To Determine the Role of the Platelet Activating Factor - Receptor in FOLFIRINOX Therapy-mediated Microvesicles Particle Generation

Krishna Awasthi
Wright State University

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TO DETERMINE THE ROLE OF THE PLATELET ACTIVATING FACTOR - RECEPTOR IN FOLFIRINOX THERAPY-MEDIATED MICROVESICLES PARTICLE GENERATION.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

by

KRISHNA AWASTHI

B. Pharm., Pokhara University, Nepal, 2016

2023

Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Krishna Awasthi ENTITLED To determine the role of the Platelet activating factor - receptor in FOLFIRINOX therapy-mediated microvesicles particle generation BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

________________________
Ravi P. Sahu, Ph.D.
Thesis Director

________________________
Jeffrey B. Travers, M.D., Ph.D.
Department Chair

Committee on Final Examination:

_______________________________
Jeffrey B. Travers, M.D., Ph.D.

_______________________________
Michael G. Kemp, Ph.D.

_______________________________
Yong-Jie Xu, M.D., Ph.D.

_______________________________
Anita Thyagarajan, Ph.D.

_______________________________
Shu Schiller, Ph.D.
Interim Dean, College of
Graduate Programs & Honors Studies
ABSTRACT
Awasthi, Krishna. M.S., Department of Pharmacology and Toxicology, Wright State University, 2023. To determine the role of the Platelet activating factor - receptor in FOLFIRINOX therapy-mediated microvesicles particle generation.

Given that Pancreatic cancer continues to be one of the primary causes of cancer-related death and that existing treatment options have a poor success rate, it is important to investigate the role of potential factor(s) involved in pancreatic cancer growth or diminishing the effectiveness of chemotherapeutic agents. An alternative regimen for pancreatic cancer treatment called FOLFIRINOX has shown to increase the survival rate and improve Eastern Cooperative Oncology Group (ECOG) performance status score among the pancreatic cancer patients. However, FOLFIRINOX therapy is associated with several side effects. Through mechanisms involving the secretion of microvesicle particles (MVP), studies, including ours have demonstrated the crucial roles of a G-protein coupled receptor called Platelet-activating factor-receptor (PAFR) in promoting tumor growth or reducing the effectiveness of chemotherapeutic drugs through the modulation of different signaling pathways in the cancer microenvironment. Following this concept, we investigated the notion that components of FOLFIRINOX regimen via their pro-oxidative stress ability will result in MVP generation in pancreatic cancer cell lines. Our in vitro studies demonstrated that FOLFIRINOX components induce MVP release in a dose dependent pattern from PAFR-expressing, but not from PAFR deficient cell lines. The FOLFIRINOX components-induced MVP release were significantly blocked by the pretreatment with PAFR antagonist and inhibitor of acid sphingomyelinase (aSMase) enzyme. To confirm the PAFR involvement, transfection studies using PAFR specific siRNA confirmed that PAFR expression is necessary for MVP generation by the FOLFIRINOX components. To determine if FOLFIRINOX therapy can exert similar effects on normal organs of epithelial origin, we performed studies where
FOLFIRINOX components were topically applied on human and murine skin with or without PAFR antagonist and aSMase inhibitor. We observed similar findings that FOLFIRINOX components stimulate a dose-dependent increased in MVP release in the skin of the wild type mice and human skin, naturally acquired with PAF receptor. To validate the mechanism, we utilized PAFR deficient (Ptafir<sup>-/-</sup>) and aSMase enzyme deficient (Smpd<sup>-/-</sup>) mouse models and treated with the topical application of FOLFIRINOX components. Pertaining to the result obtained in wild type mice and human skin, we found that FOLFIRINOX components-induced MVP release was dampened in PAFR deficient (Ptafir<sup>-/-</sup>) and Smpd<sup>-/-</sup> mice. The overall findings suggest that PAFR expression is required for FOLFIRINOX components-induced MVP generation in in vitro, in vivo, and ex vivo models. Additionally, our study shows a mechanistic model through which the PAFR-aSMase axis can generate crucial bioactive substances called MVP as a bystander effect, which are believed to limit the efficacy of chemotherapeutic drugs and pose serious risk of adverse drug reaction associated with the chemotherapeutic drugs.
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CHAPTER-1 INTRODUCTION

1.1 Cancer

Cancer develops when a few body cells multiply out of control and begin to spread to other body parts. Such abnormal growth and differentiation of the cells are usually accompanied by genetic alteration and modification in wide range of cellular signaling pathways (1,2). Normally, the human body is known to be made up of trillions of cells where a distinct group of cells have distinct growth patterns. These human cells grow by the process called cell division. However, some genetic modification and alteration in the cell signaling pathways promote abnormal cell division and give rise to the condition called cancer, usually predisposed to cancer tumors (1,2,3,4). Invading nearby tissues and expanding to other parts of the body, telling blood vessels to grow toward tumors, attempting to hide from the immune system, relying on different varieties of nutrients than normal cells, and accumulating multiple chromosome changes seem to be just a handful of the different manners that cancer cells differ from normal cells (1,3,6). Such property exhibiting tumors are divided into benign, premalignant, and malignant tumors according to their cell of origin, appearance, size, and function or activity. A benign tumor doesn't invade nearby healthy tissue or spread to other parts of the body; it stays in its original spot. However, a malignant tumor has the propensity to both disseminate throughout the body through the circulatory or lymphatic systems and invade nearby normal tissue (1,3,6). There are three distinct types of evolutionary mutations that influence the progression of the disease since they can be inherited or acquired from interaction with any genotoxic substances throughout an individual's life (1,4). Reflecting upon the genetic basis behind the cause of cancer, Proto-oncogenes are considered as an important regulator of biochemical processes and are present in normal cells. Proto-oncogenes can act as nuclear transcription factors, signal transducers in cells, and growth factors. Based on
genetic alteration in the human proto-oncogenes which are usually encoded by different proteins, it is known that normal cells are primarily transformed into cancer cells by oncogenes, whereas tumor-suppressive genes prevent the growth of cancer. (1,4,6).

Based on the cell types that they originated from, cancers are divided into different categories. For instance, carcinomas, the most prevalent type of cancer, are caused by changes in the epithelial cells. Sarcomas are malignant growths that develop in the bones, muscles, and connective tissues. White blood cell abnormalities are termed as leukemia. Lymphoma is the term used to define the malignant type of lymphatic cells or system that develops from the bone marrow. Similar to how abnormal plasma cells grow in the bone marrow, myeloma or multiple myeloma is a disorder in which these cells replicate themselves at an accelerated rate (1,6). Gender can also affect the likelihood that a person will develop cancer. Men are slightly more likely than women to receive an invasive cancer diagnosis in their lifetime. (1,7)

Figure 1: Types of cancers (1, 5).
1.2 Cancer epidemiology

The annual incidence and mortality rates linked to the most prevalent malignancies in the United States are estimated in the American Cancer Society's cancer statistics report (7,8). This report revealed that approximate 64,050 people (33,130 men and 30,920 women) will be diagnosed with pancreatic cancer in the United States alone in 2023 and around 50,000 people are predicted to die because of pancreatic cancer in 2023 in the United States alone (7,8). Everyone is at risk of getting cancer, but the incidence rises significantly with age; in the US, 80% of cancer diagnoses are made in patients 55 or older, and 57% are patients 65 or older (8). The American Cancer Society (ACS) reported similar trend as reported by National Cancer Institute in 2020 that prostate, lung, and colorectal cancer will account 48% of total cancer cases diagnosed in men in 2023 whereas breast, lung, and colorectal cancer will account 52% of total cancer cases in women in 2023 (7,9). More importantly, the report by American Cancer Society revised in 2023 suggest that pancreatic cancer accounts for about 3% of all cancers in the United States and about 7% of all cancer deaths. The seventh-highest cause of cancer-related death worldwide is pancreatic cancer, which itself is identified as the 14th most prominent malignancy (10). According to projections from Globocan, there were 458,918 pancreatic cancer diagnoses and 432,242 deaths worldwide in 2018 (10). Another study by Lola et. al reported that pancreatic ductal adenocarcinoma (PDAC) will be the second leading reason of cancer death in the United States in the year 2030 (11).

Pancreatic Ductal Adenocarcinoma is the most common type of pancreatic cancer that develops from the exocrine cells of the pancreas and accounts for more than 90% of pancreatic cancer cases (12). As reported by Cancer Research UK, incidence rates for pancreatic cancer have risen by about a tenth (9%) in the UK during the past ten years, and male rates have climbed by around a tenth (11%) whereas female rates have increased by more than a twentieth (7%) (13). The overall
data reported by different health organizations, policy-making units, and pilot study suggest that pancreatic cancer is of severe threat to the global world and needs a great attention from the research and clinical grounds (10,11,12).

Figure 2: Pancreatic cancer incidence in both sexes reported by Globocan (10)

1.3 Pancreatic Cancer

The third most prevalent cause of cancer-related fatalities in the United States today, following lung cancer and colon cancer, is pancreatic cancer, a lethal disorder that can develop in either the exocrine or endocrine component of the pancreas (10,12). It has a dismal patient prognosis and a meager 5% five-year survival rate (14,15). Numerous risk factors have been reported for pancreatic cancer which includes smoking, older age, diabetes, obesity, chronic pancreatitis, Liver cirrhosis, bacterial infection, exposure to toxic chemical compounds including carcinogens, family history
and many more (10,17). Additionally, ten percent of cases are genetically based, either due to genetic abnormalities or because they are linked to disorders like Lynch syndrome, Peutz-Jeghers syndrome, Von Hippel-Lindau syndrome, or MEN1 (multiple endocrine neoplasia type 1) (16). Notably, whilst the non-modifiable risk factors are typically unavoidable, the modifiable risk factors can be avoided (10,17,18).

**Figure 3: Risk factors for Pancreatic Cancer (17)**

Oncogene activation, tumor suppressor gene inactivation, and cell cycle dysregulation all play critical roles in the sequential progression of pancreatic cancer development. In particular, noninvasive pancreatic neoplasia comes in three morphologic types, each with unique biological and clinical characteristics (4, 19). Firstly, the pancreatic duct's main duct or one of its major
branches is home to the intraductal papillary mucinous neoplasm (IPMN), which is made up of mucin-producing neoplastic cells. Secondly, another mucinous cystic neoplasm that does not connect to the pancreatic ductal system is called mucinous cystic neoplasm (MCN), and it falls into one of three groups (benign, borderline, and malignant) \( (18,19,20) \). Lastly, pancreatic intraepithelial neoplasia (PanIN) is the most typical precursor to PC in humans \( (18,19) \). Exocrine tumors make up the majority of pancreatic tumors; these include pancreatic mucinous cystic neoplasm, ductal adenocarcinoma, acinar cell carcinoma, cystadenocarcinoma, adenosquamous carcinoma, signet ring cell carcinoma, hepatoid carcinoma, colloid carcinoma, undifferentiated carcinoma, and ductal adenocarcinoma. Importantly, the majority of pancreatic tumors, \( (80–90\% \) of cases) are ductal adenocarcinomas, which are characterized by moderately to poorly differentiated glandular features \( (18,19) \).

**Figure 4: Types of Pancreatic Neoplasm along with incidence rate \( (20) \)**

The type and stage of the pancreatic cancer, potential side effects, the patient's preferences, and their general health are just a few of the variables that can affect the recommended course of treatment \( (18) \). Based on the mentioned variable, the patient can be treated by a surgical procedure that involves the removal of all or a particular portion of the pancreas, radiation therapy, therapies
involving the use of precision medications, chemotherapy, targeted therapy and immunotherapy (14,17,18,19). The current standard of care in the advanced setting was established more than ten years ago when gemcitabine, as compared to 5-fluorouracil, improved symptoms and lengthened survival in a phase III trial when compared with the patient treated with 5-fluorouracil (21). It has been challenging to expand on gemcitabine as an anchor medication, nevertheless. Recent phase III trials combining gemcitabine with oxaliplatin, bevacizumab, or cetuximab failed (23,24,25). When it was demonstrated that erlotinib added to gemcitabine extended survival rate relative to gemcitabine alone, it represented a minor advance (21,22,23,24). In a recent clinical trial result published in The New England Journal of Medicine in 2011, a newly combined regimen called FOLFIRINOX (combination of leucovorin, 5-fluorouracil, irinotecan and oxaliplatin) showed overall progressive survival by 4.3 months compared with group that was treated with gemcitabine group (26). Additionally, there was improvement in ECOG (Eastern Cooperative Oncology Group) performance status score. However, FOLFIRINOX therapy resulted in increased incidence of grade 3 or 4 neutropenia, thrombocytopenia, diarrhea and sensory neuropathy including grade 2 alopecia (26).
Figure 5: Overall and Progression-free survival reported by clinical trial (26)

1.4 Platelet-activating factor (PAF)

A biochemical component produced by active basophils that enabled platelets to aggregate was initially referred to as "platelet-activating factor" by Benveniste and colleagues in their seminal Journal of Experimental Medicine publication in 1972 (28). It was identified as a modulator of various physiological and pathological processes involving phosphoglycerylether lipids (28). Later several groups discovered the role of PAF in mediating various biological and pathological activities (28,29,30). Notably, studies, including ours, have demonstrated that a range of immune and non-immune cells may produce PAF lipids from membrane glycerophosphocholine (GPCs) moieties by both enzymatic and non-enzymatic means (29,30). In addition to activating platelets, PAF also has numerous other functions in physiological processes such as hemostasis, inflammation, and several aspects of reproduction. PAF increases vascular leakage in living organisms, along with hypotension, lower cardiac output, acceleration of uterine contraction, digestive disorders, acute vascular constriction, and leukocyte adhesion to endothelial cells (30).
PAF has been identified in sepsis patients, and PAF receptor (PAF-R) antagonists significantly reduce the severity of sepsis in animal models (30,31).

![PAF's chemical composition and the PAF-R schematic structure](image)

**Figure 6: PAF's chemical composition and the PAF-R schematic structure**

Importantly, numerous studies, including ours, have suggested that PAF lipids produced by chemotherapy and radiation therapy (also known as PAFR agonists) may influence tumor behavior and/or anti-tumor immune responses in a variety of cancer models (32,66,67)). The investigation of PAF-PAFR signaling in the development of cancer was justified by the identification of many malignant cells that express PAFR and its capacity to trigger strong systemic pro-inflammatory, pro-proliferative, and delayed immune suppressive responses that were implicated in various pathological conditions (32,33,34,35,36).

According to several studies, mitogen-activated protein kinase (MAPK) signaling promotes pancreatic cancer cell growth and carcinogenesis when PAF is overexpressed. Additionally, it has been demonstrated that ectopic PAF can result in the activation of the mammalian target of rapamycin activator 3 complex pathway, which activates the late endosomes/lysosomes adaptor, MAPK, and the phospholipid-regulating mitogen-activated protein kinase signaling pathway, leading to neoplasia in pancreatic cancer (37).
1.5 PAFR and Pancreatic Cancer

Similar to other human malignancies, pancreatic cancer has a poor prognosis, is challenging to cure, and has a high fatality rate in the United States (7,8,9). It has been demonstrated that numerous cell types, notably tumor cells, produce oxidized lipids with PAFR agonist activity when exposed to pro-oxidative stressors, including therapeutic treatments (32,68). The PAFR activation is likely crucial for the etiology of many diseases, including the progression of cancer (32,68). Activation of the PAFR has been demonstrated to have impacts in a variety of human cancer models, but not much is known about how it affects pancreatic cancer models (47,48,65). A study carried out by our team demonstrated that chemotherapeutic agents like gemcitabine modifies the MAPK pathway to cause MVP generation in a PAFR-dependent manner in pancreatic cancer cells (47). According to Jun et al., studies published in 2013 suggested that ectopic PAF stimulation of the MAPK signaling is led by the activation of the LAM TOR3 pathway, which led to tumorigenesis in pancreatic cancer (88). Following that, a study published in 2018 by Lou et al., which showed that the mainstay treatment for pancreatic cancer since 1990 known as gemcitabine, currently overshadowed by FOLFIRINOX, activates PAFR and subsequently increases the phosphorylated NF-κB/p65 expression, and upregulates NF-κB activity in pancreatic cancer in vitro model (89). These findings suggested that PAFR activation in pancreatic cancer can modulate the tumor microenvironment and lead to the chemoresistance, including limiting the chemotherapeutic efficacy of the therapeutic interventions (47,88,89).
1.6 Microvesicle Particles (MVP)

The mechanisms by which cell(s) send signals to other cell(s) in another organ (i.e., cell-to-cell interaction) have remained relatively unknown in the past few decades, although crosstalk between assorted signaling pathways that modulate cancer growth and/or treatment efficacy has been intensively investigated. Although the discharge of extracellular vesicles (EV) or oncosomes into the environment by numerous high or confrontational forms of human cancer cells is now widely acknowledged as a feature of tumor biology, our understanding of how these structures are produced and their significance in the progression of cancer is still in its infancy (38,39,40). In particular, extracellular vesicles called microvesicle particles (MVPs) are tiny, membrane-enclosed entities that circulate throughout the body and contain a variety of chemicals (41,42). Microvesicles are directly produced from the plasma membrane and range in size from 200 to more than 1,000 nm (40,41). They can have different forms - directly derived from the parent cell's plasma membrane, microvesicles circulate throughout the body in sacs that are surrounded by membranes before releasing either their extracellular or intracellular contents (41,42,43).

When MVP were discovered in spleen nodules as well as lymph nodes of a male patient having Hodgkin disease, they were the first evidence of their presence in cancer patients in 1978 (41). Depicted in Figure 7, recent research suggests that MVP comprise bioactive molecules, including lipids, proteins, mRNAs, miRNAs, and nucleic acids, which are highly dependent on the cell types from which they originate and/or in response to various stimuli (38,39,40,41). Our group's prior research has shown that a variety of cell types can release MVP in response to ROS-generating stimuli, such as chemotherapy, in a PAFR-dependent manner that involves the enzyme acid sphingomyelinase (aSMase) (47,48,73). Importantly, PAFR antagonists and the aSMase-specific
inhibitor imipramine can both prevent the release of MVP in response to such stimuli (44,45,46,47,48).

Figure 7: Numerous facets of cancer growth influenced by tumor-derived microvesicles (49)
1.7 Hypothesis:

Given that the components of FOLFIRINOX therapy act as a pro-oxidative stressor and its associated adverse effects could be due to the epithelial cell cytotoxicity, we anticipate that the activation of PAFR and aSMase pathways could be involved in modulating FOLFIRINOX effects. We proposed two specific aims to test our hypothesis depicted in Figure 8 that PAFR expression modulates the cytotoxic effects of FOLFIRINOX therapy, and induces MVP release via stimulating aSMase enzyme in pancreatic cancer cell lines, as well as human and murine skin.

Figure 8: Anticipated hypothesis behind the current project

**Specific Aim 1:** To determine the roles of PAFR and aSMase signaling in FOLFIRINOX-induced MVP release in pancreatic cancer cell lines.
**Specific Aim 2:** To determine the effects of topical FOLFIRINOX treatment on MVP release in murine and human skin.
CHAPTER-2 METHODS AND MATERIALS

2.1 Reagents
The culture media was procured from GE Healthcare Biosciences (Marlborough, USA). Fetal bovine serum (FBS) was from Corning (Corning, NY, USA), antibiotic-antimycotic was from Gibco (Gaithersburg, MD, USA), and penicillin-streptomycin was purchased from Hyclone (Logan, UT, USA). The PAF-receptor agonist (Carbamyl PAF [CPAF]), PAF-receptor antagonist (WEB 2086), Scrambled siRNA control, as well as the inhibitor of acid sphingomyelinase (aSMase) enzyme imipramine were all acquired from Cayman Chemicals Co. (Ann Arbor, MI, USA). PAFR siRNAs clones (FlexiTube) were from Qiagen (Germantown, MD, USA) and lipofectamine 3000 was from Invitrogen (Thermo Fisher Scientific, Carlsbad, CA, USA). 5-fluorouracil was acquired from Sigma Aldrich (St. Louis, MO, USA) while oxaliplatin was purchased from Combi Blocks (San Diego, CA, USA). Similarly, irinotecan hydrochloride Trihydrate was purchased from TCI (Portland, OR, USA) and leucovorin calcium (Folinic acid) was obtained from Sigma Aldrich (St. Louis, MO, USA).

2.2 Cell Culture
Murine pancreatic cancer cell lines 6608 PDA (PAFR-expressing) and PancO2 (PAFR-deficient) were used as primary cell lines in this research project. Additionally, human pancreatic cancer cell line Panc-1, which expresses endogenous PAFR was used as a secondary cell line in this research study. The 6608 PDA cells were cultured in RPMI-1640 medium that contains 10% FBS, 2.5 mL antibiotic/antimycotic, 2.5 mL penicillin/streptomycin, 2.25 mL of 40% glucose, and 5 mL of 100 mM sodium pyruvate in a 500 mL media. On the other hand, PancO2 and Panc-1 cell lines were cultured in DMEM media that was enriched with 10% fetal bovine serum and a 100 µg/mL
combination of penicillin and streptomycin. These three cell lines were cultured in an incubator that was maintained at 37°C with 5% CO₂ and 95% humidity.

2.3  Human Skin

The Human skin was obtained by contouring procedures using anonymously discarded abdominoplasties and brachioplasties. Skin was cleaned, fat removed, and cut into 2x2 cm pieces, and then put in PBS-containing dishes. Three different components of FOLFIRINOX therapy excluding folinic acid (i.e. 5-fluorouracil (79, 80, 81), oxaliplatin (82, 83, 84) and irinotecan hydrochloride (85, 86, 87)) at different concentrations with reference to doses as indicated in the cited literature was applied topically to the dorsal skin samples. EtOH (0.1%) was used as a vehicle control. Positive controls included PMA and CPAF treatments at 100 µM concentration. Skin explants were pretreated with WEB2086, a PAFR antagonist (50 µM), or imipramine, a selective aSMase inhibitor (500 µM) for 1 hour followed by the treatments with CPAF (100 µM) and PMA (100 µM) at the above given dose, 5-fluorouracil (6 µg/ml) and oxaliplatin (5 mg/kg) to ascertain the roles of the PAFR and aSMase.

2.4  Mice Skin

The PAFR-deficient (PAFR-KO; Ptafr⁻/⁻) mice on a C57BL/6J background were a generous gift from Professor Takao Shimizu (Department of Biochemistry, University of Tokyo), and PAFR-expressing C57BL/6J wildtype (WT) mice were produced by inbreeding. Dr. Irina Petr-ache’s lab at National Jewish Medical Center (Denver, Colorado, USA) provided the Smpd1⁺/- heterozygous mice, which were originally from Dr. Edward Schuchman's lab. The heterozygous littermates of the aSMase KO (Smpd⁻/-) mice were mated with the mutant mice. All mice were kept in the animal care and use facility at Wright State University under particular pathogen-free conditions, kept on
a 12-hour light/dark cycle, and given standard animal food and water *ad libitum*. They were utilized between 7- and 10-weeks age. The mice were euthanized by CO₂ asphyxiation and cervical dislocation, and the dorsal skin was removed. Multiple treatments like EtOH, components of FOLFIRINOX regimen, excluding folinic acid, PAFR agonist CPAF, and PMA were then topically applied at the given doses, and the mice skin tissues were then kept in a water bath at 37°C to incubate for 4 hours. Skin samples were pretreated with WEB2086, a PAFR antagonist, or imipramine, a selective aSMase inhibitor, and then treated with three different components of FOLFIRINOX excluding folinic acid, CPAF, and PMA, followed by a 4-hour incubation period, to determine the roles of the PAFR and aSMase. The similarly designed experiments were carried out in *Ptafr*⁻/⁻ and *Smpd1*⁻/⁻ mice to confirm the involvement of the PAFR and aSMase.

### 2.5 Cell Survival Assay

Cell survival assay was examined by SRB (Sulforhodamine assay). A total of 3*10^3* pancreatic cancer cells per well were seeded overnight in a 96 well microtiter plate with 100 µL media. On the following day, the media was decanted out the 96 well microtiter plate. After that, all the wells were treated with components of FOLFIRINOX therapy that includes 5-fluorouracil (50,51,52), oxaliplatin (53,54,55), irinotecan hydrochloride (56,57,58) and folinic acid (59,60,61) at different concentration with reference to doses mentioned in different literatures. Cells treated with 0.1% DMSO served as control group and incubated for 2 different time points (i.e. 24 hours, and 48 hours). On the other hand, cell survival assay was done for three different time point i.e. 24 hours, 48 hours, and 72 hours for the human PAFR-expressing pancreatic cancer cell line, Panc-1.

After the allotted time point of treatment with FOLFIRINOX regimen, cells were monitored under the microscope and then fixed with 100 µl of 10% trichloroacetic acid (TCA) by using multi-
channel pipette and then placed at 4°C for 1 hour. After that 96 well plate was washed thrice with distilled water and subsequently, 100 µL 0.4% (weight/volume) SRB (dissolved in 1% acetic acid) was added for staining purpose and incubated in the dark condition for 15 minutes. After that the unbound dye was flicked off and the 96 well plate was washed with double distilled water containing 1% acetic acid. Finally, protein-bound dye was dissolved by 150 µL of 10 mM unbuffered Tris base [tris (hydroxymethyl)aminomethane] for 5-10 minutes on shaker. At the end, final absorbance was taken at 570 nm with SYNERGY H1 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) and the treated groups were normalized with the control group.

2.6 Assessment of Microvesicle particle release from Cells, Human Skin and Mice

A total of 0.3 million cells were plated in 6 well plates followed by addition of 2 mL of the respective culture media and kept further for incubation for 24 hours in an incubator maintained at 5% CO₂ and 95% humidity. On the following day, these cells were treated with three different components of FOLFIRINOX regimen (excluding folinic acid) with the IC₁₂₅, IC₂₅, and IC₅₀ concentrations that were resulted from cell survival assays (i.e. SRB assay) and incubated for 4 hours. The culture medium was taken after 4 hours, and cells and debris were removed by centrifugation at 2000 x g for 20 minutes. The isolated MVPs were obtained from the pelleted supernatant after centrifugation at 20,000 x g for 70 minutes. The Nano Sight NS300 device (Nano Sight Ltd.) was used to measure the MVP concentration, which was expressed as the number of MVPs per million cells. Similar protocol was used for the assessment of MVP release from the human and mice skin. Baseline MVP release varies between 10⁸ and 10⁹ from vehicle (control) treatment on murine and human skin and pancreatic cancer cell lines per analyzed dilution of sample.
2.7 siRNA Transfection

0.3 million PAFR expressing cells were plated in a 6 well plate and seeded overnight in CO\textsubscript{2} incubator maintained at 37°C with 5% CO\textsubscript{2} and 95% humidity. On the next day, the cells were washed thrice with PBS and transfected using scramble RNA or PAFR-specific siRNA and were supplemented with 2 mL of FBS-containing RPMI 1640 media. After 48 hours of incubation, the media was removed, and the cells were again washed thrice with 1 mL of HBSS and 500 µL of 1% BSA was added in each well. Finally, cells were treated with 100 nM CPAF, 100 nM PMA, and IC\textsubscript{50} dose of 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride resulted from cell survival – SRB assay and incubated for 4-hour time point.

After 4 hours of treatment with various agents, the supernatants were extracted, and cells and debris were removed by centrifugation at 2000 g for 20 min. The isolated MVPs were obtained from the pellets after centrifuging the supernatants at 20,000 g for 70 minutes. A Nano Sight NS300 device (Nano Sight Ltd.) was used to measure the MVP concentration, which was expressed as the number of MVPs per million cells. Baseline MVP release varies between 10\textsuperscript{8} and 10\textsuperscript{9} from vehicle (control) for the PAFR expressing 6608 PDA cell line.

2.8 Statistical Analysis

The statistical analysis was evaluated using GraphPad Prism version 7.0. (GraphPad Software, San Diego, CA, USA). The one-way ANOVA with post hoc Tukey multiple comparison test was used to examine the data. It was considered that a statistically significant difference between the two groups existed when the p-value was less than 0.05.
CHAPTER 3 - RESULTS

3.1 Specific Aim 1: To determine the roles of PAFR and aSMase signaling in FOLFIRINOX-induced MVP release in pancreatic cancer cell lines.

3.1.1 FOLFIRINOX Regimen (excluding folinic acid) decrease the survival rate in murine and pancreatic cancer cell lines in a time- and dose-dependent manner

Murine pancreatic cancer cell line 6608 PDA and PancO2 were treated with various concentration of FOLFIRINOX regimen i.e. 5-flurouracil, oxaliplatin, irinotecan hydrochloride, and folinic acid (leucovorin) at two different time points (i.e. 24 hours and 48 hours). SRB staining assay was performed to determine the cell viability. The cell viability in both the cell lines showed a decreasing trend with increasing concentrations of 5-flurouracil, oxaliplatin and irinotecan hydrochloride. The half-maximal inhibitory concentration (IC$_{50}$) of 5-flurouracil, oxaliplatin, and irinotecan hydrochloride was found to be 25 µM, 50 µM, and 100µM at 48 hours (Figure 9A-D) in PAFR expressing murine pancreatic cancer 6608 PDA cell line while the IC$_{50}$ of 5-flurouracil, oxaliplatin, and irinotecan hydrochloride was found to be 12.5 µM, 12.5 µM, and 50 µM at 48 hours in the PAFR-deficient murine pancreatic cancer PancO2 cell line (Figure 9E-H). However, we did not notice any significant decrease in cell viability in both murine pancreatic cancer cell lines treated with folinic acid (leucovorin). Furthermore, our study showed that higher concentrations of 5-flurouracil, oxaliplatin, and irinotecan hydrochloride are required to achieve IC$_{50}$ in PAFR-expressing 6606 PDA cell line as compared to PAFR deficient murine pancreatic cancer PancO2 cell line.

Additionally, the IC$_{50}$ of cell survival for 5-flurouracil was found to be 50 µM at 72 hours in PAFR expressing human Panc-1 pancreatic cancer cell line (Figure 9I). The Panc-1 cells showed a similar trend (i.e. decreased cell viability with increasing concentration of 5-flurouracil).
IC50 = 25 µM at 48 Hrs.

IC50 = 50 µM at 48 Hrs.

IC50 = 100 µM at 48 Hrs.
IC50 = 50 µM at 48 Hrs.

IC50 = 12.5 µM at 48 Hrs.
Figure 9: Effect of FOLFIRINOX regimen on cell survival of human and murine pancreatic cell line.

(A, B, C and D) 6608 PDA cells were treated with either vehicle (0.1% DMSO) or different concentrations of FOLFIRINOX regimen for 24 hrs. and 48 hrs. (E, F, G and H) PancO2 cells were treated with either vehicle (0.1% DMSO) or different concentrations of FOLFIRINOX regimen for 24 hrs- and 48 hrs. (I) Human pancreatic cell line Panc-1 was treated with either vehicle (0.1% DMSO) or different concentrations of 5-fluorouracil and incubated for 24 hrs-, 48 hrs- and 72 hrs. The cell survival was measured following incubation using a sulforhodamine-B assay. Data are expressed as the mean ± SE of three independent experiments and are presented as the percent survival against FOLFIRINOX treatments. DMSO (dimethyl sulfoxide).

3.1.2 FOLFIRINOX treatment stimulates MVP release from PAFR-expressing cell lines in a process blocked by PAFR antagonist and acid sphingomyelinase inhibitor

After optimizing the different components of the FOLFIRINOX regimen in PAFR-expressing and PAFR-deficient pancreatic cancer cell lines, our next study determined the effect of PAFR activation in FOLFIRINOX components’ mediated MVP release (excluding folinic acid). To that end, we treated PAFR expressing 6608 PDA and Panc-1 cell line using IC12.5, IC25, and IC50 doses of FOLFIRINOX component. Regarding this experiment, cells treated with 0.1% ethanol vehicle were considered as negative control whereas cells treated with CPAF and PMA were considered as positive control. As a result, we noticed that components of FOLFIRINOX regimen induce
MVP release from PAFR-expressing cell lines in a concentration-dependent manner (Figure 10A-D). Similarly, the cells treated with positive control CPAF and PMA (phorbol 12-myristate 13-acetate, a PAFR-independent agonist) showed an increased level of MVP release from the PAFR-expressing cell lines (Figure 10A-D). In a separate experiment, PAFR-expressing cell lines were pre-treated with the PAFR antagonist WEB2086, and inhibitor of acid sphingomyelilase (aSMase) enzyme, imipramine, for 1 hour and then treated with components of FOLFIRINOX regimen. We noticed that the induced MVP release through these FOLFIRINOX components was blocked significantly by the PAFR antagonist and imipramine (Figure 10A-D). These results suggest that the PAFR may be involved in MVP release caused by FOLFIRINOX components. Additionally, our results demonstrating that PAFR antagonist inhibits MVP release induced by FOLFIRINOX components indicate that PAFR activation is one of the potential factors that contributes to MVP release from PAFR-expressing cells. More importantly, the suppression of MVP by imipramine indicates the role of an aSMase enzyme in PAFR-dependent MVP release by FOLFIRINOX.

A.
Figure 10: Effects of FOLFIRINOX regimen on microvesicle particle (MVP) secretion on PAF-R expressing cell line
(A, B and C) 6608 PDA cells were treated with vehicle as control (0.1% EtOH or DMSO), PAFR agonist (CPAF, 100 nM), phorbol 12-myristate 13-acetate (PMA, 100 nM), 5-fluorouracil (6.25 µM, 12.5 µM, 25 µM), oxaliplatin (12.5 µM, 25 µM, 50 µM) and irinotecan hydrochloride (25 µM, 50 µM and 100 µM). (D) Panc-1 cells were treated with vehicle as control (0.1% EtOH or DMSO), phorbol 12-myristate 13-acetate (PMA 100 nM), PAFR agonist (CPAF 100 nM) and 5-fluorouracil (12.5 µM, 25 µM, 50 µM). In a separate experiment, 6608 PDA and Panc-1 cells were pretreated with WEB2086 (10 µM) or imipramine (IMI, 20 µM) for 1 hour followed by treatment with CPAF and PMA (100 nM each), IC50 dose of 5-fluorouracil, oxaliplatin and irinotecan hydrochloride. After 4 hours, MVP were isolated and analyzed as described in method section. Data are representative of mean ± SE of three independent experiments. The statistically significant differences were observed between the control group with CPAF, PMA, 5-fluorouracil, oxaliplatin and irinotecan hydrochloride treated groups (*= p<0.05, **= p<0.005, ***= p<0.001, ****= p<0.0001) and 5-fluorouracil, oxaliplatin and irinotecan hydrochloride treated group with CPAF, PMA, WEB and imipramine ($= p<0.05, @= p<0.05, \lambda= p<0.005$ and $#= p<0.05$).

3.1.3 FOLFIRINOX regimen is incapable of generating microvesicle particle in PAF-R deficient cell lines.

For the purpose of determining the role of PAFR activation in FOLFIRINOX induced MVP release, we utilized PAFR deficient murine pancreatic cancer cell line, PancO2. Similar to the PAFR positive cell line 6608 PDA, these cells were treated with the CPAF, PMA, and IC50 dose of 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride as resulted by the cell survival assay (Figure 9E-H). In a separate experiment, these PancO2 cells were pretreated with imipramine 20 µM and then treated with PMA (100 nM) as PMA was used as a positive (PAFR-independent) control for PancO2 cells. After 4 hours of incubation, the supernatants were collected and analyzed for MVP release. We found that only PMA, PAFR-independent agonist, showed significantly increased level of MVP release whereas there was no significant increase in MVP release in the CPAF, 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride treated groups (Figure 11A-C). Similarly, pretreatment with imipramine was able to block the PMA-induced MVP release. Additionally, our prior published research has shown that PAFR antagonist called WEB2086 is unable to block the MVP release that is induced by the treatment with the PAFR independent agonist called as PMA (47). This confirms that FOLFIRINOX regimen does not generate MVP in the PAFR deficient cell
lines, which suggest the involvement of the PAFR signaling in the generation of MVP by FOLFIRINOX regimen.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Figure 11: Effects of FOLFIRINOX regimen on microvesicle particle (MVP) secretion on PAFR deficient cell line**

(A, B and C) PancO2 cells were treated with vehicle as control (0.1% EtOH or DMSO), PAFR agonist (CPAF, 100 nM), phorbol 12-myristate 13-acetate (PMA, 100 nM), 5-fluorouracil (3.125 µM, 6.25 µM, 12.5 µM), oxaliplatin (3.125 µM, 6.25 µM, 12.5 µM) and irinotecan hydrochloride (12.5 µM, 25 µM and 50 µM). In a separate experiment, PancO2 cells were pretreated with imipramine (20 µM) for 1 hour followed by treatment PMA (100 nM). After 4 hours, MVP were isolated and analyzed as described in method section. Data are representative of mean ± SE of three independent experiment. The statistically significant differences were observed between the Ctrl-EtOH and PMA 100 nM (****= p<0.0001); PMA 100 nM vs
Imi + PMA 100 nM (\#= p<0.0001). The NS indicates no any significant difference in the microvesicle particle release compared with control group.

3.1.4 PAFR silencing by specific siRNA attenuates FOLFIRINOX regimen-induced MVP release in PAFR expressing cell line.

With the help of PAFR-specific siRNA, we silenced PAFR expression to determine if PAFR signaling is necessary for FOLFIRINOX-induced MVP release. To silence the PAFR gene, we transfected 6608 PDA cell line with Scr-ctrl or PAFR-specific siRNA and then treated with the IC\textsubscript{50} concentration of 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride. After 4 hours of incubation, supernatants were collected and MVPs were analyzed. We observed that CPAF, 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride-induced MVP release was significantly reduced when the PAFR was knocked down as opposed to when these treatments were used alone (Figure 12). In contrast to PMA alone or the vehicle control treatments in the Scr-ctrl, siRNA did not exert any significant effect on PMA-induced MVP release (Figure 12). These results show that the release of MVP by the components of FOLFIRINOX treatments requires PAFR signaling.

![Figure 12: Effect of the PAFR specific siRNA of FOLFIRINOX regimen induced MVP release on PAF-R expressing cell line.](image_url)
6608 PDA cells were transfected with scramble control or PAFR siRNA and after 4hrs. treated with 0.1% EtOH + DMSO as vehicle control, 100 nM CPAF, 100 nM PMA as positive controls, IC$_{50}$ dose of 5-fluorouracil, oxaliplatin and irinotecan hydrochloride (i.e. 5-fluorouracil 25 µM, oxaliplatin 50 µM and irinotecan hydrochloride 100 µM). After 4hrs of incubation, MVPs are isolated and analyzed as described in method section. Data are representative of mean ± SE of three independent experiment. The statistically significant differences were observed between the Ctrl-EtOH vs. CPAF 100 nM, PMA 100 nM, 5-Fu 25 µM, Oxa 25 µM, Irino 100 µM for the scrambled RNA transfected group (****= p<0.0001). The statistically significant differences were observed between the Ctrl-EtOH vs PMA 100 nM for the PAFR siRNA transfected group (****= p<0.0001). The NS indicates no any significant difference in the MVP release compared with control group for the PAFR siRNA transfected group.
3.2 Specific Aim 2: To test the effects of topical FOLFIRINOX treatment on MVP release in murine and human skin.

3.2.1 Topical FOLFIRINOX (excluding folinic acid) treatment generate MVP release in murine skin and human skin.

In addition to inducing cytotoxic effects on tumor cells, FOLFIRINOX therapy has been shown to cause deleterious effects on normal tissues of epithelial origin (74, 75, 76). Thus, to determine the roles of the PAFR and aSMase enzyme in FOLFIRINOX-mediated effects on epithelial cells, we employed human and murine skin explants as models of epithelial origin. To that end, 5-fluorouracil (3 µg/ml, 6 µg/ml and 12 µg/ml), oxaliplatin (2.5 mg/kg, 5 mg/kg and 7.5 mg/kg) and irinotecan hydrochloride (5 mg/kg, 10 mg/kg, 20 mg/kg) was applied topically on the murine and human skin explants (79, 80, 81, 82, 83, 84, 85, 86, 87). Murine and human skin treated with ethanol 0.1% served as a negative vehicle control whereas murine and human skin treated with PAFR agonist CPAF (100 µM) and PAFR independent agonist PMA (100 µM) served as positive controls for this experiment. After 4 hours incubation, 6mm skin punch biopsies were collected, and the samples were then prepared for MVP extraction and analysis as described in the material and method section. Our studies demonstrate that FOLFIRINOX treatment induces MVP release in a dose-dependent pattern when compared to vehicle control treatment (Figure 13A-C). Similar increase in MVP release was demonstrated in the murine and human skin explant samples that were treated with CPAF and PMA (Figure 14D-E).

To identify the mechanism, we then conducted a separate experiment where we pretreated murine and human skin explants with a specific antagonist of PAFR called WEB2086 (50 µM) and specific inhibitor of aSMase enzyme (imipramine, 500 µM) for 1 hour and then treated with CPAF, PMA, 5-fluorouracil 6 µg/ml, oxaliplatin 5 mg/kg, and irinotecan hydrochloride 10 mg/kg for 4 hours. Following the MVP analysis, we observed that WEB2086 and imipramine blocked MVP
release induced by CPAF, PMA, 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride (Figure 13A-C). However, PMA-induced MVP release was unaltered by the WEB2086 compound (Figure 13A-C).

Overall, the findings we observed was that the PAFR antagonist inhibits MVP release induced by FOLFIRINOX components. These findings indicate that PAFR signaling may be playing an important role in this event. Additionally, blockade of FOLFIRINOX-induced MVP release by imipramine, an inhibitor of aSMase enzyme (Figure 13A-C), suggest that regardless of type of stimuli employed, inhibition of aSMase activity prevents MVP release, demonstrating involvement of the aSMase enzyme.
Figure 13: Effect of topical FOLFIRINOX regimen on MVP release in WT murine skin

(A, B and C) Wild-type murine skin (six mice per group) was treated topically with 0.1%EtOH as vehicle control, CPAF 100 µM, PMA 100 µM, 5-fluorouracil (3 µg/ml, 6 µg/ml and 12 µg/ml), oxaliplatin (2.5 mg/kg, 5 mg/kg and 7.5 mg/kg) and irinotecan hydrochloride (5 mg/kg, 10 mg/kg, 20 mg/kg) and then incubated for 4 hrs. In a separate experiment, murine skin was pretreated with WEB 2086 (50 µM) and imipramine (IMI, 500 µM) for 1 hr. and then treated with 5-fluorouracil 6µg/ml, oxaliplatin 5 mg/kg and irinotecan hydrochloride 10 mg/kg and incubated for 4 hrs. Following incubation, fat was removed and 6 mm punch biopsies was taken (2 were collected for each treatment or group) then sections were cut into minute pieces, and collagenase/dispase (5 mg/ml) was added for digestion for overnight at 37°C. As described in the technique section, MVPs were separated and extracted. Data are representative of mean ± SE of three independent experiment. The statistically significant differences were observed between the control group with CPAF, PMA, 5-fluorouracil, oxaliplatin and irinotecan hydrochloride treated groups (***= p<0.002, ****= p<0.0001) and 5-fluorouracil, oxaliplatin and irinotecan hydrochloride treated group with CPAF, PMA, WEB and imipramine ($= p<0.0001, @= p<0.0001, and #= p<0.0001)
Figure 14: Effect of topical FOLFIRINOX regimen (5-FU and Oxaliplatin) on MVP release in Human skin explants.

(D and E) Human skin was treated topically with 0.1% EtOH as vehicle control, CPAF 100 µM, PMA 100 µM, 5-fluorouracil (3 µg/ml, 6 µg/ml and 12 µg/ml; two biopsies per treatment groups from 3 human skin) and oxaliplatin (2.5 mg/kg, 5 mg/kg and 7.5 mg/kg; two biopsies per treatment groups from 2 human skin) and then incubated for 4 hrs. In a separate experiment, human skin was pretreated with WEB2086 (50 µM) and imipramine (IMI, 500 µM) for 1 hr. and then treated with 5-fluorouracil 6µg/ml and oxaliplatin 5mg/kg and incubated for 4 hrs. Following incubation, fat was removed and 6 mm punch biopsies was taken then sections were cut into minute pieces, and collagenase/dispase (5 mg/ml) was added for digestion for overnight at 37°C. As described in the technique section, MVPs were separated and extracted as described in method section. Data are representative of mean ± SE of three and two independent experiment for 5-
fluorouracil and oxaliplatin respectively. The statistically significant differences were observed between the control group with CPAF, PMA, 5-fluorouracil and oxaliplatin treated groups (*= p<0.05, **= p<0.005, ***= p<0.001, ****= p<0.0001) and 5-fluorouracil and oxaliplatin treated group with CPAF, PMA, WEB and imipramine ($= p<0.05, @= p<0.05, \lambda = p<0.005$ and #= p<0.05)

3.2.2 Topical FOLFIRINOX regimen (excluding Folinic acid)-induced MVP release in murine skin is suppressed by PAFR blockade.

Our next studies took advantage of the PAFR-deficient \((Ptafir^{-/-})\) mice to further characterize the involvement of the PAFR in FOLFIRINOX-induced MVP release. For this purpose, we applied 0.1% EtOH, CPAF, PMA, 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride topically on the dorsal skin of \(Ptafir^{-/-}\)mice and incubated the samples for 4 hours. Skin punch biopsies were taken and prepared for MVP extraction and analysis as described above. We noticed that only the samples treated with PMA resulted in increased MVP generation whereas we did not notice any significant increase in MVP generation from the skin samples that were treated with CPAF and components of FOLFIRINOX regimen i.e., 5-fluorouracil, oxaliplatin and irinotecan hydrochloride (Figure 15A-C). These findings suggest that PAFR signaling plays a critical role in the generation of MVPs by FOLFIRINOX components.
The dorsal skin section of PAFR knock out mice (six mice per groups) was topically treated with EtOH 0.1% serving as a control treatment, CPAF (100 µM), PMA (100 µM), 5-fluorouracil (6 µg/ml), oxaliplatin (5 mg/kg) and irinotecan hydrochloride (10 mg/kg). Following incubation of 4 hrs, two punch biopsies per each treatment was taken followed by removal of layer of fat and chopped into fine pieces in collagenase dispase (5 mg/ml) and kept in a shaker for 24 hrs. for the purpose of digesting of finely chopped skin. Following 24hrs. time period, samples were processed further for MVP analysis as described in method section. Data are expressed as the mean ± SE and statistically significant differences were observed between control and PMA (**** = p<0.0001).

3.2.3 Topical FOLFIRINOX regimen (excluding Folinic acid)-induced MVP release in murine skin is suppressed by aSMase enzyme.

Our next studies taking advantage of the aSMase enzyme deficient (Smpd1−/−) mice further characterized the involvement of this enzyme in FOLFIRINOX-induced MVP release. For this purpose, we applied 0.1% EtOH, CPAF, PMA, C-2 Ceramide (20 µM), 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride at given doses topically on the dorsal skin of Smpd1−/− mice and incubated for 4 hours. Skin punch biopsies were taken and prepared for MVP extraction and evaluation as previously described. We noticed that only samples treated with an aSMase enzymatic reaction's byproduct called C-2 Ceramide resulted in increased MVP generation.

Figure 15: Effect of topical FOLFIRINOX regimen on MVP release from PAFR KO murine skin

(A, B and C) The dorsal skin section of PAFR knock out mice (six mice per groups) was topically treated with EtOH 0.1% serving as a control treatment, CPAF (100 µM), PMA (100 µM), 5-fluorouracil (6 µg/ml), oxaliplatin (5 mg/kg) and irinotecan hydrochloride (10 mg/kg). Following incubation of 4 hrs, two punch biopsies per each treatment was taken followed by removal of layer of fat and chopped into fine pieces in collagenase dispase (5 mg/ml) and kept in a shaker for 24 hrs. for the purpose of digesting of finely chopped skin. Following 24hrs. time period, samples were processed further for MVP analysis as described in method section. Data are expressed as the mean ± SE and statistically significant differences were observed between control and PMA (**** = p<0.0001).
whereas we did not notice any significant increase in MVP generation from the skin samples that were treated with CPAF, PMA, and components of FOLFIRINOX regimen (i.e., 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride) (Figure 16A-C). These findings suggest that aSMase, an enzyme required for the biogenesis of MVP, plays a critical role in the generation of MVP by three components of FOLFIRINOX regimen.

Figure 16: Effect of topical FOLFIRINOX regimen on MVP release from acid sphingomyelinase deficient murine skin
(A, B and C) The dorsal skin section of Smpd1−/− mice (six mice per group) was topically treated with 0.1% EtOH serving as a control treatment, CPAF (100 µM), PMA (100 µM), C-2 ceramide (20 µM), 5-fluorouracil (6 µg/ml), oxaliplatin (5 mg/kg) and irinotecan hydrochloride (10 mg/kg). Following incubation of 4 hrs., two punch biopsies per each treatment was taken followed by removing of layer of fat and chopped into fine pieces in collagenase dispase (5 mg/ml) and kept in a shaker for 24 hrs. for the purpose of digesting of finely chopped skin. Following 24hrs. time period, samples were processed further for MVP analysis as described in method section. Data are expressed as the mean ± SE and statistically significant differences were observed between control and C-2 ceramide (**** = p<0.0001).
CHAPTER 4 – DISCUSSION

The bioactive phospholipid termed platelet activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is crucial in inflammatory and immunological disorders (62,63,64). PAF binds to the G protein-coupled, PAFR, which is found on a variety of immune cells, including mast cells and basophils (63,64). Researchers have proposed that, despite the fact that PAF mediates chronic inflammation in response to allergens and infectious events, PAF-mediated physiological effects can turn pathogenic event with excessive activity or dysregulation (62,63,64). Besides, PAFR activation plays an important role in several cancer models (65,66). Several studies, including ours, have shown that PAFR agonists are produced from experimental cell lines in response to chemotherapeutic drugs and these chemotherapeutic drugs-induced PAFR agonists favor the growth of cancer tumors in a PAFR dependent pattern (65,66). Highlighting the few contributions of the PAF in tumor microenvironment, a study has shown that through the activation of the PAF/PAFR signaling pathway, high PAF concentrations in the tumor microenvironment can encourage ovarian cancer cells to be stem cells both in vitro and in vivo models through the activation, phosphorylation and increased expression of CK2 (Casein Kinase 2), which ultimately leads to the activation of NF-κB (67). A study highlighting the potential role of PAF suggested that PAF-like molecules produced by radiotherapy can act on cancer cells to protect them from radiation-induced apoptosis and favor tumor development through inducing immunosuppression by regulating macrophages differentiation (68). Since most of the chemotherapeutic drugs are considered as pro-oxidative stressor due to their ability to induce the generation of ROS resulting in release of bioactive molecules called MVPs which are enriched with different bioactive components, including DNA, RNA lipids and proteins (47,48).
A newly combined regimen for the treatment of pancreatic cancer called FOLFIRINOX (a combination of leucovorin, 5-fluorouracil, irinotecan and oxaliplatin) showed overall improved survival by 4.3 months and improved ECOG (Eastern Cooperative Oncology Group) performance status score compared with the group that was treated with gemcitabine alone (26). However, FOLFIRINOX therapy resulted in increased incidence of grade 3 or 4 neutropenia, thrombocytopenia, diarrhea, sensory neuropathy, including grade 2 alopecia and other side effects (26,69,70). These different chemotherapeutic agents in FOLFIRINOX regimen are considered as pro-oxidative stressors (90,91,92,93,94,95), and our studies have shown that such pro-oxidative stressors can generate MVP that have potential to exhibit several properties such as contributing to the chemoresistance and mediated pathophysiological conditions, including negatively affecting the efficacy of cancer drugs (49,71,72).

Firstly, using murine and human pancreatic cancer cell lines, we have shown that components of FOLFIRINOX regimen (excluding folinic acid) exert a significant decrease in the pancreatic cancer cell viability at two different time points. However, we did not notice any substantial decrease in pancreatic cancer cell viability by the folinic acid in both PAFR-expressing and PAFR-deficient cell lines. A study published in 2017 by Wang et al. reported that there is no inherent cytotoxic action of folinic acid and that tetrahydrofolic acid's 5-formyl derivative, folinic acid, is metabolized to other diminished folic acid derivatives, such as tetrahydrofolate, and as a result, leucovorin has vitamin activity comparable to that of folic acid (60). Similarly, another study reported the effectiveness of 5-fluorouracil is increased by using folinic acid to raise the intracellular concentration of 5,10-CH2FH4, despite the fact that leucovorin itself has no anticancer activity (61).
Secondly, using PAFR expressing murine and human pancreatic cell lines, we found that PAFR expression is required for the generation of MVP by the components of FOLFIRINOX regimen. Our hypothesis was that components of FOLFIRINOX regimen (excluding folinic acid), through its pro-oxidative stressor ability, would generate PAFR agonists and activate PAFR, which would subsequently stimulate the aSMase enzyme to induce MVPs that carry PAFR agonists. To that approach, we treated PAFR-positive and PAFR-deficient cell lines with the half maximal inhibitory concentration (IC$_{50}$) doses of 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride as revealed by our cell viability assay. As a result of the MVP analysis, we found that these components of FOLFIRINOX regimen generate MVP from the PAFR-expressing cell lines in a dose dependent pattern. However, we did not notice any increase in the MVP release from the PAFR deficient cell line. These results were consistent with our previous studies that was done with another chemotherapeutic agent called gemcitabine (47). With proven evidence that PAFR plays a critical role in mediating the effects of numerous stimuli, we further assessed the crucial role of the PAFR and aSMase enzyme in FOLFIRINOX components induced MVP release. We performed this experiment using pharmacological tools that are readily accessible, including the well-known PAFR antagonist, WEB2086 and the particular aSMase inhibitor, imipramine. Our studies demonstrated that pretreatment with WEB2086 and imipramine significantly blocked the MVP release induced by the components of FOLFIRINOX regimen (excluding folinic acid). These results are aligned to our previous studies highlighting the role of PAFR and aSMase in MVP release phenomenon (47,48,73). The fact that WEB2086 could block CPAF-mediated MVP release but not PMA-mediated MVP release demonstrated the importance of the PAFR signaling in this event. Along similar lines, the ability of imipramine to block the release of MVP caused by CPAF and PMA as well as 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride further
supported the hypothesis that an aSMase enzyme is involved in MVP generation from PAFR-expressing cell lines. Similarly, there was no significant increase in the MVP by CPAF, 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride from PAFR-deficient cells confirming that PAFR signaling is required in MVP generation by FOLFIRINOX components. To further confirm the involvement of PAFR in the generation of MVP from these pancreatic cancer cell lines, our transfection studies using PAFR expressing cell line revealed that silencing PAFR with siRNA did not affect MVP generation with PMA treatment, but significantly decreased MVP release by the treatments with PAF-agonist CPAF and other components of FOLFIRINOX regimen.

Various side effects like allergic contact dermatitis and inflammatory colitis, dihydropyrimidine dehydrogenase deficiency, neurotoxicity, pulmonary toxicity, and diarrhea are caused by the topical application of 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride (74,75,76). Notably, our recently published research aimed to ascertain if MVP release could be involved in the pathologic effects of topical gemcitabine treatment (74,75,76,77,78). To that end, similar to our cell line studies, we hypothesized that components of FOLFIRINOX regimen via their pro-oxidative stressors’ ability will generate PAF agonists and activate the PAFR to stimulate MVP release via aSMase enzyme. Similar to our results we perceived in our cell lines study, we found that topical application of 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride induced MVP release in dose dependent pattern in PAFR-expressing wildtype mice. Subsequently, we found that these induced MVPs by the FOLFIRINOX components were blocked by the PAFR antagonist, WEB 2086 and aSMase inhibitor, imipramine. Moving further, we employed Ptafr−/− mice and Smpd1−/− mice to confirm the role of the PAFR-aSMase axis in the MVP generation from the topical application of FOLFIRINOX components. As a result, we noticed that only mice skin treated with PMA, PAFR independent agonist resulted in increased MVP generation whereas there was no
significant MVP release from the murine skin samples treated with CPAF, 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride. Simultaneously, only Smpd1<sup>−/−</sup> mice skin treated with a by-product aSMase enzyme known as C2 ceramide that bypasses aSMase-induced MVP generation, resulted in increased MVP release.

In summary, our results from this study done on pancreatic cancer cell lines suggest that components of FOLFIRINOX i.e. 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride induce MVP release in a process that is blocked by the PAFR antagonist and inhibitor of aSMase enzyme. These results were exactly same when done with topical application of 5-fluorouracil, oxaliplatin, and irinotecan hydrochloride in wild type mice skin. Considering that PAFR activation mediates both acute pro-inflammatory and delayed systemic immunosuppressive effects, these results suggest that topical FOLFIRINOX components-induced PAFR agonists-containing MVP release could be one of the causes involved in not only restricting the therapeutic efficacy, but may also be linked to FOLFIRINOX components-mediated cutaneous toxic effects. Overall, our animal studies suggest a working model by which topical application of FOLFIRINOX components can produce bioactive vesicles called MVP as a byproduct of a mechanism that depends on the PAFR-aSMase axis.
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