Potential Involvement of Micro vesicle Particles in the Synergistic Effects of Ultraviolet-B Radiation and Platelet -Activating Factor Receptor Agonists on Cytokine Production

A Thesis submitted in partial fulfillment of the

requirements for the degree of

Master of Science

by

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY **Shweta Bhadri** ENTITLED Potential Involvement of Microvesicle Particles in the Synergistic Effects of Ultraviolet-B Radiation and Platelet -Activating Factor Receptor Agonists on Cytokine Production BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF **Master of Science.**

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ABSTRACT

Bhadri Shweta, M.S., Department of Pharmacology and Toxicology, Wright State University, 2021, *Potential Involvement of Microvesicle Particles in the Synergistic Effects of Ultraviolet-B Radiation and Platelet -Activating Factor Receptor Agonists on Cytokine Production*

Cytokines play a pivotal role in regulating inflammation, which is a condition that makes the tissue vulnerable to different pathological and physiological conditions. Thus, how cytokines are regulated is an important area of study. Skin that receives ultraviolet B radiation (UVB), a major pro-oxidative stressor, results in the release of multiple cytokines and chemokines like tumor necrosis factor (TNF)-alpha and interleukin (IL)-8. Previous studies from our group and others have demonstrated synergistic release of TNF-alpha when UVB is combined with IL-1 or the lipid mediator Platelet-activating factor (PAF). Of interest, subcellular microvesicle particles (MVP) have been proposed to play an important role in intercellular communication. Moreover, UVB and PAF agonists cause MVP release in keratinocytes. Therefore, we believe that understanding the role of MVP in these inflammatory responses could be insightful for photosensitivity mechanisms and to suppress inflammation. The current study focuses on the combination of low concentrations of PAF agonist and UVB in-vitro and ex-vivo to observe potential synergism in the release of cytokines and MVP. We also studied the effects of acid sphingomyelinase (aSMase) inhibitor imipramine, for its ability to modulate both MVP and cytokine release. The application of aSMase inhibitor inhibited the synergistic response of MVP and cytokines allows us to conclude the potential involvement of MVP in the exaggerated response of cytokines from combining UVB and PAF. These studies have potential relevance in understanding abnormal skin reactions such as photosensitivity.

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1. INTRODUCTION

1.1 Statement of Problem

Extracellular vesicles belong to a heterogeneous group of particles that contain lipid bilayers. These particles are thought to play a role in intercellular communication as they are packed with cellular components such as nucleic acids (DNA, RNA), proteins. MVP belongs to the group of particles ranging from 100-1000 nm. In pathological conditions or response to cellular stress, the release of these particles is elevated. In this scenario, MVPs could prove a potential target or a biomarker in the future.

Here, we investigated the synergism induced by two distinct stimuli which have in common the ability to generate MVP and cytokines by themselves. We addressed the synergism by combining CPAF and UVB in very low concentrations in-vitro as well as ex-vivo.

To evaluate the role of MVP we considered aSMase inhibitor imipramine and studied its effects on MVP release and cytokine release.

1.2 Hypothesis

We hypothesized that when two distinct stimuli, such as UVB and PAF-R agonists, are combined in very low concentrations in-vitro and ex-vivo gives a synergistic response in MVP and release of cytokines IL-8 and TNF-alpha. We also hypothesized that aSMase inhibitor imipramine inhibits this exaggerated response of MVP and cytokines.

1.3 Assumptions

We made assumptions that all the cellular models we used would respond the same way as the human body does.

2. REVIEW OF LITERATURE

2.1 UVR Radiation

UVR plays a major role as an environmental stressor. It is composed of three different radiations according to the wavelength, which are UVC (200–280 nm), UVB (280–315 nm), and UVA (315–400 nm). Skin is the largest organ of the body and external, gets the most exposure to UV radiation. Out of these 3 radiations, UVB and UVA manage to reach the earth's surface and UVB tends to reach and affect the outer layer of the skin. While UVA reaches the middle layer of the skin (dermis).

They all cause different inflammatory responses in the skin due to variation in their penetration in the skin tissue (Lee et al., 2017). UVB induces an acute pro-inflammatory phenotype in the skin. Pro-inflammatory phenotypic mediators include the generation of arachidonic acid, as well as protein and lipid-derived cytokines, and mast-derived mediators such as histamine. Induction of these pro-inflammatory factors in keratinocytes has been linked to the increased risk of UVB to cause skin cancers.

Mechanisms through which UVB induces inflammation are relatively unexplored. However, UVB induces oxidative stress in the keratinocyte cells. UVB is associated with increased reactive oxygen species (ROS) production (de Jager et al., 2017). ROS in general when produced in larger amounts acts as a mediator of inflammation. ROS combines with nitric oxide (NO) at a much faster rate and generates reactive nitrogen species. This all together creates a pro-inflammatory environment in the skin tissue.

Our group previously has shown that UVB follows the PAF-R activation pathway. PAF is a glycerophosphocholine-derived lipid mediator which is involved in numerous process, especially in inflammatory responses (Travers, 2020). UVB as a pro-oxidative stressor generates high amounts of PAF and ROS in the keratinocytes. The exaggerated levels of ROS and PAF act on PAF-R which releases pro-inflammatory cytokines and subcellular vesicles.

2.2 Platelet activating Factor

PAF (1- 1-O-alkyl-2-acetyl-sn- glycerol-3-phosphocholine), an autocoid, is a potent lipid mediator and causes pro-inflammatory responses like activation of immunological cells such as neutrophils, macrophages, eosinophils (Damiani et al., 2017). PAF is found on many cells such as epithelial cells, mast cells, leukocyte and it binds to G-protein coupled receptor, the PAF-R (Puebla-Osorio et al., 2015). The presence of PAF-R on human keratinocytes, HaCaT cells, and A-431 cells was proved by immunofluorescence studies (Liu, L. 2020).The activation of PAF-R is associated with oxidative damage and various infections.

PAF is synthesized in two ways: de novo synthesis and remodeling pathway. Stimulation of the cells through stressors activates the remodeling pathway, whereas without any outside stimulation PAF is generated through de novo synthesis.

UVB has been shown to produce PAF and PAF-mimicking molecules like oxidized GPC eventually activating PAF-R (Travers, 2020). Activation of PAF-R leads to the generation of various pro-inflammatory cytokines including TNF- alpha, IL-8, IL-6, IL-10 which reinforces inflammation.

In the current studies we have used CPAF Carbamyl-PAF as PAF-R agonist (Bihl et al., 2016). As it and PAF-R agonist, it is also used as a negative control on PAF-R negative cell line (KBM cell line).

2.3 Micro vesicle Particles

Micro vesicle particles are circular membranous particles released by cells in pathological or physiological conditions. These are distinguished into two kinds based on their size and mechanism for their production. Microvesicle particles (MVP) are 150- 500 nm in size and are generated through blebbing of the plasma membrane of the cell. In contrast, exosomes range from 20-100 nm formed intracellularly (Richards et al., 2018) .

There have been different biochemical pathways involved in the generation of MVPs noted in the literature (Verhoef et al., 2003). For example, as seen in **Figure 1.** P2X7R is an ATP gated ion channel and when activated by ATP it induces a rapid movement of ion influx or efflux. Ions like Ca+2, Na+, K+ start to flow through the membrane. Following that larger molecules of around 800 Da enter through the membrane. Continuous activation of P2X7 allows the release of IL-1B in increased amounts. This results in micro vesiculation and blebbing of the plasma membrane. Blebbing is the protruding of vesicles that are bound by a membrane. This process involves the movement of large numbers of Ca+2. This MVP shedding is associated with the activation of acid sphingomyelinase (aSMase) (Bianco et al., 2009). This enzyme transfers itself from the lysosome to the membrane. It hydrolyzes sphingomyelinase and forms ceramide and hence it modifies the plasmas membrane which can encourage the blebbing response. (Verhoef et al., 2003). Hence, our studies included the use of aSMase inhibitor imipramine (Bianco et al., 2009) as a tool to understand its role in the intercellular communication of cytokines via MVP.

MVP has been known to play an important role in intercellular communication. They serve as messengers where they tend to transport RNA, proteins, DNA. Literature shows that these vesicles have been transporting various proteins from one cell type to another cell type for example from senescent fibroblasts to keratinocytes (Terlecki-Zaniewicz et al., 2019). This behavior of the vesicles could explain the transport of various cytokines such as TNF-alpha to the neighboring epithelial cells. In this study supported by previous studies, we investigated the involvement of MVP in the exaggerated response of cytokines following combination treatments of PAF agonist and UVB.

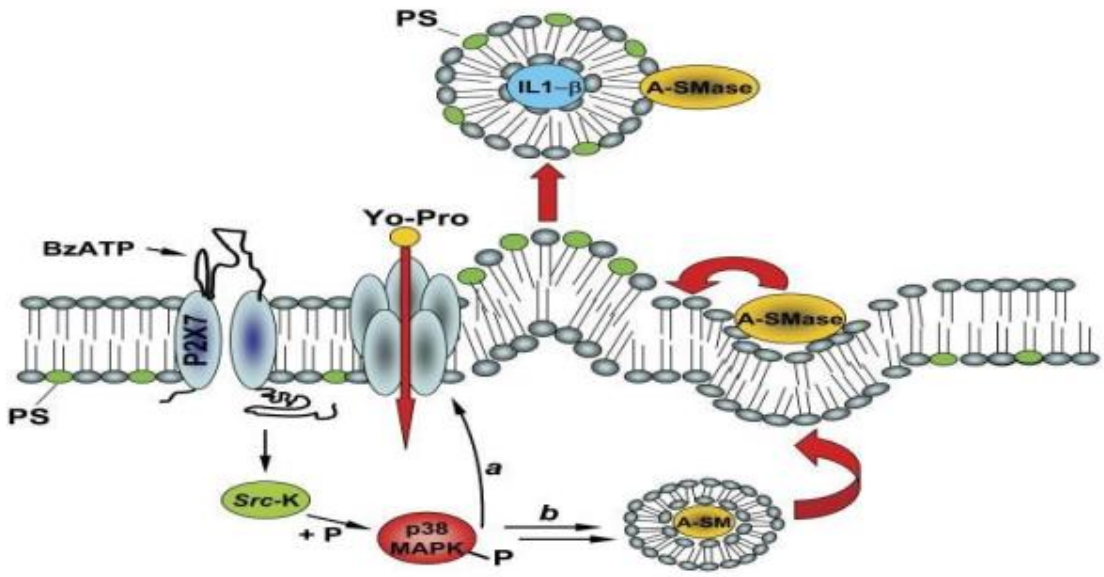

Figure 1. Model for signaling pathways involving P2X7 for MVP generation (Bianco et al., 2009)

2.4 Toxic Epidermal Necrolysis

Toxic Epidermal Necrolysis (TEN) is a very rare disorder but is an adverse reaction to drugs and it affects the epidermal layer of the skin. TEN is characterized by the separation of the epidermal layer of the skin from the dermis, severe pain, inflammation, bacterial infections. Analysis of the blister fluid also showed higher amounts of TNF-alpha, IL-10 (Holbrook et al., 2019; Nassif et al., 2004).

In a case study of a 22-year-old female subject, she developed TEN after a series of events that involved UVR (Gatsen et al., 2011). The condition was characterized by severe inflammation, redness of the skin, pain, vaginal infection. The patient had a history of use of drugs like Ibuprofen. Before the condition, she ingested about 200 mg of Ibuprofen. She observed an inflammatory response after the ingestion of Ibuprofen. While she also visited a tanning bed, which gave an immediate and severe reaction, and observed large amounts of apoptosis, destruction of the epidermis of the skin. This can be observed in **Figure 2 (a) (b)**.

Histology of the affected skin biopsy showed presence of apoptotic keratinocytes **Figure 2 (c)**. Immunohistochemistry of the affected skin biopsy stained with TNF-alpha protein shows higher amounts of TNF-alpha as compared to normal skin tissue **Figure 2 (d) (e)**.

Supporting that a study was conducted on keratinocytes with a combination of UVA or UVB and IL-1a or PMA which resulted in the synergistic release of TNF-alpha (Gatson et al., 2011). Concluding to which, we believe that UVB affected the abnormal drug reaction and caused an aggravated, destructive response like TEN.

This current study investigated the combined effects of PAF agonist and UVB on the release of pro-inflammatory cytokines and MVP to understanding the role of MVP in the cytokine response.

Mechanistic pathways caused for these destructive inflammatory responses through this study could provide ways to block these responses.

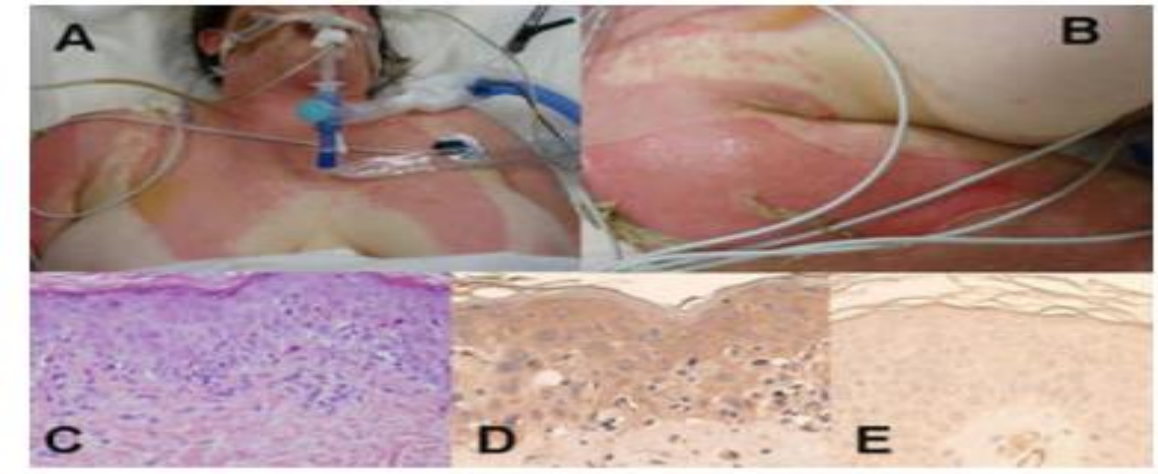

Figure 2. Clinical Photographs of a female subject with an aggravated response due to Toxic Epidermal Necrolysis (Gatson et al., 2011)

2.5 Tumor Necrosis Factor (TNF-alpha)

TNF-alpha is a pleiotropic cytokine secreted by different types of cells such as macrophages, microglia, astroglia, epithelial cells, Langerhans (Musco & van Staden, 2010). TNF-alpha is secreted by cells in stress caused by lipopolysaccharide (LPS) or UV radiation. TNF-alpha has also a unique property that when UV radiation induces the production of TNF-alpha in keratinocytes, they induce nearby cells and hence proliferate the inflammatory phenotypic cells (Bashir et al., 2009).

TNF-alpha follows signaling pathways such as NF-kB. Previous studies have shown induction of TNF-alpha by UVB through the higher amount of ROS production (Travers, 2020). Also, our group has showed increased amounts of TNF-alpha when PAF agonist in its optimal dose is

combined with a low fluence of UVB in keratinocyte-derived HaCaT cells. The focus of the current study was to analyze combination effects on low concentrations of PAF agonists and UVB on the TNF-alpha release in keratinocyte-derived HaCaT cells.

The goal of the study to analyze TNF-alpha levels would be to study the mechanisms through which cells under stress create a pro-inflammatory environment. This would also provide insights into the involvement of MVP as they are stated as messengers in intercellular communication.

2.6 Interleukin-8 (IL-8)

Interleukin-8 (IL-8) is a pro-inflammatory cytokine also known as chemokine. It belongs to the CXC chemokine family. Studies have shown upregulation of IL-8 upon UVB stimulation in-vitro (Storey et al., 2005) (Kang et al., 2007). Literature shows that higher amounts of IL8 are analyzed in psoriatic lesions (Storey et al., 2005). The mechanism for UV-induced IL-8 release could be autocrine/paracrine i.e. TNF-alpha could possibly induce IL-8 expression in keratinocytes.

Our study focused on the effect of the combination of low concentrations of CPAF and low fluence of UVB or CPAF/TPA/UVB alone on the IL-8 release in epithelial (KB cells and HaCaT) cell lines. The potential involvement of MVP in the UV- induced IL8 release and intercellular talk was the goal of investigation.

2.7 Imipramine

Acid Sphingomyelinase (aSMase) is an enzyme present in the lysosomal compartment, it catalyzes the hydrolysis of sphingomyelin to ceramide. Ceramide induces the generation of plasma membrane platforms.(Beckmann et al., 2017). The activation of aSMase is a regulating step in the

formation of plasma membrane vesicles. It can be activated by different stimuli for example ROS, stress inducers. Imipramine is a tricyclic depressant and has been proved to inhibit acid sphingomyelinase enzyme (Beckmann et al., 2014).

Previously it has been shown that UV-C induces the activation and translocation of aSMase and ceramide formation. The pathway of aSMase activation plays an important role in the Micro vesicle formation. (Charruyer et al., 2005).Therefore, to evaluate the role of Micro vesicle particles in the UVB-induced inflammatory responses, inhibition of aSMase activation is a defined approach.

3. PREVIOUS STUDIES

Previously, studies were conducted on the KB cell line and HaCaT cell line to investigate the kinetics of the release of MVPs. UVB-induced PAF-R activation leads to an increased release in MVP at 4 hrs. TNF-alpha which is a pro-inflammatory cytokine was released in a concentration-dependent manner. Combination of optimal concentration of CPAF (100 nM) and a low dose of UVB 600 J/m^2 induced an aggravated response to MVP and TNF-alpha release. But, a low concentration of CPAF (1 nM/10 nM) which individually cannot produce a significant response in the MVP or cytokine release when combined with another PAF-R stimulus of low fluence was unexplored. Pro-inflammatory cytokines such as IL-8 also remain to be unexplored.

In this study, we tried to investigate the effect of the combination of two disparate stimuli at their low concentrations on the release of MVP and cytokines.

4. HYPOTHETICAL MODEL

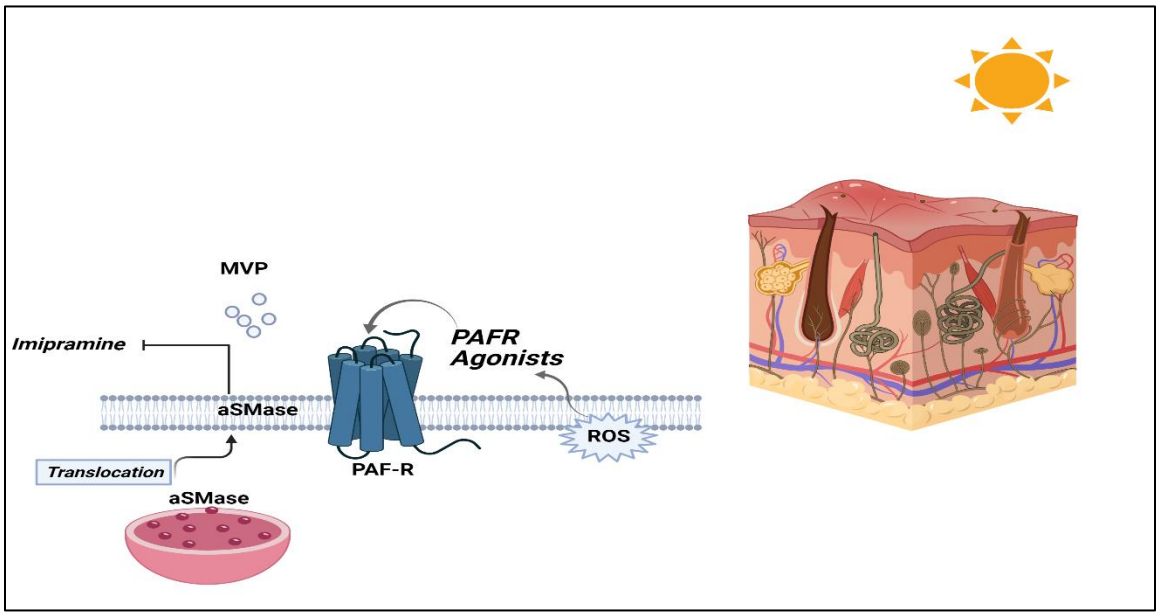

Figure 3. Hypothetical model showing UVB-induced MVP release through PAF-R activation pathway. This hypothetical model (Liu et al., 2021) (Created with BioRender.com) depicts the UVB-induced formation of Microvesicles. Skin, the most exposed part of the human body to UV radiation absorbs UVB in the epidermal region. It induces the production of higher amounts of ROS (Reactive oxygen species) which form PAF-R agonists or ox-GPC. These PAF-R agonists activate the G-protein coupled receptor PAF-R which allows aSMase to translocate from lysosomal compartment to plasma membrane. This translocation leads to ceramide formation and thereby assisting in the plasma membrane blebbing process. (Charruyer et al., 2005)

5. MATERIALS AND METHODS

5.1 Cell culture

The three cell lines KBM, KBP, and HaCaT cells were grown in DMEM high glucose media. To make complete media, in a bottle of Dulbecco's modified Eagle's medium DMEM High glucose media 50 ml of FBS, 5 ml of Penicillin/0.1 mg/ml streptomycin (5 ml), and 2 mM glutamine. Media was removed from the cell culture plates and discarded. The plates were then washed with 1X filtered PBS 3 times and discarded it. Ten mL of fresh media was then added to the cell culture plates.

5.2 Passaging Cells

HaCaT/ KB cells were maintained at a 37˚ C incubator with 5 % CO_2. For culturing of cells, at 80 % confluency media was discarded from the culture plate and washed with 1X filtered PBS for 3 times. It was then trypsinized with 2 ml of 0.25% trypsin. These plates were then kept in the incubator for 5-10 mins to separate the cells from the plate. To make up the volume till 10 ml for 10 cm^2 plate 8 ml of media was added and triturated. For an equal amount of cells to be put in all the treatment groups, cell count was performed and viability assessed by Trypan blue test.

5.3 Cell Count

To get the cell count/ ml HaCaT/ KB cells were trypsinized by adding 2 ml of trypsin 0.25 % in a 10 cm^2 plate and incubating for 5 -10 mins. Followed by 8 ml of DMEM high glucose media to make it up to 10 ml and triturate it to separate the cells. Ten µL of this cell suspension and 10 µL

of Trypan blue was added in a 1.5 ml of Eppendorf tube. Ten µL of this mixture was then analyzed by the Countess II Automated Cell Counter.

5.4 Treatments

HaCaT cells and KB cells were cultured as previously reported (Liu et al., 2021). Keeping the seeding density $5*10^5$ cells/10 cm^2 plate HaCaT cells were passaged and were treated two days after it reached 80% confluency. For KBM and KBP cells, the seeding density was kept at $0.8*10^6$ cells/10 cm^2 plate. For the treatment of the cells, culture plates with 80 % confluency were washed with 1 X filtered PBS for 3 times followed by the addition of 4 mL of Hank's Balanced Salt Solution + 10mg/ml fatty acid-free BSA with the required concentration of CPAF, TPA, Imipramine. These treated cell culture plates were then incubated for a specific length of time. For Sham culture plates (used as negative control) only 4 mL of Hank's Balanced Salt solution + BSA was added and incubated for the same period as others.

For UVB treatment, culture plates with 80 % confluency were washed with 1 X filtered PBS for 3 times 4 mL of Hank's Balanced Salt Solution + BSA was added to the cell culture plates. According to the UVB fluence, the culture plates with their lids open were kept under Philips UVB light source with an emission of 4.66 J/m^2/sec fluence rate. For 100 J/m^2 -21 sec 200 J/m^2 – 44 sec 400 J/m^2 – 1 min 26 sec 600 J/m^2 - 2 min 8 sec 1000 J/m^2 – 3 min 34 sec.

5.5 MVP Isolation

MVP were isolated as previously reported (Liu et al., 2021). Briefly, after the treatment of the cells for a specific period, the supernatants were transferred to 2 ml centrifuge tubes. The supernatants were then centrifuged for 2,000 X g for 20 min at 4˚C to separate the cell debris followed by 20,000 X g for 70 min at 4˚C to isolate Microvesicle particles. After the centrifugation,

the cell pellet was air-dried and resuspended with 100 µL of filtered PBS. MVPs were analyzed by Nanosight 300 instrument by 70x dilution of the re-suspended pellet in 1X filtered PBS.

5.6 MVP Analysis

The resuspended pellet was diluted by 70X dilution with 1X filtered PBS and then analyzed by Nanosight 300. The particles/ml reading from the Nanosight was multiplied by the dilution and divided by the cell number. The normalized cell count of MVP was then calculated for 100,000 cells. To calculate the fold change CPAF/TPA was normalized to MVP count of Vehicle group and UVB was normalized to No treatment group.

5.7 Skin Explants

Our group has a history of using discarded skin tissue (Liu et al., 2021). The specimens were obtained from de-identified patients after abdominoplasty surgery from Miami Valley Hospital, Dayton Ohio. The skin samples were washed with water and cut into 4 cm^2 squares and fat was trimmed. To keep the skin tissue hydrated, 1 ml of PBS was added to the plates. CPAF/TPA concentrations were prepared in DMSO 90% : EtOH 10%. The skin tissue was treated with either No treatment or vehicle (DMSO 90%: EtOH 10%) or various concentrations of CPAF (1 µM), TPA (1 µM) and treated with CPAF (1 µM)/ TPA (1 µM) for 1 hour of pre-incubation for Combination treatments. For UVB treatments, the skin tissue was irradiated with UVB fluences of 500 J/m^2, 1000 $J/m^{2,}$ or 2500 J/m^2 and incubated for 4 hours.

5.7 Punch Biopsies

After 4 hours of incubation, punch biopsies of 6 mm were taken and weighed. The biopsies were cut into small pieces and digested in an Eppendorf® tube containing 1 ml of 5 mg/ml collagenase and kept on a shaker at 37˚C overnight.

5.8 MVP isolation for Skin Explants

The following day after digestion of the skin tissue, MVPs were isolated by performing centrifugation steps of 2000 X g for 20 min. The supernatant was transferred to 20,000 for 10 min and 20,000 for 70 min.

5.9 ELISA

After treating the cells with CPAF/TPA/UVB or combination treatments supernatants were taken and centrifuged for 5 min at 5000 rpm to separate the cell debris. These samples were stored at -20˚C and analyzed within a week. Calculations were done by comparing with the standard concentrations and normalized to cell counts.

6 . RESULTS

6.1 Concentration-dependence of UVB treatment on IL-8 and TNF-alpha release in KBP cells. To address the mechanistic pathway of UVB we used PAF-R positive (KBP) and PAF-R negative (KBM) cell lines. Concentration dependent response of IL-8 and TNF-alpha release for UVB was studied. KBP and KBM cells were treated with UVB fluences of 100 J/m^2, 200 J/m^2, 400 J/m^2, 600 J/m^2, 1000 J/m^2. KBP and KBM cell culture plates with 80% confluency were treated with 4ml HBSS+BSA and irradiated with UVB fluences of 100 J/m^2, 200 J/m^2, 400 J/m^2, 600 J/m^2, 1000 J/m^2 through a UVB light source of intensity 4.66 J/m^2/sec fluence rate followed by incubation for 4 hours. Supernatants were collected and analyzed accordingly for ELISA. As shown in **Figures 4 and 5**, UVB treatment resulted in these cytokines (IL-8 and TNF-alpha) in KBP cells, which was maximal at ~400J/m^2. UVB treatment of PAF-R-negative KBM cells did not result in cytokine release (data not shown).

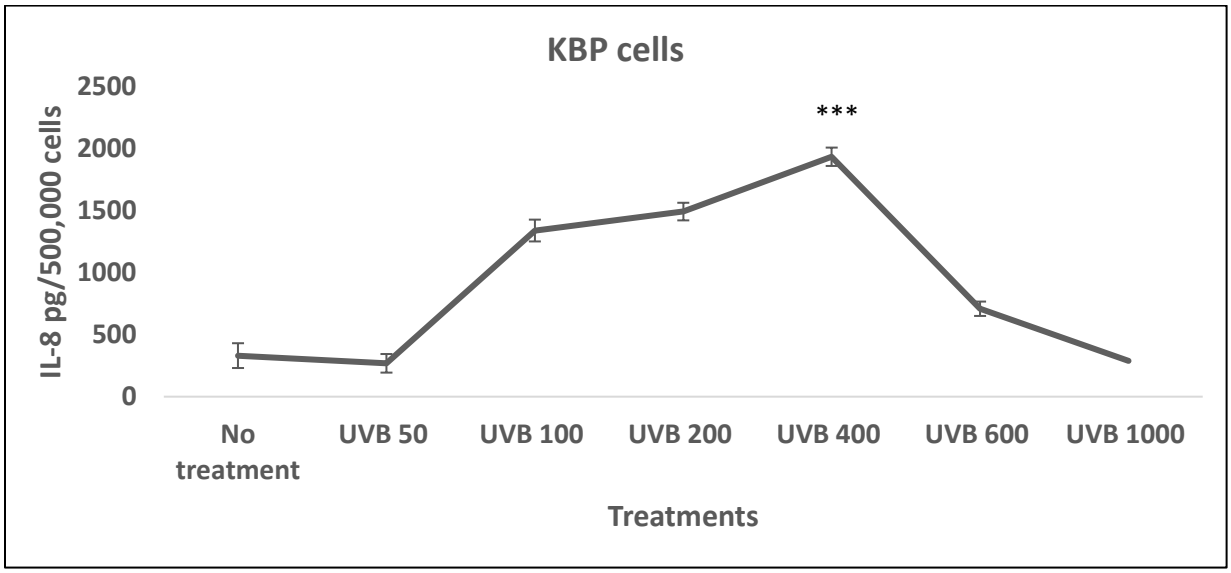

Figure 4. Concentration-dependence of UVB treatment on IL- 8 release in KBP cells. KBP cells were treated with either no treatment or various doses of UVB ranging from 100 J/m^2, 200

J/m^2, 400 J/m^2, 600 J/m^2, 1000 J/m^2, and incubated for 4 hours. Supernatants were removed and IL8 was analyzed normalized per cell count. The data are the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with *** P <.001. Statistical analysis was performed by one-way ANOVA. Differences in the samples were considered significant if the P-value is less than 0.05

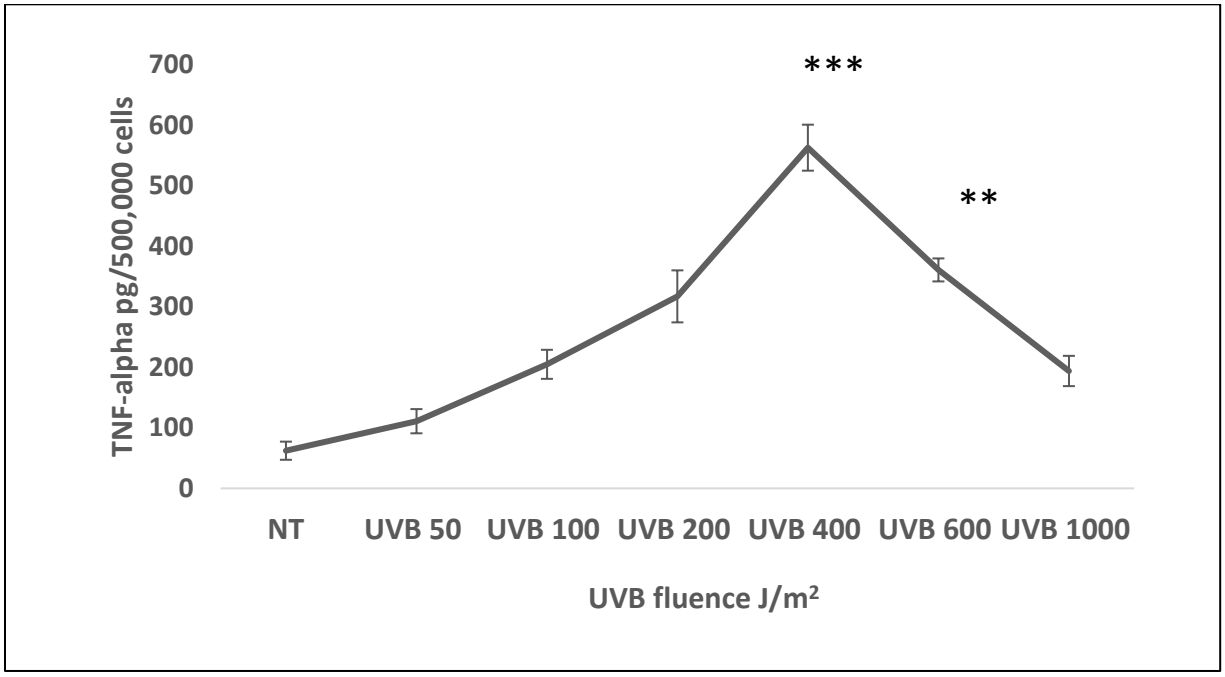

Figure 5. Concentration-dependence of UVB treatment on TNF-alpha release in KBP cells. KBP cells were treated with either No treatment or various doses of UVB ranging from 50 J/m^2, 100 J/ m^2, 200 J/ m^2, 400 J/ m^2, 600 J/ m^2, 1000 J/ m^2, and incubated for 4 hours. Supernatants were removed and TNF-alpha was analyzed normalized per cell count. The data are the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with **P <0.01,*** P <0.001. Statistical analysis was performed by one-way ANOVA. Differences in the samples were considered significant if the P-value is less than 0.05.

6.2 Concentration-dependence of CPAF treatment on cytokine release in KBP cells. To address the optimal concentrations of CPAF on KBP cells for the release of cytokines IL-8 and TNF-alpha we used PAF-R positive (KBP) cell line and PAF-R negative (KBM) cell line. The concentration-dependent response of IL8 and TNF-alpha for CPAF was studied. KBP and KBM cells were treated with with CPAF concentrations (1 nM, 10 nM, 50 nM, 100 nM and 250 nM). KBP and KBM cell culture plates with 80% confluency were treated with 4ml HBSS+BSA and treated with 1 nM, 10 nM, 50 nM, 100 nM and 250 nM) followed by incubation for 4 hours. Supernatants were collected and analyzed accordingly for ELISA.

As observed in **Figures 6 and 7**, the optimal response for TNF-alpha and IL-8 release is at 100 nM concentration of CPAF. In accordance to previous studies, KBM cells did not respond to CPAF treatment in the cytokine release (data not shown) (Bihl et al., 2016).

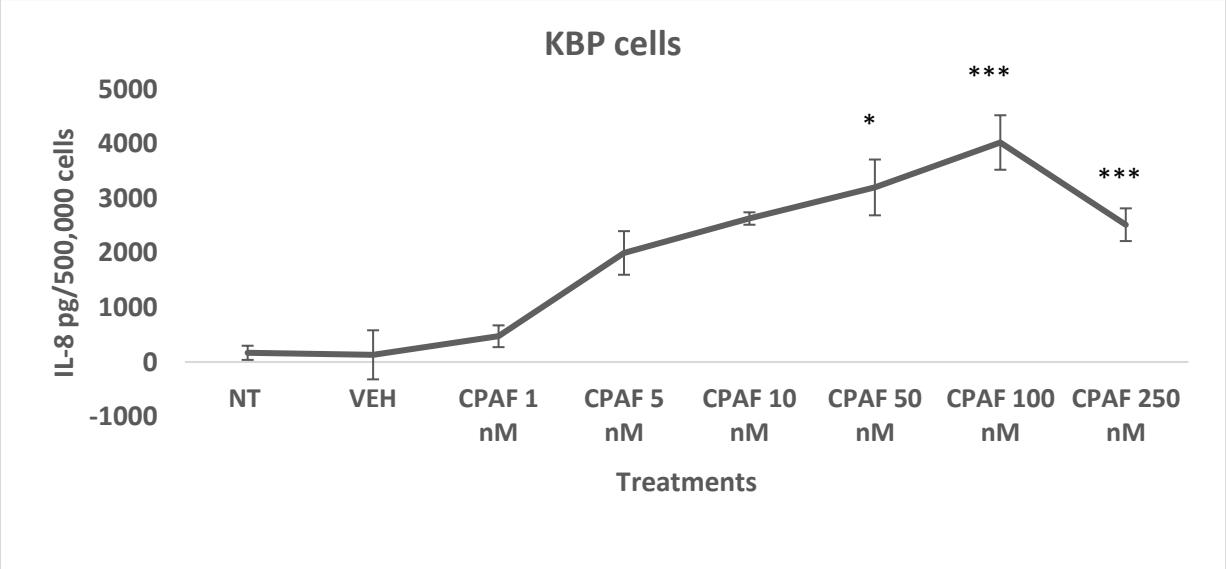

Figure 6. Concentration-dependence of CPAF treatment on IL-8 release in KBP cells. KBP cells were treated with either no treatment (NT), 0.01% EtOH vehicle (VEH) or various concentrations of CPAF at 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 250 nM and incubated for 4

hours. Supernatants were removed and IL-8 was analyzed normalized per cell count. The data are the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with * P < 0.05, ** P < 0.01, *** P <0.001.

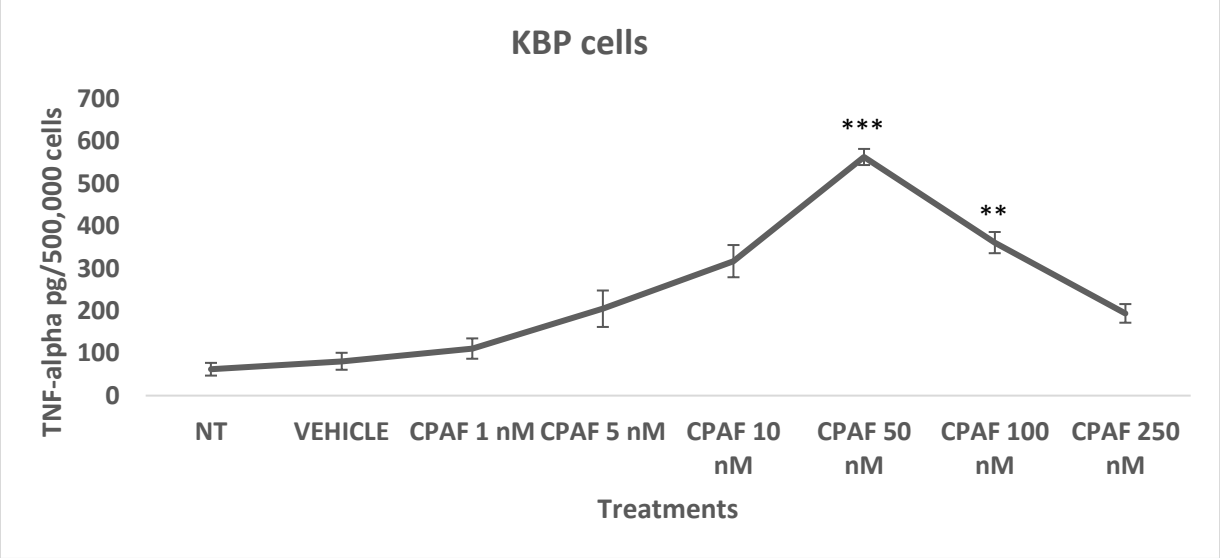

Figure 7. Concentration-dependence of CPAF treatment on TNF-alpha release in KBP cells. KBP cells were treated with either no treatment (NT), 0.01% EtOH vehicles (VEH) or various concentrations of CPAF at 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 250 nM and incubated for 4 hours. Supernatants were removed and TNF-alpha was analyzed normalized per cell count. The data are the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with ** P < 0.01, *** P <.001.

6.3 Concentration dependence of CPAF on MVP release in KBP cells

To determine the optimal, sub-optimal concentration of CPAF at which the PAF receptor is activated and leads to the release of MVPs we performed a concentration-dependent curve by treating keratinocyte-derived KBP cells with CPAF concentrations (1 nM to 250 nM). The purpose of treating the cells with as low as 1 nM was to see a minimal effect on the cells in the release of microvesicle particles. KBP cells were treated with CPAF (1 nM, 10 nM, 50 nM, 100 nM, 250

nM) and incubated for 4 hours. This step was followed by centrifugation. As seen in **Figure 8**. a sigmoidal curve was obtained with the optimal release at 100 nM of CPAF. This result is linear to the optimal dose obtained in the release of cytokines (IL-8 and TNF-alpha)(**Figures 6 and 7**)

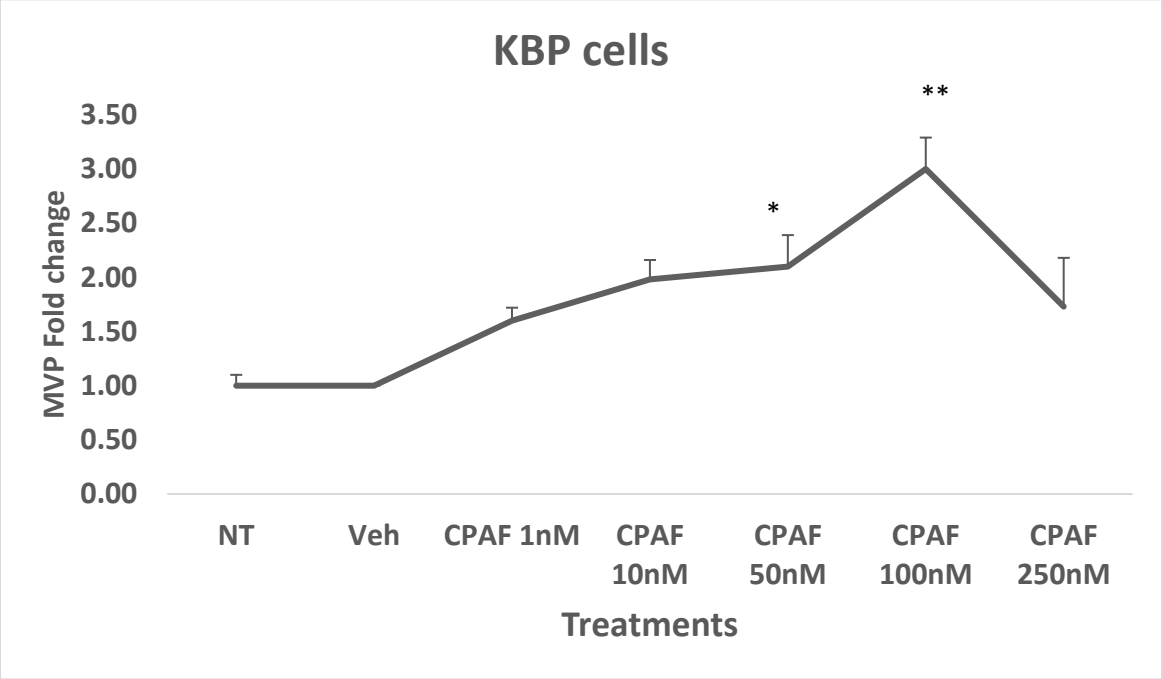

Figure 8. Concentration dependence of CPAF treatment on MVP release in KBP cells

KBP cells were treated with either No treatment or various doses of CPAF ranging from 1 nM, 10 nM, 50 nM, 100 nM, 250 nM, and incubated for 4 hours. Supernatants were removed and MVPs were analyzed particles/ml were normalized to Vehicle particles/ml per cell count. The data is the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with * $P < 0.05$, ** $P < 0.01$. Statistical analysis was performed by one-way ANOVA. Differences in the samples were considered significant if the P-value is less than 0.05.

6.4 Concentration dependence of CPAF on HaCaT cells on MVP release

To determine the optimal, sub-optimal concentration of CPAF at which the PAF receptor is activated and leads to the release of MVPs, cytokines we performed a concentration-dependent curve by treating keratinocyte-derived HaCaT cells with CPAF concentrations (1 nM to 250 nM). The purpose of treating the cells with as low as 1 nM was to see a minimal effect on the cells in the release of microvesicle particles. Keratinocyte derived HaCaT cells were treated with CPAF (1 nM, 10 nM, 50 nM, 100 nM, 250 nM) and incubated for 4 hours. This step was followed by centrifugation. As seen in **Figure 9** a sigmoidal curve was obtained with the optimal release at 100 nM of CPAF.

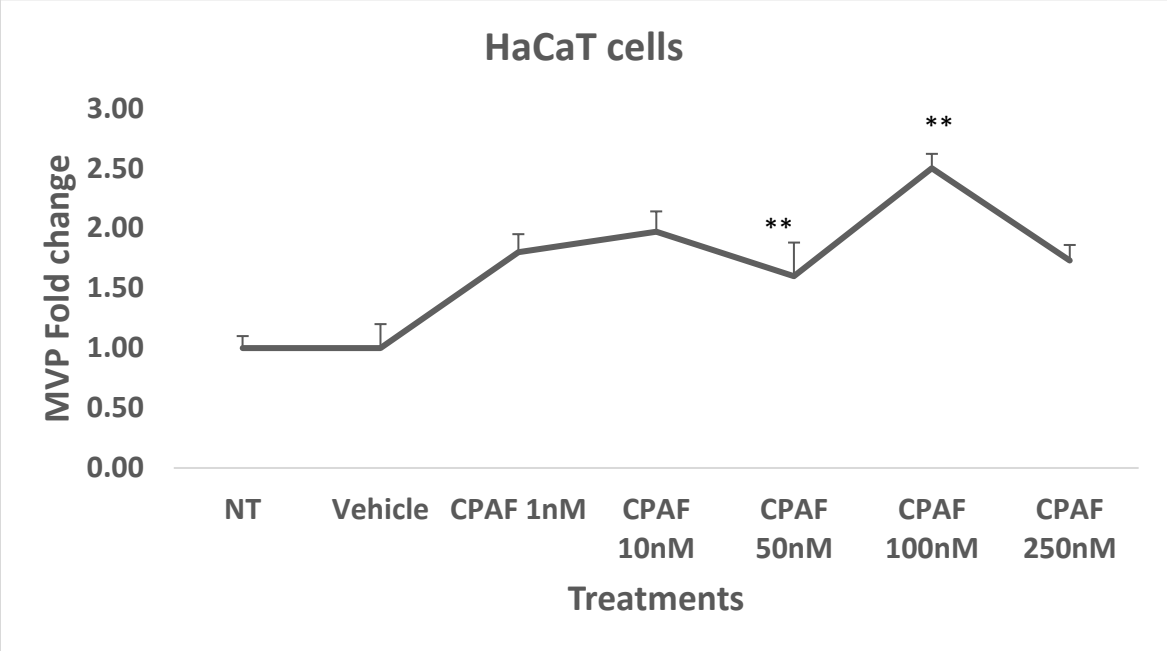

Figure 9. Concentration dependence of CPAF treatment on MVP release in HaCaT cells

HaCaT cells were treated with either No treatment or various doses of CPAF ranging from 1 nM, 10 nM, 50 nM, 100 nM, 250 nM and incubated for 4 hours. MVPs were removed from the supernatant and particles/ml were normalized to Vehicle particles/ml per cell count. The data is the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with * $P < 0.05$, ** $P < 0.01$. Statistical analysis was performed

by one-way ANOVA. Differences in the samples were considered significant if the P-value is less than 0.05.

6.5 Time Response Studies of CPAF/TPA/UVB on the release of MVP in HaCaT cells

To report the kinetics of treatment of CPAF/TPA/UVB on the release of MVP, three-time points were considered. HaCaT cells were treated with CPAF concentrations ((1 nM, 10 nM, 50 nM, 100 nM, 250 nM) and incubated for 4 hours. Supernatants were collected and analyzed for MVP by NTA. For 8 and 24 hr time points, fresh Hank's Balanced Salt Solution + BSA was added and supernatants were collected at those time points.

As observed in the **Figure 9**, the effect of optimal concentration of CPAF/TPA/UVB on the release of MVP remained constant at 4, 8 hr but it gradually decreases at 24 hrs. This supports us to establish that when treated with CPAF/TPA/UVB the release of MVP is optimal before 8 hrs.

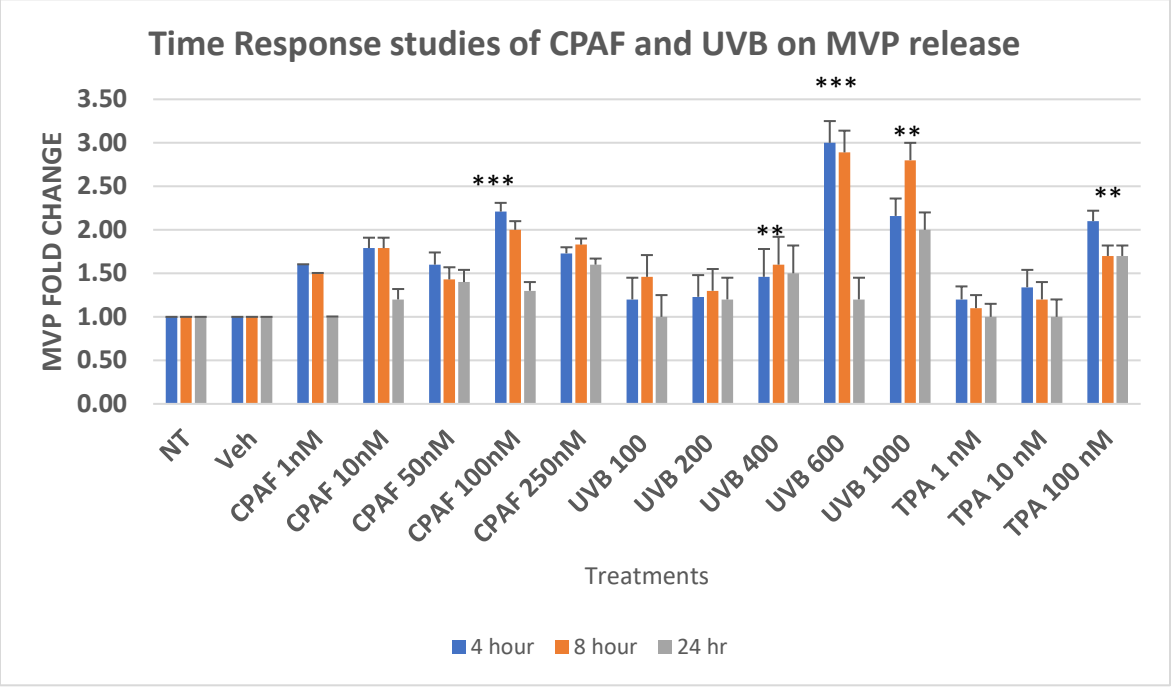

Figure 10 Time response study of CPAF/UVB/TPA cells for MVP release on HaCaT cells

HaCaT cells were treated with either No treatment or various doses of CPAF ranging from 1 nM, 10 nM, 50 nM, 100 nM, 250 nM UVB 50 J/m^2, 100 J/m^2, 200 J/ m^2, 400 J/ m^2, 600 J/ m^2, 1000 J/

m^2, TPA 1 nM, 10 nM, 100 nM and incubated for 4,8,24 hours. Supernatants were removed and MVPs were analyzed particles/ml were normalized to Vehicle particles/ml per cell count. The data is the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with ** $P < 0.01$, *** $P < .001$. Statistical analysis was performed by one-way ANOVA. Differences in the samples were considered significant if P-value is less than 0.05.

6.6 Combination of low concentration of CPAF and gradually increasing UVB fluences results in synergistic response in IL-8; MVP

The next studies were designed to be able to establish the effects of low concentration of CPAF and different variants of UVB fluences on HaCaT cell lines in vitro. We investigated the effects of combination of low concentration CPAF and low fluence of UVB on the release of MVP using a 4 hour incubation time. HaCaT cells at 80% confluency were treated with CPAF (1 nM) for one hour followed by various fluences (100 J/m^2, 200 J/m^2 or 400 J/m^2) of UVB. Similarly, we tested slightly higher concentration of CPAF (10 nM) along with UVB.

MVPs were analyzed by Nanoparticle tracking analysis and normalized to the cell count. As seen in **Figures 10 and 11**, CPAF at low concentration and UVB at low fluence alone do not generate a significant response in MVP release. However, when combined together generate a statistically significant exaggerated response in MVP release. This response can be called a synergistic response by HaCaT cells in response to CPAF at low concentration and UVB at low fluence. In contrast to the 10nM CPAF + UVB, IL-8 did not show any response in the combination of CPAF (1 nM) at low concentration and UVB at low fluence (data not shown).

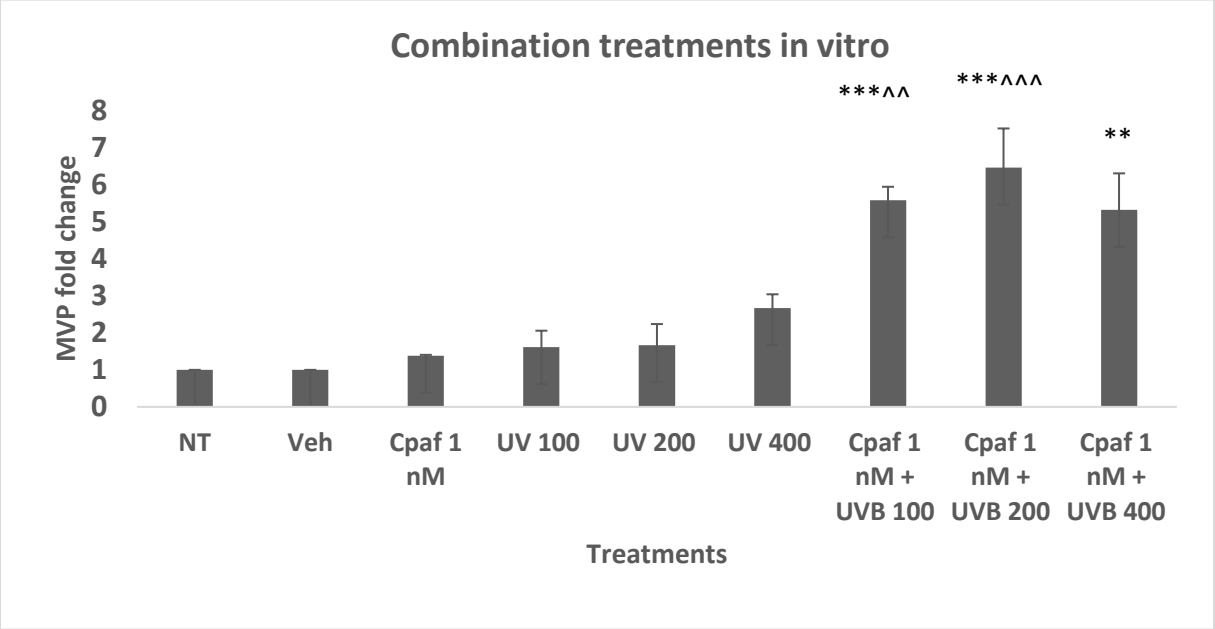

Figure 11. Effects of a combination of low concentration CPAF and low fluence of UVB for MVP release in HaCaT cells.

HaCaT cells were treated with either No treatment or treatments like CPAF 1 nM or UVB 100 J/m^2, 200 J/m^2, 400 J/m^2 and combination of CPAF 1 nM + UVB 100 J/m^2/ 200 J/m^2 or 400 J/$m^2$and then incubated for 4 hours. Supernatants were removed and MVPs were analyzed particles/ml were normalized to Vehicle particles/ml per cell count. The data is the mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with ** P < 0.01, *** P <.001. Asterisks * indicate difference compared to CPAF and ^ indicate difference compared to UVB.

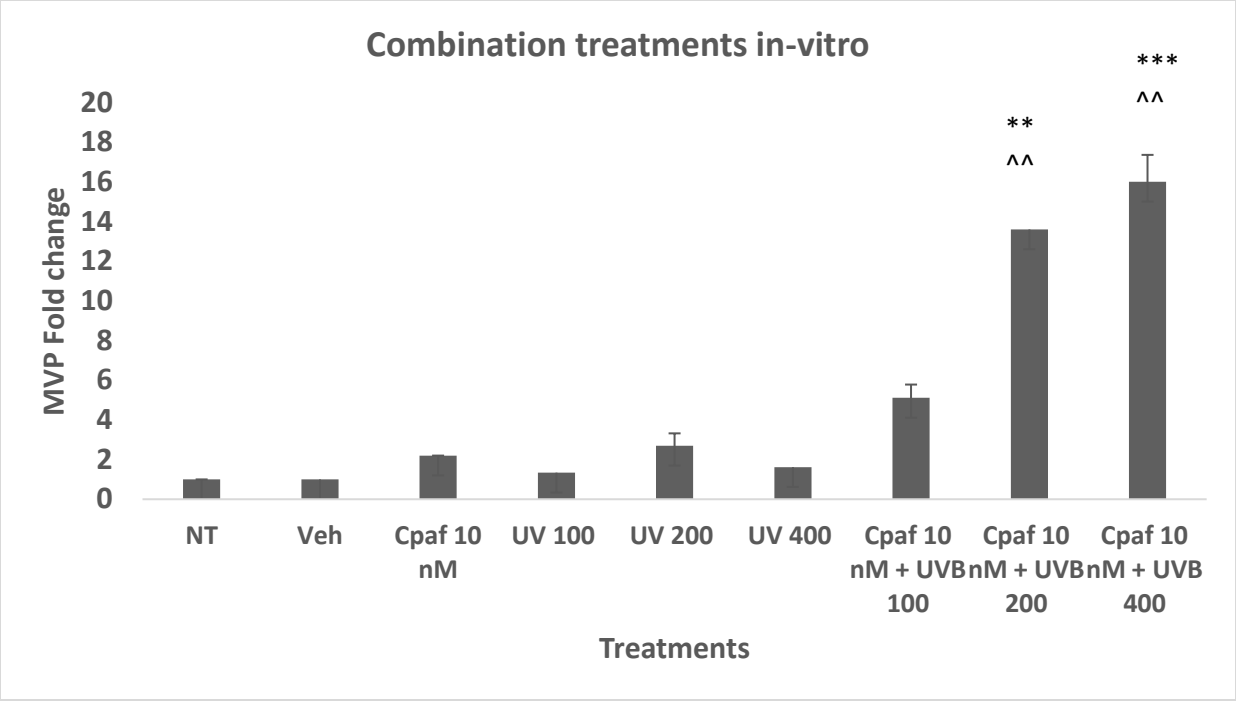

Figure 12. Effects of a combination of low dose CPAF and a low dose of UVB for MVP release in HaCaT cells

HaCaT cells were treated with either No treatment or treatments like CPAF 10 nM or UVB 100 J/m^2, 200 J/m^2, 400 J/m^2 and combination of CPAF 10 nM + UVB 100 J/m^2/ 200 J/m^2 or 400 J J/m^{2}and then incubated for 4 hours. Supernatants were removed and MVPs were analyzed particles/ml were normalized to Vehicle particles/ml per cell count. The data is the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with ** P < 0.01, *** P <.001. Differences * mark indicate difference compared to CPAF and ^ indicate difference compared to UVB. Fold Change for CPAF/TPA treatments was calculated by normalizing to Vehicle (0.1% EtOH) and UVB with no treatment.

6.7 Effects of the combination of low concentration CPAF and low fluence of UVB on IL8 release in HaCaT cells.

Since we found that low levels of CPAF and UVB were synergistic on MVP release, we next tested their effects on IL-8 release in HaCaT cells. As shown in **Figure 12**, combining 10nM CPAF with UVB resulted in enhanced levels of IL-8 protein release.

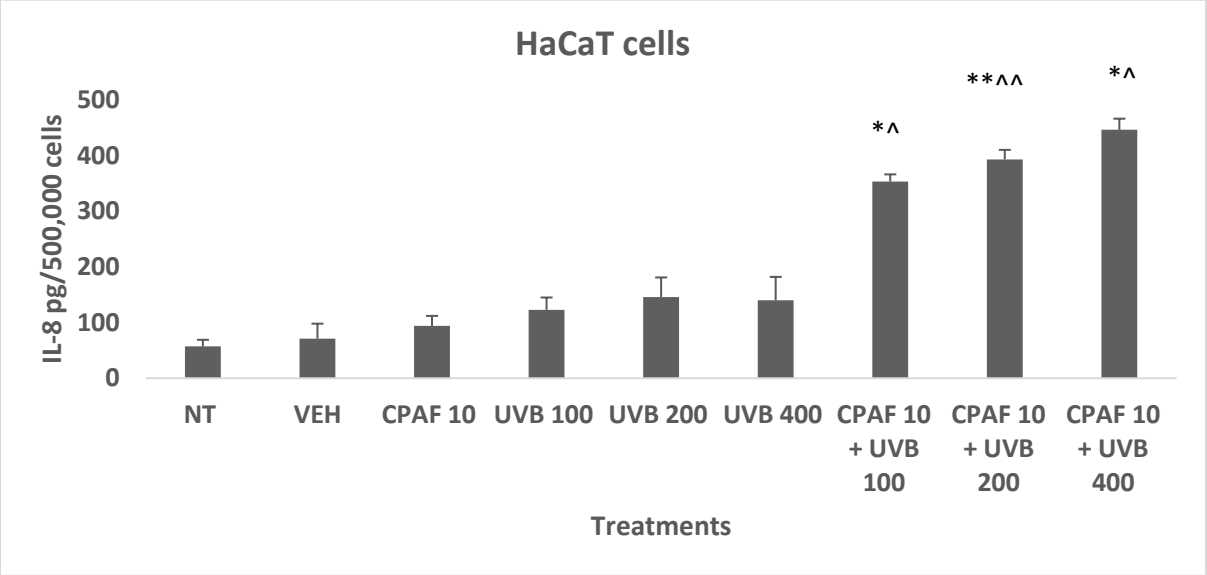

Figure 13. Effects of the combination of low dose CPAF and a low dose of UVB for IL8 release on HaCaT cells.

HaCaT cells were treated with either no treatment or treatments like CPAF 10 nM or UVB 100 J/m^2, 200 J/m^2, 400 J/m^2 and combination of CPAF 10 nM + UVB 100 J/m^2/ 200 J/m^2 or 400 J/m^2 and then incubated for 4 hours. Supernatants were removed and IL8 was analyzed by ELISA and normalized to cell count. The data is the mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with * $P < 0.05$, ** $P < 0.01$ Asterisks * indicate difference compared to CPAF and ^ indicate difference compared to UVB.

6.8 Combination of low concentration of CPAF and gradually increasing UVB fluences results in Synergistic response on MVP release in Skin explants

We investigated the effect of low concentration of CPAF and different variants of UVB fluences on MVP release in the ex-vivo skin explants at 4 hour incubation times. Skin punch biopsies (6 mm) were taken after 4 hours of incubation, weighed, and digested in 5 mg/ml of collagenase overnight. Following day centrifugations were performed with the addition of 1 ml 1X PBS at 2000 X g for 20 min, 20000 X g for 10 min, 20000 X g for 70 min at 4˚C.

The combination of low concentration of CPAF and low fluence of UVB do exert a significant effect on the skin explants in the MVP release. A similar behavior is observed here between skin explant tissue and that of keratinocyte derived HaCaT cells which suggests that the HaCaT cells are an appropriate model for skin.

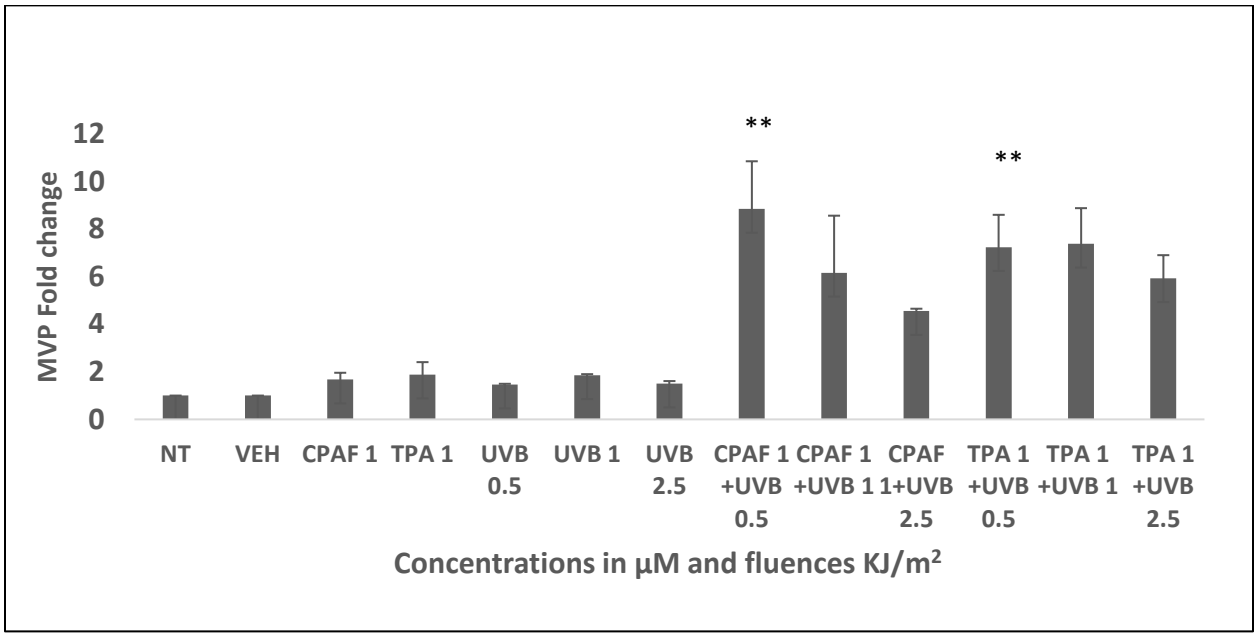

Figure 14. Effects of combination of low dose CPAF and low dose of UVB for MVP release on Skin explants ex-vivo

Human Skin Explants were treated with either No treatment or treatments like CPAF 1 µM /TPA 1 µM or UVB 500 J/m^2, 1000 J/m^2, 2500 J/m^2 and combination of CPAF 1 µM/ TPA 1 µM + UVB 500 J/m^2/ 1000 J/m^2 or 2500 J/m^2 and then incubated for 4 hours. MVPs were extracted from treated punch biopsies and normalized to Vehicle/ No treatment particles/g. The data is the Mean +/- SE from two separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with * $P < 0.05$, ** $P < 0.01$, *** $P < .001$. Differences * mark indicate difference compared to CPAF and ^ indicate difference compared to UVB. Fold Change for CPAF/TPA treatments were calculated by normalizing to Vehicle (0.1% EtOH) and UVB with no treatment**.**

6.9 Effect of Imipramine on combination treatment on the release of MVP and cytokines

Pharmacologic blocking aSMase gives a directive of role of MVPs in intercellular communication. We investigated the role of imipramine in the combination of low concentrations of CPAF and UVB on the release of MVPs and IL-8. Cells were treated with either No treatment or with combination treatments CPAF (10 nM)/UVB 400 J/m^2 and for combination treatments preincubation of CPAF (10 nM) for 1 hour + UVB (400 J/m^2) and combination treatments preincubation of CPAF (10 nM) for 1 hour + UVB (400 J/m^2) followed by 50 µM of imipramine, incubated for 4 hours, 8 hours and 24 hours. Supernatants were collected and subjected to centrifugations of 2000 X g for 20 mins followed by 20000 X g. As shown in **Figure 15**, imipramine which is an aSMase inhibitor completely suppressed the increased MVP release.

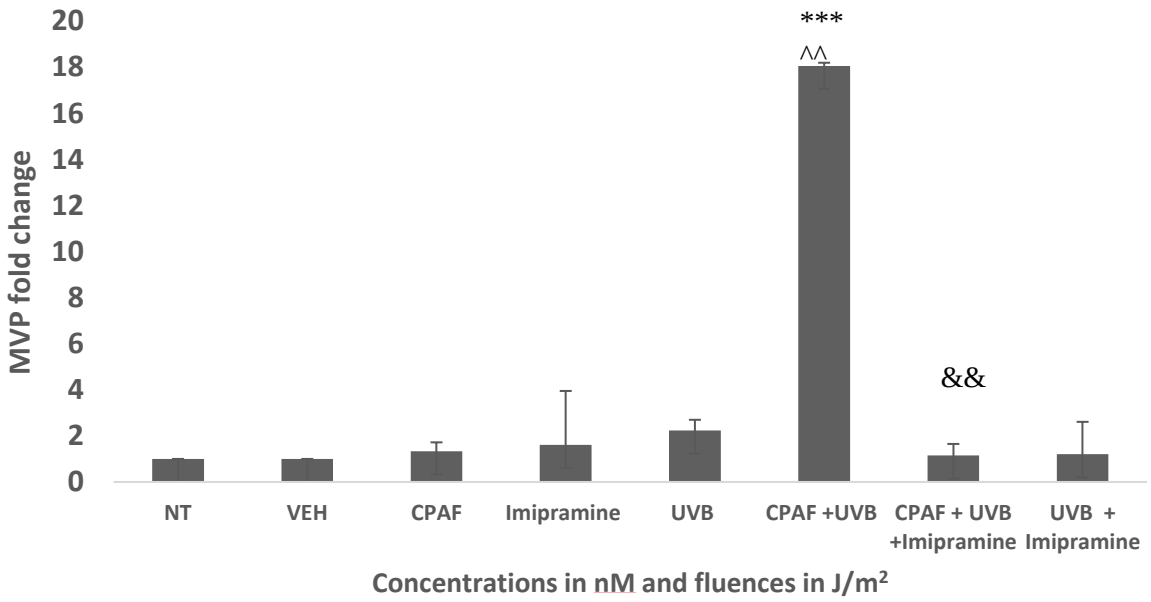

Figure 15. Effects of Imipramine on the combination of low dose CPAF and a low dose of UVB on MVP release

HaCaT cells were treated with either No treatment or treatment with Imipramine 50 µM ,CPAF 10 nM or UVB 400 J/m^2 and combination of CPAF 10 nM + UVB 400 J/m^2 or immediate treatment with imipramine 50 µM incubated for 4 hours. Supernatants were removed and MVP was analyzed normalized to cell count and vehicle particles/g. The data is the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with *** $P < 0.001$ as compared to CPAF and ^^ $P< 0.01$ as compared to UVB and &&

$P< 0.01$ indicates differences between the combination treatment group and the combination treated + Imipramine group. Fold change for CPAF/TPA treatments was calculated by normalizing to Vehicle (0.1% EtOH) and UVB with no treatment.

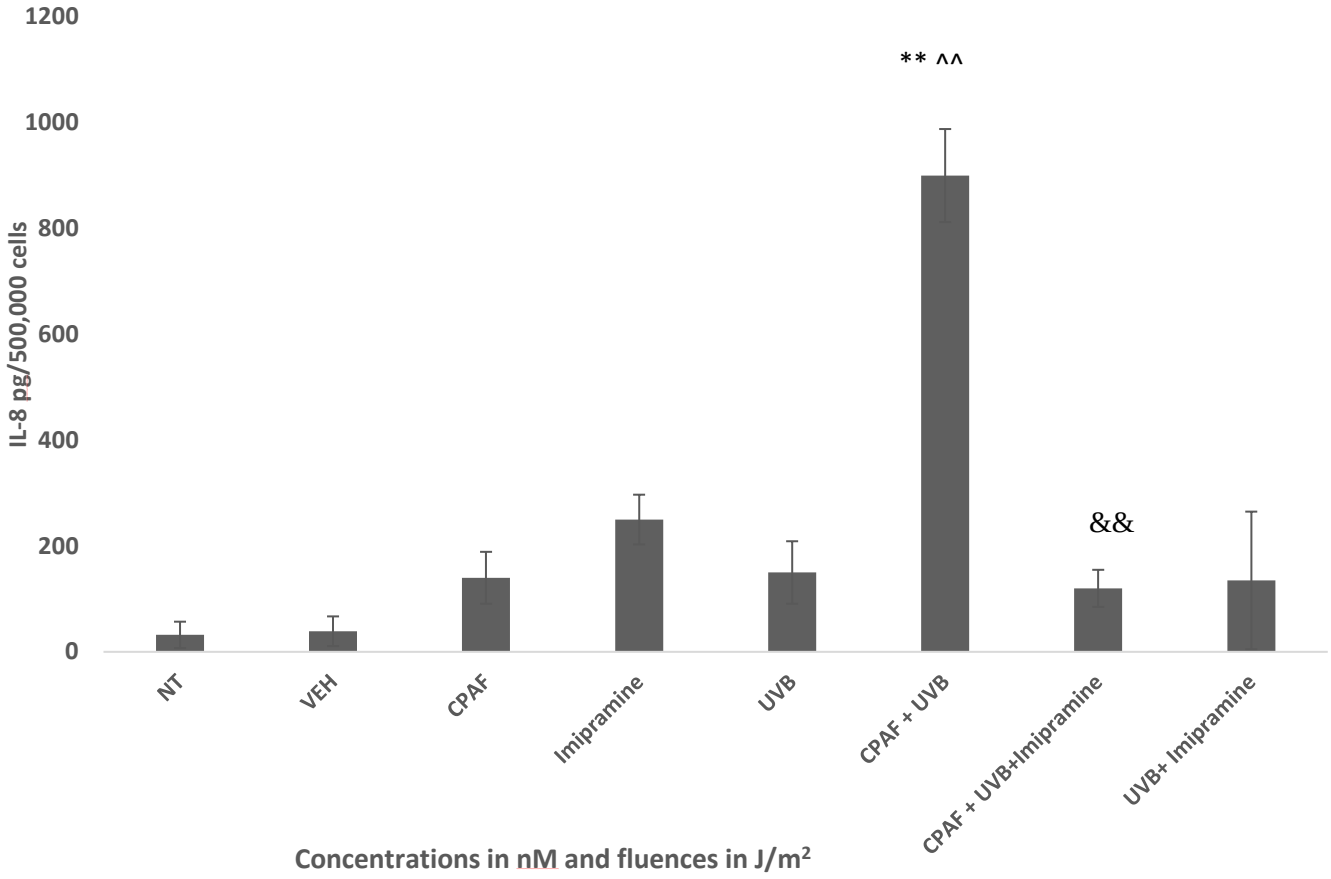

Figure 16. Effects of Imipramine on the combination of low dose CPAF and a low dose of UVB on IL-8 release

HaCaT cells were treated with either No treatment or treatment with Imipramine 50 µM ,CPAF 10 nM or UVB 400 J/ m^2 and combination of CPAF 10 nM + UVB 400 J/ m^2 or immediate treatment with Imipramine 50 µM incubated for 4 hours. Supernatants were removed and IL8 was analyzed by ELISA and normalized to cell count. The data is the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with ** $P < 0.01$ as compared to CPAF and ^^ $P< 0.01$ as compared to UVB and && $P< 0.01$ indicates differences between the combination treatment group and the combination treated + Imipramine group.

7. DISCUSSION

7.1 Summary

UVB radiation is a potent stimulator of cellular stress responses in epithelial cells (Travers, 2020). These UVB-induced responses include the generation of higher amounts of pro-inflammatory cytokines as well as MVP. Our group has previously shown that UVB-induced stress has systemic effects on rodents (Travers, 2020). These systemic effects of UVB in mice models have shown increased levels of MVP when irradiated with UVB.

The results from this study established that UVB induces stress responses in the cells in the form of the release of MVPs and cytokines. Of importance, recent studies by our group have documented that MVP released by thermal burn injury of UVB contains the lipid mediator PAF (Travers, 2020). Hence, MVP could be one mechanism by which keratinocytes could release the metabolically labile PAF to allow it to activate G-coupled receptor PAF-Rs in the body. Activation of PAF-R due to UVB also induced the release of pro-inflammatory cytokines including IL-8. From the results above we observed UVB-induced release of pro-inflammatory cytokine in a concentration-dependent manner in KB cells expressing the PAF-R (KBP) which indicates that UVB generates lipid mediator PAF and it follows the PAF-R activation pathway.

To study the kinetics of MVP when treated with CPAF/TPA/UVB we studied the time course of release of MVP. For the optimal concentration of CPAF 100 nM, the release of MVP was optimal for 4 – 8 hours. There was a gradual decrease in MVP release for 24 hours timepoint. This explains that UVB-induced release of MVP is an immediate release but not an extended-release.

Synergism is observed when two drugs or agonists when combined give a response greater than the individual responses combined. We obtained a synergistic effect by combining UVB and PAF

agonist CPAF at very low concentrations. Several lines of evidence suggest the synergism between the combination of CPAF and UVB. First, a combination of low concentration of CPAF and low fluence of UVB which produce minimal response individually gave a synergistic response and generated around 6-7 fold change of MVP. This synergistic response was also observed in the IL-8 and TNF-alpha release. Second, another minimal concentration of CPAF (10 nM) was then combined with UVB (400 J/m^2) which resulted in a synergistic response of around 18 fold change in MVP release. This indicates synergism in keratinocyte-derived HaCaT cells for the combination of low concentrations of CPAF and UVB. Third, human skin explants treated with low concentration CPAF (1 µM) and low fluence UVB (500 J/m^2) showed a synergistic response when combined together. Hence, similar synergism was observed in skin explants ex-vivo. This indicates a correlation of results between the two models.

This synergism indicates that even drugs/agonists that generate PAF in very low quantities when combined with mere sunlight or tanning beds can cause an aggravated response of cytokines and MVP. This was potentially observed in the toxic epidermal necrolysis case in **Figure 1**. The mechanism is not well understood for this synergistic effect but we believe phospholipase A2 (PLA2) could possibly play a role. Previously, our group has reported that synergistic cytokine production between UVB and CPAF was blocked by protein kinase C (PKC) inhibitors {Wolverton et al. 2010}. This synergism may contribute to the fact that photosensitivity reacts in the response to cellular stressors and the phenotype in inflammation. Photosensitivity is related to a condition where the individual's reactivity to UV radiation is exaggerated in inflammatory responses as compared to the control group. Our group has studied photosensitivity affects local and systemic effects in mice by using XPA KO mice. While in this study the HaCaT cells or

Human skin explants were conditioned such that it produced an exaggerated response similar to that of photosensitive cells or mice. By this, it indicates that photosensitivity has pro-inflammatory phenotypes and that MVP plays a major role in the inflammatory response. Systemic effects of the combination of low concentrations of CPAF and UVB are yet to be determined.

To study the role of MVP in pro-inflammatory cytokine responses, we used aSMase inhibitor imipramine to see the effects on cytokine release. Imipramine blocked MVP release when treated immediately after the combination treatment of CPAF and UVB. Imipramine also blocked the IL-8 levels. This provides evidence that MVP may be partly responsible for the transport of these chemokines. The present studies have clinical applications in that finding that MVP can be involved in this synergistic effect of UVB and other types of stimuli could propel the use of novel agents such as imipramine as therapeutics.

Future studies that can build upon the current ones include testing the combination of low-concentration UVB and stimuli in mice. Moreover, these types of studies could even be pursued in human subjects.

7.2 Future Studies:

The future studies would be to analyze TNF-alpha release as it is a pro-inflammatory cytokine in the combination treated HaCaT cells to build a directive of the combination of PAF-R agonist and UVB effects on cytokine release. Studies could also be investigated for combination of low concentrations of PAF-R agonist and UVB in mice and humans.

7.3 Conclusion

The results from the above studies give us a strong evidence that the combination of PAF-R agonists and UVB can cause a synergistic effect in IL-8 and MVP release in-vitro and ex-vivo. Inhibition of aSMase enzyme which blocks MVP release also inhibited the IL-8 release, suggesting a potential role of MVP in the increased cytokines released in response to these agents.

SUPPLEMENTARY FIGURES

Figure 17. Skin Preparation for treatments

Effect of Imipramine on Combination treatment in the release of MVP at 8 hr and 24 hr

Blocking of Acid sphingomyelinase gives a directive of the role of MVPs in intercellular communication. We investigated the role of Imipramine in the combination of low concentrations of CPAF and UVB on the release of MVPs, cytokines. Cells were treated with either No treatment or with combination treatments CPAF (10 nM)/UVB 400 J/m^{2}and for combination treatments preincubation of CPAF (10 nM) for 1 hour + UVB (400 J/m^2) and combination treatments preincubation of CPAF (10 nM) for 1 hour + UVB (400 J/m^2) followed by 50 µM of Imipramine (reference **Figure 13**) 8 and 24 hr time points, fresh Hank's Balanced Salt Solution + BSA was added and supernatants were collected at those time points. Supernatants were collected and subjected to centrifugations of 2000 X g for 20 mins 4˚C followed by 20000 X g for 70 min 4˚C. There was a synergistic response observed at 8 hr time point but it was not observed at 24 hrs which explains the Time response studies of MVP conducted. As reference to **figure** 13, the MVP release was blocked by Imipramine at 8 hrs.

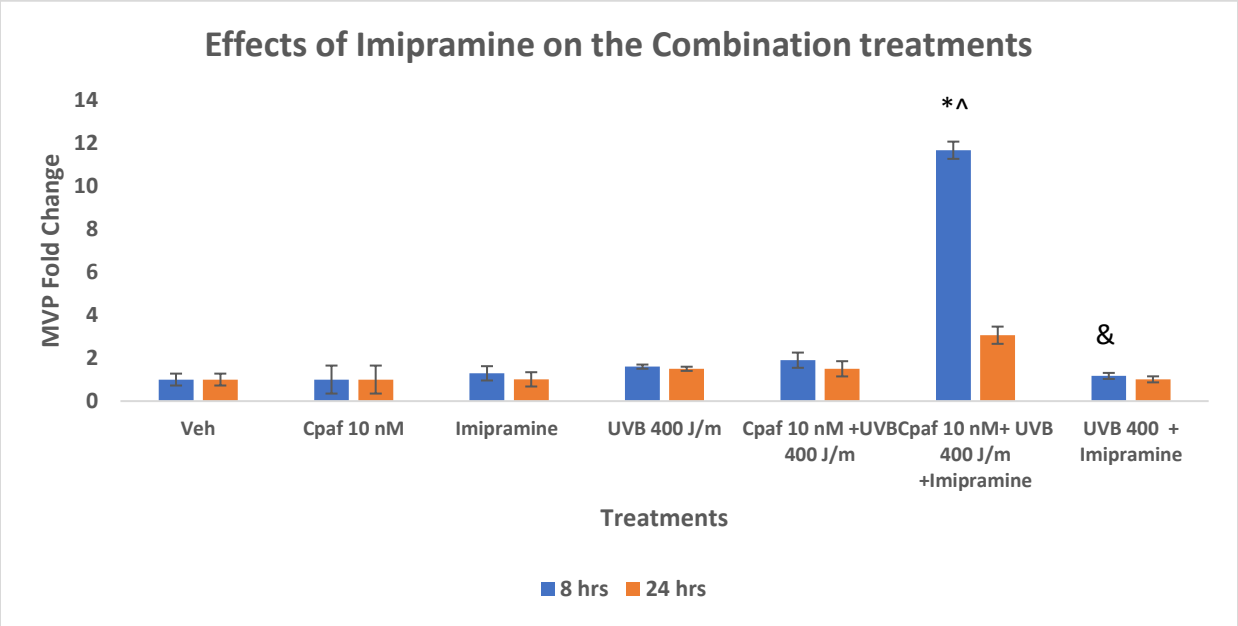

Figure 18. Effects of Imipramine on the combination of low dose CPAF and a low dose of UVB on MVP release at 8 hr and 24 hrs

HaCaT cells were treated with either No treatment or treatment with Imipramine 50 µM ,CPAF 10 nM or UVB 400 J/ m^2 and combination of CPAF 10 nM + UVB 400 J/ m^2 or immediate treatment with Imipramine 50 µM incubated for 8 hours and 24 hours. Supernatants were removed and MVP was analyzed normalized to cell count and vehicle particles/g. The data is the Mean +/- SE from 3 separate experiments using duplicate samples. Statistical analysis was performed by one-way ANOVA, with *** $P < 0.001$ as compared to CPAF and ^^ $P< 0.01$ as compared to UVB and && $P< 0.01$ indicates differences between the combination treatment group and the combination treated + Imipramine group. Fold Change for CPAF/TPA treatments was calculated by normalizing to Vehicle (0.1% EtOH) and UVB with no treatment.

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