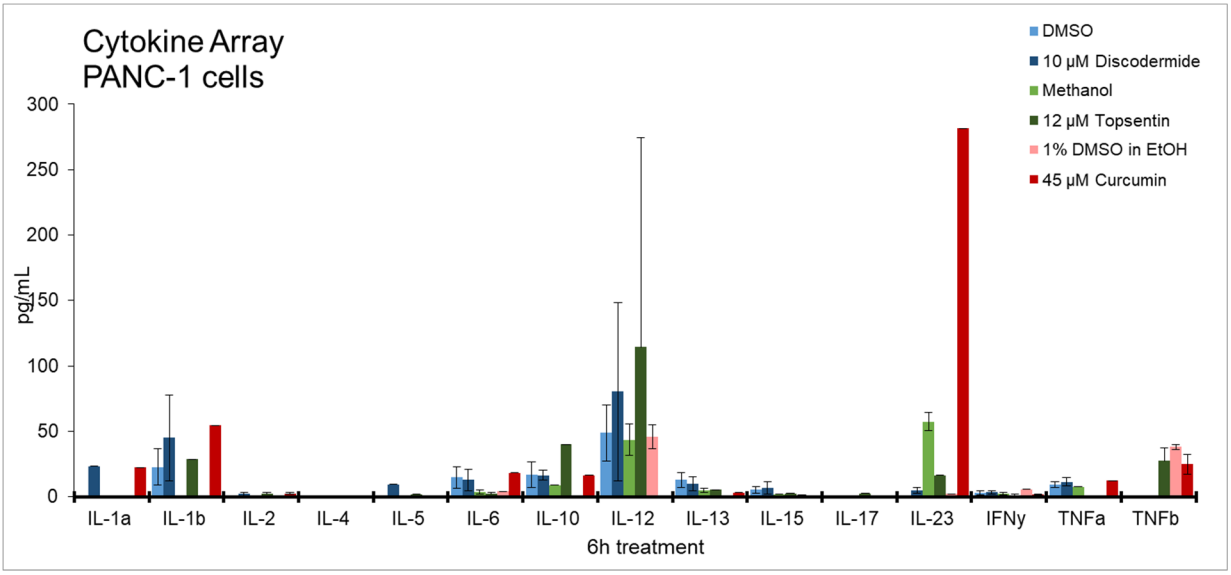

Figure 7. PANC-1 results from ELISA array.

DISCUSSION

Since the number of deaths from pancreatic cancer is steadily increasing, there is a need for new treatments (American Cancer Society, 2018). Previous experiments conducted in the Guzmán lab showed that HB-131 inhibits CCL-2 secretion by PANC-1 cells and that HB-018 inhibits degranulation of mast cells (Guzmán 2018, personal communication). One aim of this project was to determine if these compounds affect other cytokines by assessing their effects on 15 other cytokines. This was achieved by performing a sandwich ELISA array. The other aim of this project was to determine MMP expression and activity and assess whether these compounds lower metastatic potential. MMP expression was determined using western blotting and MMP activity was determined using zymography.

Since all of the controls on the viability assay were approximately 100%, there were minimal pipetting errors while plating. All of the compounds were also near 100%, showing that they were not cytotoxic.

Although there were difficulties getting successful western blots, they did show MMP-2 expression in one sample. The bands that were visible show that there was MMP-2 in conditioned media from PANC-1 cells. Another result from running the westerns in this project was how to modify the original protocol. It is now known that adding a water wash causes loss of the majority of proteins and is not advised. The best results were obtained allowing complete evaporation of the ethanol. A modification that should be made to the protocol is that the cells to be tested should be treated with media that does not require fetal bovine serum (FBS) to get rid of the serum albumin presence on the membrane seen around 58 kDa.

Since the zymograms showed a decrease in the percentage of MMP-2 activity caused by treatment of PANC-1 cells with HB-131 and HB-018 compared to treatment with their solvent

controls in 2 out of the 3 gels, it was concluded that these compounds did decrease MMP-2 activity in PANC-1 cells. This experiment needs to be repeated at least one more time to perform statistics and determine if the decrease is significant and to obtain tighter replicates. However, the data obtained to date suggests that both of these compounds exhibit anti-metastatic activity *in vitro*.

The ELISA cytokine array produced interesting and sometimes surprising results. This array contains a combination of both pro- and anti-inflammatory cytokines whose functions are summarized in Table 1.

Cytokine	Function
IL-1α	innate immunity; primes T cells during contact hypersensitivity and allergic responses.
IL-1β	innate immunity; important for induction of fever
IL-2	regulates the activities of white blood cells (especially T cells)
IL-4	promotes Th2 lymphocyte development; inhibition of LPS-induced proinflammatory cytokines synthesis
IL-5	growth and differentiation factor for both B cells and eosinophils
IL-6	induced by lipopolysaccharides (LPS) along with TNF-α and IL-1; often used as a marker for systemic activation of proinflammatory cytokines down-regulates the synthesis of IL-1 and TNF
IL-10	potent inhibitor of Th1 cytokines, including both IL-2 and IFN-γ; potent deactivator of monocyte/macrophage proinflammatory cytokine synthesis.
IL-12p70	stimulates the production of IFN-γ and TNF-α from T cells and natural killer (NK) cells, and reduces IL-4 mediated suppression of IFN-γ.
IL-13	down-regulates the production of TNF, IL-1, IL-8, and MIP-1α (CCL-3) by monocytes and has profound effects on expression of surface molecules on both monocytes and macrophages.
IL-15	induces cell proliferation of natural killer cells
IL-17	activates several signalling cascades that lead to the induction of chemokines to recruit immune cells, such as monocytes and neutrophils to the site of inflammation. Promotes inflammation in concert with TNFα and IL-1; activation of IL-17 signalling is often observed in the pathogenesis of various autoimmune disorders, such as psoriasis.
IL-23	heterodimeric cytokine composed of the p40 subunit of IL-12 plus a p19 subunit unique to IL-23. The p19 subunit has homology to both IL-6 and the p35 subunit of IL-12. Acts on memory CD4+ T cells to promote their proliferation and support their differentiation into Th1 effectors during a secondary response. Stimulates DCs (and possibly NK cells) to produce IFNγ. Primary role is speculated to be to promote sustained cell-mediated responses designed to clear persistent intracellular infections.
IFN-γ	critical for innate and adaptive immunity against viral, some bacterial and protozoal infections. IFNγ is an important activator of macrophages and inducer of Class II MHC expression. Aberrant IFNγ expression is associated with a number of autoinflammatory and autoimmune diseases.
TNF-α	Can induce fever, apoptotic cell death, cachexia, inflammation and inhibit tumorigenesis and viral replication and respond to sepsis via IL-1 & IL-6 producing cells.
TNF-β	involved in the regulation of cell survival, proliferation, differentiation, and apoptosis
Th1 cytokines-- produce the **proinflammatory** responses responsible for killing intracellular parasites and for perpetuating autoimmune responses	
Th2 cytokines--associated with the promotion of IgE and eosinophilic responses in allergic responses; has more of an **anti-inflammatory** response. In excess, Th2 responses will counteract the Th1 mediated microbicidal action.	

Table 1. Cytokine functions (courtesy of Dr. Esther Guzmán).

Curcumin increased the concentration of IL-23, a pro-inflammatory cytokine, in a very large manner. Curcumin is known as an anti-inflammatory compound and has been reported to decrease the expression of IL-23 in a previous study performed in cells (Zang et al., 2015). This study also showed increased expression on IL-17, while IL-17 was undetectable in our results.

However, given the variability in the results obtained, the experiment needs to be repeated and statistics need to be conducted to determine if our results are correct. If our results were true, the discrepancy with the previously reported values could be due to the differences in cells.

HB-131 is known to inhibit CCL-2 (Guzmán 2018, personal communication). By conducting the ELISA experiments, it was possible to ascertain its effects on other cytokines. HB-131 did not appear to affect any of the fifteen cytokines tested. We expected that it may affect some of the pro-inflammatory cytokines present in the array. Nevertheless, this lack of effects supports the idea that the inhibition of CCL-2 is specific and not due to a general inhibition of protein synthesis.

The results obtained with HB-018 support the idea that this compound has important anti-inflammatory activity. HB-018 decreased the concentration of IL-23, a pro-inflammatory cytokine, produced by PANC-1 cells. It also increased the concentration of IL-10, an anti-inflammatory cytokine. It is important to note that statistical significance of the ELISA array could not be determined because there were not enough duplicate values. Therefore, this data needs to be repeated to be validated. Both HB-131 and HB-018 showed anti-inflammatory activity in pancreatic cancer cells. Inhibition of CCL-2 by HB-131 seems to be specific, and the lowering of MMP-2 activity suggests that this compound has anti-metastatic activity in vitro. HB-018 not only inhibits mast cell degranulation, but it lowers the metastatic potential of PANC-1 cells through inhibiting MMP-2 activity. Furthermore, it inhibits pro-inflammatory cytokines and increases anti-inflammatory cytokines. From these findings, HB-131 and HB-018 are interesting compounds with anti-inflammatory activities in pancreatic cancer cells that merit further development.

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