Treatment of Prostate Cancer with Fucoidan of Low Molecular Weights in Combination with Guanine-rich Oligonucleotide Therapy

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A thesis submitted to

Auckland University of Technology

in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

2017

School of Science

Treatment of Prostate Cancer with Fucoidan of Low Molecular Weights in Combination with Guanine-rich Oligonucleotide Therapy

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List of Abbreviations

EGFR/ErbB-1/HER1: The Epidermal Growth Factor Receptor

Ras/MAPK: Mitogen Activated Protein Kinase

FCSPs: Fucose-Containing Sulfated Polysaccharides

LMWF: Low-Molecular-Weight Fucoidan

SF: Sigma Fucoidan

MW: Molecular Weight

MMP: Mitochondrial Membrane Potential

Ras/Raf/MAPK pathway: Signal-Regulated Kinase (ERK) pathway

CDDP: Cis-platin

TAM: Tamoxifen

GRO/GroA/AS1411: Guanosine-Rich Oligonucleotide

MTT: Methylthiazol-diphenyl-tetrazolium

DMSO: Dimethyl Sulfoxide

EMEM: Eagle's Minimum Essential Medium

FBS: Fetal Bovine Serum

PDT: Cells Population Doubling Time

PI: Propidium Iodide

RNAse A: Ribonuclease A

DDW: Double Distilled Water

μM: Micromoles per liter (μmol/L)

RNAse: Ribonuclease

S.D: Stand Deviation

Log: Logarithm

IC_{50}: The half maximal inhibitory concentration

TRITC: Tetraethyl Rhodamine Isothiocyanate,

FITC: Fluorescein Isothiocyanate

GC: Guanine & Cytosine

ERK: Extracellular Regulated Protein Kinases

NK: Natural Killer

LLC: Lewis Lung Carcinoma

ECM: Extra Cellular Matrix

AIF: Apoptosis-Inducing Factor

FADD: Fas-Associated Death Domain

IG: Indukantha Ghritha

PDGFR: Platelet-Derived Growth Factor Receptor

VEGF: Vascular Endothelial Growth Factor

PBS: Phosphate Buffered Saline

ERK: Extracellular Regulated Kinase

Attestation of Authorship

I hereby declare that this submission is my own work and that, to be the best of my knowledge and belief, 'Different Molecular Weight Fucoidan in Combination with GroA Therapy in the Treatment of Prostate Cancer', contains no material previously published or written by another person (except where explicitly defined in the acknowledgements) nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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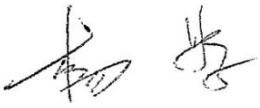
Signed: … 

Date: ……20-06-2017……..

Acknowledgements

First of all, I greatly appreciate my primary supervisor A/Prof. Jun Lu, for giving me the opportunity to work on this subject. He is knowledgeable and possesses great knowledge about anti-cancer research. In the course of my study, he has guided me patiently, giving me a lot of good advice, so that I could avoid making too many detours in my research. He gave me careful and valuable guidance for the data analysis and thesis writing. He played an important role in the accomplishment of my project.

I also need to express my thanks to my secondary supervisor Dr. Yan Li. He gave me a lot of technical guidance about cell culture, cell mechanism studies and helpful advice with the results review. I am thankful for the support I received from him.

Additionally, I need to thank Kelvin Wang who has helped our lab with our of flow cytometry equipment. He gave lots of technical guidance about flow cytometry to me, including the operation of the machine, sample preparation and data analysis. I would not be able to finish the cell apoptosis and cell cycle analysis part without his help and support. I also appreciate the help I received from the colleagues in my lab.

Special thanks should be given to Brid Lorigan and Sonya Popoff for helping me order all the experimental materials and supply a lot of other important lab stuffs. Also, I give many thanks to all staff members in the laboratories of the Faculty of Health and Environmental Sciences in Auckland University of Technology.

Finally, I would like to thank my parents and other family members for their support.

Abstract

Three oncogenes, ErbB receptors, Ras proteins and nucleolin, may contribute to malignant transformation of healthy cells. Previously, mutant Ras was shown to be capable of activating ErbB receptors in a ligand-independent manner. Moreover, nucleolin, a transcriptional regulator and ribosome biogenesis factor, could bind both K-Ras and the cytoplasmic tail of ErbB receptors to enhance ErbB receptor activation. Therefore, focus on ErbB receptors and nucleolin are important in improving cancer patient survival.

Fucoidan is one of the Fucose-Containing Sulfated Polysaccharides (FCSPs) isolated from various brown seaweeds which are known to possess essential bioactive properties, notably growth inhibitory effects on tumor cells. FCSPs also possess sulfated galactofucans, with backbones built of (1→6)- β -D-galacto- and/or (1→2)- β -D-mannopyranosyl units. In addition to sulfate groups these backbone residues may be substituted with fucosides, single fucose substitutions, and/or glucuronic acid, xylose or glucose substitutions. Fucoidan has remarkable biological activities, especially in terms of anticancer-promoting applications. Previous studies have shown that fucoidan mainly targets the EGFR (ErbB-1)-signaling pathway, which may contribute to fucoidan's observed chemo-preventive potential.

Nucleolin is a ubiquitously expressed acidic phosphoprotein that is involved in important aspects of cell proliferation and cell growth. It is localized primarily to the nucleoli, but it undergoes nuclear cytoplasmic shuttling and is also found on the cell surface of some types of cells. Cell surface nucleolin is found in a wide range of tumor cells, and it is used as a marker for cancer diagnosis. Ronit Pinkas-Kramarski's lab in Israel has identified non-nucleolar nucleolin as an ErbB receptor-interacting protein. This interaction leads to receptor dimerization and activation as well as to increased colony growth in soft agar. Recent studies have identified a crosstalk between nucleolin, ErbB1 and Ras proteins. Therefore, it seems that combining two drugs, Fucoidan (which inhibits ErbB1) and GroA/AS1411 (which specifically inhibits cell surface nucleolin) may have a better inhibitory effect on ErbB receptor activation and thereby a stronger inhibitory effect on cancer cell growth and tumorogenicity.

This study describes the effect of Low-molecular-weight fucoidan (LMWF) and Sigma Fucoidan (SF) used alone or combined with GroA on the proliferation of human

prostate cancer cell lines PC-3 and DU-145. LMWF and FS inhibited the growth of these cancer cells in a time- and dose- dependent manner in the present study, but the effect of SF was unsatisfied. Simultaneous combination of LMWF and GroA made an additive inhibitory effect on prostate cancer cell viability. In treatments using SF combined with GroA, SF was not able to enhance the inhibitory effect of GroA on proliferation of PC-3 and DU-145 cells in a concentration dependent manner. Even at very high concentrations, the results of combination SF and GroA treatments only yielded a GroA effect on cell viability. The results may suggest that LMWF can act as a synergistic anti-cancer agent in the treatment of prostate cancer cell lines DU-145 and PC-3, and has the potential to regulate MAPK activity. Combination of LMWF and GroA could have a better inhibitory effect on ErbB receptor activation, and subsequently, a stronger inhibitory effect on cancer cell growth and tumorogenicity. Cell apoptosis and cell cycle analysis conducted by using flow cytometry was also applied to study the mechanism of action of these two drugs. This study provides further evidence that LMWF can affect the cell cycle in sub-G1 phase and leads to cellular apoptosis in PC-3 and DU-145 prostate cancer cell lines.

In conclusion, LMWF is a promising natural substance that may be used in combination therapy against cancer, in particular those with ErbB overexpression, such as prostate cancer.

Chapter 1 Introduction

1.1 Background

The Epidermal Growth Factor Receptor (EGFR; ErbB-1; HER1) is a cell surface protein that binds to epidermal growth factor (Herbst, 2004). ErbB-1 is a member of the ErbB family of receptors, whose mutations have been identified as affecting ErbB-1 expression or activity and are associated with cancer (Zhang et al., 2007). Moreover, activation of ErbB receptors induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation. The Ras/MAPK (mitogen activated protein kinase) cascade is one of the most prominent signaling pathways activated by ErbB receptors. Activated Ras/MAPK can induce the release of growth factors including EGF-like ligands resulting in receptor phosphorylation via an autocrine or paracrine loop, and also induce ErbB phosphorylation in a ligand-independent manner. Therefore, targeting ErbB receptors activation can potentially reduce malignancy (Sebolt-Leopold & Herrera, 2004).

Fucoidan is one of the Fucose-Containing Sulfated Polysaccharides (FCSPs) isolated from various brown seaweeds which are known to possess essential bioactive properties, notably growth inhibitory effects on tumor cells. The molecules include sulfated galactofucans with backbones built of (1→6)-β-D-galacto- and/or (1→2)-β-D-mannopyranosyl units. In addition to sulfate these backbone residues may be substituted with fucosides, single fucose substitutions, and/or glucuronic acid, xylose or glucose substitutions (Ale, Mikkelsen, & Meyer, 2011; Duarte, Cardoso, Noseda, & Cerezo, 2001). FCSPs of different degrees of purity and composition, extracted from brown seaweeds such as Sargassum sp. and Fucus sp., have been documented to have a wide range of biological activities including anticoagulant (Nardella et al., 1996), antithrombotic (Blondin, Fischer, Boisson-Vidal, Kazatchkine, & Jozefonvicz, 1994; Jiao, Yu, Zhang, & Ewart, 2011), anti-inflammatory (Blondin et al., 1994), anti-viral (Adhikari et al., 2006) and notably anti-tumoral effects (Ale, Maruyama, Tamauchi, Mikkelsen, & Meyer, 2011). Unfractionated FCSPs have thus specifically been found to reduce cell proliferation of lung carcinoma and melanoma cells *in vitro*; to exert immunopotentiating effects in tumor bearing animals; and to activate natural killer cells in mice leading to increased anti-tumor effectiveness (Ale, Maruyama, et al., 2011; Maruyama, Tamauchi, Iizuka, & Nakano, 2006; Takahashi, 1983). Previous studies

applied a crude polysaccharide isolated from *Fucus vesiculosus*, composed predominantly of sulfated fucose, to human colon cancer cells in vitro. The crude brown seaweed polysaccharide extract induced apoptosis, and provided data that suggested that the apoptosis was induced via activation of caspases (Kim, Park, Lee, & Park, 2010). Moreover, commercially available crude FCSPs ("fucoidan") extracted from *F. vesiculosus, a Sargassum sp.* and from *F. vesiculosus*, have been reported to inhibit proliferation and induce apoptosis on human lymphoma HS-Sultan cells lines by activation of caspase-3 and induce growth inhibition and apoptosis of melanoma B16 cells *in vitro* (Aisa et al., 2005; Ale, Maruyama, et al., 2011). *In vitro* studies have demonstrated that fucoidan inhibits the growth of non-small cell bronchopulmonary carcinoma NSCLCN6 cells and human lymphoma HS-Sultan cells, and also induces apoptosis in cells derived from human lymphoma, promyelocytic leukemia, colon carcinoma, breast carcinoma, ovarian carcinoma, and hepatoma, including the prevention of angiogenesis by suppressing expression and secretion of the angiogenesis factor (VEGF) (Oh, Kim, Lu, & Rosenthal, 2014).

ErbB-1 (EGFR), one of the receptor tyrosine kinases, plays a pivotal role in regulating cell transformation, differentiation, and transformation. The effect of fucoidan on the EGF-induced phosphorylation of EGFR (ErbB-1) was tested in mouse skin epidermal JB6 cells. Cells were treated with 1 ng/mL EGF for 15 min, in the absence or presence of various concentrations of fucoidan and assayed by immunoblotting with the anti phospho-EGFR and anti-EGFR antibodies. Results indicated that treatment with fucoidan significantly decreased the phosphorylation of EGFR (ErbB-1), but not the EGFR (ErbB-1) total protein level (N. Y. Lee et al., 2008). One of the most important protein kinase cascades activated by tumor promoters, such as EGF, are the mitogen-activated protein kinases (MAPKs), following the activation of EGFR (ErbB-1). Thus, the effect of fucoidan was tested on the MEK/ERK signalling pathway and found that fucoidan significantly suppressed EGF-induced phosphorylation of extracellular regulated protein kinases (MEK), ERK1/2, and p90RSK, respectively in JB6 cells in a dose-dependent manner. Fucoidan also significantly inhibited the formation of EGF-promoted neoplastic cell transformation of JB6 cells in a dose-dependent manner (by using the soft agar assay). It was hypothesized that fucoidan may bind with EGF, thereby inhibiting EGF-induced cell transformation. One of the studies here illustrated that fucoidan exerted a strong inhibitory activity on EGF-induced phosphorylation of EGFR (ErbB-1) (Lee et al., 2008). Additionally, it was shown that

fucoidan can directly bind with EGF *in vitro* to prevent the binding of EGF to its receptor. These results suggested that fucoidan has potent anticancer-promoting activity and mainly targets the EGFR-signaling pathway, which may contribute to the chemo-preventive potential.

Previous studies have shown that ErbB1 interacts with the multifunctional protein nucleolin. This interaction leads to receptor dimerization and activation as well as increased colony growth in soft agar (Farin et al., 2011). Nucleolin is a ubiquitously expressed acidic phosphoprotein that is involved in important aspects of cell proliferation and cell growth. It is localized primarily to the nucleoli, but it undergoes nuclear cytoplasmic shuttling and is also found on the cell surface of some types of cells. Cell surface nucleolin is found in a wide range of tumor cells, and it is used as a marker for cancer diagnosis. Inhibition of cell-surface nucleolin and nucleolin activities suppresses cell and tumor growth and also results in high expression levels of ErbB receptors or activated Ras protein (Yona Goldshmit, Trangle, Kloog, & Pinkas-Kramarski, 2014; Schokoroy, Juster, Kloog, & Pinkas-Kramarski, 2013).

Nucleolin inhibitor GroA (AS1411) is a first-in-class anticancer agent, currently in Phase II clinical trials, which is a 26-base guanine-rich oligonucleotide (GRO) with an unmodified (phosphodiester) DNA backbone (Reyes-Reyes, Šalipur, Shams, Forsthoefel, & Bates, 2015) This molecule and its related analogs can inhibit proliferation and induce cell death in many types of cancer cells, but have little effect on normal cells (Hassan, Watari, AbuAlmaaty, Ohba, & Sakuragi, 2014). Previous experiments show that antiproliferative GROs such as AS1411 can build stable G-quadruplex structures and produce an unusual resistance to cellular and serum nucleases. Moreover, GROs can bind directly and selectively to nucleolin, and the growth inhibitory activity of GROs is positively correlated with their ability to bind this protein (Hassan et al., 2014). Additionally, several biological effects of AS1411 have been shown to result from its ability to alter the subcellular localization of certain nucleolin-containing complexes, or to interfere with the molecular interactions of nucleolin (Reyes-Reyes et al., 2015).

Therefore, GroA (AS1411) can act as a nucleolin inhibitor, and when combined with fucoidan, may have a better inhibitory effect on ErbB-1 receptor activation, and subsequently a stronger inhibitory effect on cancer cell growth and tumorogenicity.

1.2 Objectives of Study

The main purpose of the study is to compare two different fucoidans: Low-molecular-weight fucoidan (LMWF) and Sigma Fucoidan (SF), both of which are isolated from Undaria pinnatifida, which is thought to have inhibitory effects on prostate cancer cell lines. Additionally, it is a purpose of this study to determine whether these two fucoidans, when combined with GroA, have interactions with each other. If there are identified interactions, the results of this study may offer some references for treatment of prostate cancer patients. The specific goals in this study are summarized as the following points:

1: Testing if low-molecular-weight fucoidan (LMWF) and Sigma Fucoidan (SF) can suppress the growth of prostate cancer cell lines PC-3 and DU-145. If there is suppression then attempt to find the optimal concentration range, action time, and calculate the IC50 value of fucoidan.

2: Combining low-molecular-weight fucoidan (LMWF)/ Sigma Fucoidan (SF) with GroA in order to find the combination concentration, and then find if they have interactions with each other.

3: Using flow cytometry to find the basic action mechanism of the two fucoidan-based drugs as well as GroAon prostate cancer cells, especially fucoidan, by analysing cell cycle effects and cellular apoptosis.

1.3 Overview

This thesis consists of six chapters.

Chapter 1 is the introduction.

Chapter 2 is the literature review, which comprehensively introduces the natural products Fucoidan and GroA, as cancer treatments.. The introduction of fucoidan is followed by describing its structure, molecular weight, anti-cancer potential and relevant bioactivity. The history of fucoidan is also presented. The aptamer GroA/ AS1411 and the combination possibility are introduced. Their effects on the cell cycle, apoptosis pathway, signaling pathways, as well as combination with fucoidan as a synergistic anti-cancer agent are outlined.

Chapter 3 describes the methodology part of the study. The materials, equipment and methods used in this study are described, as well as experimental design, data analysis and statistical data analysis. The three major study methods are a cytotoxicity assay, an apoptosis assay and a cell cycle assay.

Chapter 4 presents all of the results obtained from the study, including the cell proliferation assay results, apoptosis assay results and cell cycle results.

Chapter 5 discusses the results shown in Chapter 4, and makes some assumptions about the observed mechanisms according to previous research.

Chapter 6 is the conclusion, and summarises the findings obtained from this study, puts forward the weak points of current research and provides some suggestions for future study.

Chapter 2 Literature Review

2.1 Introduction to Cancer

Cancers are multifactorial diseases of various etiologies. They arise largely as a result of acquired genetic changes that alter cell function leading neoplastic cells to gain survival or growth advantages (Cooper, 2000). For many years, cancer research has essentially focused on plants and terrestrial microorganisms, mainly because these resources are easily available and folk traditions have described beneficial effects from their use. Several different therapeutic strategies such as chemotherapy, radiation therapy, surgery or combinations have been used to treat different types of cancer. For cancer cells to survive, the generation of new blood vessels (angiogenesis) is required. Cancer leads to death mostly through the spreading of tumor cells to distal organs (metastasis). Various pathways are disrupted in tumor developments a result of unbalanced programmed cell death, disordered signaling pathways, angiogenesis and poor immune response against cancerous tissue. Most of the chemotherapeutic agents used in cancer treatment target these major deregulated pathways. Unfortunately, as many of these therapies cause severe side effects, the toxicities of the therapies limit the dose, and thus the efficacy of treatment. Therefore, there is strong interest in developing better-tolerated anti-cancer agents.

2.2 A Role for Natural Products for Cancer Treatment

Chemotherapy has been a cornerstone of standard cancer treatment regimens since the 1960s. A variety of chemicals ranging from traditional agents such as methotrexate and folic acid analogues to novel chemicals such as anthracyclines have been used in cancer treatment (Joo, Visintin, & Mor, 2013). Despite promising tumor growth-inhibitory effects in pre-clinical tests, many agents fail in clinical trials when adverse unexpected side effects are revealed. Traditionally anti-cancer chemotherapy targets rapidly dividing and proliferating cells. Therefore, even normal cells which have high-proliferating potential are also affected. Concerns over toxicity, tumor cell resistance and development of secondary cancers from chemotherapeutic chemicals have generated interest in exploiting natural products for cancer treatment. Natural products are also being tested as adjuvants for use in synergy with chemotherapeutic

agents. For example, Indukantha Ghritha (IG) is a polyherbal preparation consisting of 17 plant components widely prescribed by ayurvedic physicians for various ailments. IG is also used as an adjuvant in cyclophosphamide cancer chemotherapy and shown to stimulate the hematopoietic system and induce leukopoiesis in tumor-bearing mice. When administrated in combination with cyclophosphamide, it reversed myelosuppression induced by cyclophosphamide, suggesting its potential to minimize or reverse chemotherapy-induced leukopenia (George, Rajesh, Kumar, Sulekha, & Balaram, 2008).

Polysaccharides include a large family of diverse biopolymers. They consist of monosaccharide residues linked together by O-glycosidic bonds that are found in natural and semi-synthetic structures (Caliceti, Salmaso, & Bersani, 2010). So far, polysaccharides have been widely used in pharmaceutical technology as first choice excipients for production of traditional formulations. Due to structural diversity, polysaccharides display the highest biological properties among macromolecules. Many natural polysaccharides obtained from natural sources such as plants and algae have anti-cancer properties, especially fucoidan, which is well documented. Natural polysaccharides' anti-cancer activity combined with natural biodiversity will allow the development of a new generation of therapeutic measures against cancer that has been going on for years, with recent focus directed towards bioactive compounds of natural origin, including FCSPs from brown seaweeds (Caliceti et al., 2010; Zong, Cao, & Wang, 2012).

2.3 Fucoidan

Fucoidan is a polysaccharide that consists of sulfated fucose residues. The richest sources of fucoidan are marine organisms, including brown algae species such as Laminaria and Fucus (Pomin & Mourão, 2008). Various forms of fucoidan have also been recognized in some marine invertebrates such as cucumbers (Ribeiro, Vieira, Mourão, & Mulloy, 1994) and sea urchins (Mulloy, Ribeiro, Alves, Vieira, & Mourão, 1994). Fucoidan-rich brown algae have been marketed as a dietary supplement or nutraceutical.

2.3.1 Historic Overview

The use of macro algae has been based on farming of edible species or on the production of agar, carrageenan and alginate since the beginning of the last century. Of

all seaweed products, hydrocolloids have had the biggest influence on modern western societies. They have attained commercial significance through their use in various industries which exploit their physical properties such as gelling, water-retention and their ability to emulsify (Renn, 1997). Little commercial exploitation of products extracted from seaweeds occurs outside the hydrocolloid industry. However, in recent years pharmaceutical firms have started looking towards marine organisms, including seaweeds, in their search for new drugs from natural products. These products are also increasingly being used in medical and biochemical research. Prior to the 1950s, the medicinal properties of seaweeds were restricted to traditional and folk medicines (Lincoln, Strupinski, & Walker, 1991). During the 1980s and 90s, compounds with biological activities or pharmacological properties (bioactivities) were discovered in marine bacteria, invertebrates and algae (Mayer & Lehmann, 2000). According to Ireland et al.'s study, algae were the source of about 35% of the newly discovered chemicals between 1977–1987, followed by sponges (29%) and cnidarians (22%) (Ireland et al., 1993).

According to archaeological studies, evidence of the use of seaweed and sea algae by ancient societies has been excavated. It is indicated that Japanese people in the Jomon period and the Yayoi period (BC 1000-AD 300) utilized seaweed and sea algae. Granted, fucoidan is a relatively new discovery, since the technology for extracting, concentrating and processing that slimy part of seaweed has only been around in more modern times. Extraction using dilute acetic acid followed by purification was used by Prof. Kylin of Uppsala University in Sweden as early as 1913 to isolate "fucoidin" (subsequently referred to as fucoidan) from various species of *Laminaria* and *Fucus* (Kylin, 1913). In this seminal report, Kylin reported that fucoidan extracted using his method mainly contained fucose, but he also observed that the fucose occurred together with mannitol, alginic acid and laminarin (Kylin, 1913); we now know that this interpretation was a result of co-extraction of these latter contaminants with the fucoidan. Two years later, Kylin reported that fucoidan isolated from Laminaria digitata contained methylpentose, interpreted as l-fucose, as well as some other pentoses (Kylin, 1915). In 1957, Springer et al. discovered the anticoagulant function of fucoidan (Springer, Wurzel, McNeal Jr, Ansell, & Doughty, 1957). In the 1970s, articles of research on anticancer activities of extracted materials from sea algae as well as fucoidan began to be published. During this time, a reagent of fucoidan isolated from rockweed sea algae, and produced by Sigma Inc., USA, was sold at $100/gram. Since

then, technology to extract fucoidan from sea algae has improved and the study and research of fucoidan has become more active. A key research paper on the antivirus activity of sulfated polysaccharides was pubished in 1988 (Baba, Snoeck, Pauwels, & De Clercq, 1988), and later, in 1995 another landmark study on sulfated polysaccharides such as fucoidan having inhibitory activities on the growth of Helicobacter pylori bacteria was generated (Hirmo, Utt, Ringner, & Wadström, 1995).

Fucoidan has continued to gain attention in the world. Takara Shuzo Co., Ltd., a brewing company, published their research paper on anticancer activity by the apoptotic effects of fucoidan at the meeting of the 55th "Japanese Cancer Association" in 1996. One year later, Marine Products Kimuraya Co., Ltd. applied for a patent for "Antibacterial composition" regarding antibacterial actions of seasoned mozuku against E. coli O-157. Later the material which had "antibacterial composition" was found to be fucoidan. The research boom in the field of fucoidan studies is a story that spans the last two decades, with about 485 papers published between 2000 and 2010 and an additional 310 papers during 1990–2000.

In the 21st century, fucoidan or FCSPs from seaweed biomass have been the subject of many scientific studies aiming at assessing their potential biological activities, including antitumor and immunomodulatory mechanisms (Alekseyenko et al., 2007), antiviral activity (Makarenkova, Deriabin, L'vov, Zviagintseva, & Besednova, 2009), antithrombotic and anticoagulant functions (Zhu et al., 2010), anti-inflammatory [(Semenov et al., 1997), and antioxidant effects (Wang, Zhang, Zhang, Song, & Li, 2010), as well as their effects against various renal (Veena, Josephine, Preetha, Varalakshmi, & Sundarapandiyan, 2006), hepatic (Hayakawa & Nagamine, 2009) and uropathic disorders (Zhang et al., 2005).

2.3.2 Structure

Since Kylin first isolated fucoidan in 1913, the structures of fucoidans from different brown seaweeds have been investigated. Fucoidans from several species of brown seaweed, for example Fucus vesiculosus, have simple chemical compositions, mainly being composed of fucose and sulfate. However the chemical compositions of most fucoidans are complex.

The fucose-containing sulfated polysaccharides (FCSPs) principally consist of a backbone of (1→3) - and (1→4)-linked α -l-fucopyranose residues, that may be

organized in stretches of (1→3)-α-fucan or of alternating α(1→3)- and α(1→4)-bonded l-fucopyranose residues. The l-fucopyranose residues may be substituted with sulfate (SO_3^-) on the C-2 or C-4 (rarely on C-3), with single l-fucosyl residues and/or with short fucoside (fuco-oligosaccharide) side chains. If present, the fucoside side chains are usually O-4 linked to the α-l-fucopyranose backbone residues. However, as FCSPs structures of more brown seaweeds are being elucidated (as discussed further in the present review), it appears that FCSPs cover a broader range of complex polysaccharides than only those having fucan backbones. Apart from variations in the sulfate content and substitutions, the monosaccharide composition of FCSPs also varies among different species of brown seaweeds. Hence, in addition to fucose, different types of FCSPs may also contain galactose, mannose, xylose, glucose and/or glucuronic acid—usually in minor amounts (Jiao et al., 2011).

The fucoidan structure and monosaccharide composition vary depending on different factors such as the source of fucoidan, the time and location of harvesting and the extraction method, which can affect the fucoidan's bioactivities. In addition, fucoidans extracted by different methods may also have different structures. Ponce et al. reported that fucoidan of Adenocytis utricularis extracted at room temperature was composed of mainly fucose, galactose and sulfate ester (the "galactofucan"). The fucoidan extracted at 70°C was composed mainly of fucose, accompanied by other monosaccharides (mostly mannose, but also glucose, xylose, rhamnose and galactose), as well as significant amounts of uronic acids and low proportions of sulfate ester, namely "uronofucoidan" (Ponce, Pujol, Damonte, Flores, & Stortz, 2003).

2.3.3 Fucoidan's Anti-Cancer Potential

Fucoidan is unlikely to be used as a sole agent for cancer treatment where there are known effective therapies, yet as a non-toxic edible product it is an easily deliverable bioactive compound. Here, we will discuss research into the potential for orally delivered fucoidan as an "adjunct" therapy to conventional treatment. Fucoidan may find a role either to reduce side effects, to enhance the therapeutic effects of conventional therapy, or to address cancer for which there are no known therapy options.

The anti-cancer property of fucoidan has been demonstrated in vivo and in vitro in different types of cancers. Nevertheless, it has been rarely investigated for its anti-cancer properties in clinical trials. Fucoidan mediates its activity through various mechanisms such as induction of cell cycle arrest, apoptosis and immune system

activation. Additional activities of fucoidan have been reported that may be linked to the observed anti-cancer properties and these include induction of inflammation through immune system, oxidative stress and stem cell mobilization (Kwak, 2014).

2.3.3.1 Effect of Fucoidan on Cell Cycle

The fundamental processes of progression through the cell cycle and of programmed cell death involve the complex interaction of several families of proteins in a systematic and coordinated manner. They are separate, distinct processes that are intimately related and together play an important role in the sensitivity of malignant cells to chemotherapy. The cell cycle is the mechanism by which cells divide. It is a high energy demanding process that requires an encompassed and ordered series of events to guarantee the correct duplication and segregation of the genome. This process involves four sequential phases that go from quiescence (G0 phase) to proliferation (G1, S, G2, and M phases) and back to quiescence (Norbury & Nurse, 1992). Increasing knowledge on cell cycle deregulations in cancers has promoted the introduction of marine bioactive compounds, which can either modulate signaling pathways leading to cell cycle regulation or directly alter cell cycle regulatory molecules, in cancer therapy.

Fucoidan treatment results in sub G0/G1 cell accumulation (suggestive of dead cells/apoptotic cells) in a variety of cell types (Aisa et al., 2005; Z. Zhang, Teruya, Eto, & Shirahata, 2011). It can also induce cell cycle arrest in other phases; Riou et al. (Riou et al., 1995) and Mourea et al. (Moreau et al., 2006) reported arrest in G1 phase in a chemo-resistant non-small-cell bronchopulmonary carcinoma line by fucoidan from Ascophyllum nodosum and Bifurcaria bifurcate, respectively.

In an investigation of its mechanism of action, fucoidan demonstrated significant down-regulation of cyclin D1, cyclin D2 and CDK4 in cancer cells (Banafa & Roshan, 2013; Boo et al., 2013; Haneji et al., 2005). The crude fucoidan from *Fucus vesiculosus* increased the level of p21/WAF1/CIP1 in PC3 cells and down-regulated E2F, a transcription factor that controls progression of cells from G1 to S phase (Boo et al., 2013).

In a recent study, fucoidan down-regulated cyclin E, CDK2, and CDK4 resulting in G0/G1 arrest in human bladder cancer 5637 cells. Furthermore, immunoprecipitation assays revealed a significant increase in the binding of p21/WAF1/CIP1 to CDK2 and CDK4 in cells treated with fucoidan, suggesting that the induced G0/G1 arrest is due to

suppression of CDK activity following direct binding of this CDK inhibitor to CDKs 2 and 4 (T.-M. Cho, Kim, & Moon, 2014). Table 1 summarizes findings of studies examining the effects of fucoidan on the cell cycle.

Table 1. Effects of fucoidan on cell cycle and apoptosis molecules.

Cell Type	Fucoidan Source	Dose (µg/mL)	Effects on Cell Cycle	Effects on Apoptosis Pathways Extrinsic Intrinsic Common		
Human lymphoma HS-sultan cells (Ale et al.,2011)	*F. vesiculosus*	100	• ↑ sub G0/G1 • No G0/G1 or G2/M arrest		• ↓ MMP	• Caspase 3 activation
HTLV-1 infected T-cell HUT-102-cells (Haneji et al.,2005)	*C. okamurans*	3000	• G1 arrest		• Caspase 9 activation	• Apoptosis was reversed by caspase 3 inhibitor
			• ↓ cyclin D2, c-myc	• Apoptosis was reversed by caspase 8 inhibitor	• No changes in Bcl-2 and Bcl-XL	
			• No changes in p21,p53		• ↓ survivin, cIAP-2	
Human hepatocellular carcinoma cells (Nagamine et al.,2015)	*Okinawa mozuku*	22.5	• ↑ G2/M phase in HAK-1A, KYN-2, KYN-3 cell lines		• No clear caspase 9 activation in HAK-1B cell line	• No clear caspase 3 activation in HAK-1B cells
Human breast cancer MCF7	Not	1000		• Caspase 8 activation	• Caspase 9 activation	• Caspase 7

cells (Yamasaki-Miyamoto et al.,2009)	mentioned		• ↑ sub-G1 fraction			activation
				• Caspase inhibitors blocked apoptosis completely	• ↓ Bid, cytosolic Bax	• PARP cleavage
					• ↑ whole lysate Bax, cytosolic cytochrome C	
Human acute leukemia NB4 and HL-60 cells (Atashrazm et al.,2016)	*F. vesiculosus*	150	• ↑ sub-G1 fraction	• Caspase 8 activation	• caspase 9 activation	• PARP cleavage
					• No changes in Bcl-2 or Bax	• Caspase 3 activation
					• ↓ Mcl-1, ↑ cytochrome C	
Human colon cancer HT-29 and HCT116 cells (Kim et al.,2010)	*F. vesiculosus*	-	-	• Caspase 8 activation	• Caspase 9 activation	• PARP cleavage
				• ↑ Fas, DR5, TRAIL	• ↑ cytochrome C, Smac/Diablo, Bak, t-Bid	• Caspase 3 and 7 activation
				• No significant effects on FasL and DR4	• No changes in Bcl-2, Bcl-xL, Bax, Bad, Bim, Bik	
					• ↓ XIAP, survivin	
Human lung cancer A549	*U. pinnatifida*	50, 100, 200	• ↑ Sub-G1frction	-	• Caspase-9 activation	• ↓ procaspase-3

cells (Boo et al.,2011)					• ↓ Bcl-2, ↑ Bax	• PARP cleavage
Human breast cancer MCF-7 cells (Zang et al.,2011)	*Cladosiphon novae-caledoniae*	82, 410, 820	• ↑ Sub-G1	• No changes in caspase-8	• Mitochondrial dysfunction	• No activation of PARP and caspase-7
			• No significant changes in cell cycle distribution		• AIF and cytochrome C release	
					• No cleavage of caspase-9 and Bid.	• All caspase inhibitors failed to attenuate FE-induced apoptosis
					• ↓ Bcl-2, Bcl-xl ,↑ Bax, Bad	
Hela cells (Costa et al.,2011)	*Sargassum filipendula*	1500	-	-	• No effect on caspase 9 activation	• No effect on caspase 3 (Caspase independent)
					• ↑ cytosol AIF	
Human breast cancer MCF-7 cells (Kasai et al.,2015)	*F. vesiculosus*	400, 800, 1000	• G1 phase arrest	• Caspase-8 activation	• ↓ Bcl-2	• Caspase-dependent pathway
			• ↑ Sub G0/G1 ↓ cyclin D1 and CDK-4 gene expression		• ↑ Bax	
					• Release of cytochrome C and APAf-1	
Human prostate cancer PC-3 cells	*U. pinnatifida*	100	• G0/G1 phase arrest	• DR5, caspase-8	• ↓ Bcl-2	• Caspase-3 activation
			• ↓ E2F-1		• ↑ Bax,	
			• ↑		• Caspase 9	

(Boo et al., 2013)			p21Cip1/ Waf	activation	activation	• PARP cleavage
Human Hepatocellular Carcinoma SMMC-7721 cells (Yang et al.,2013)	*U. pinnatifida*	1000	• Non-significant accumulation in S-phase	• Caspase-8 activation	• Caspase-9 activation • MMP dissipation, Cytochrome C release • ↓ Bcl-2, ↑ Bax • ↓ XIAP, livin mRNA expression	• Caspase-3 activation

2.3.3.2 Effect of Fucoidan on the Apoptosis Pathway

Apoptosis is critically important for the survival of multicellular organisms. The process of programmed cell death, or apoptosis, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms, characterized by cytoplasmic shrinkage and chromatin condensation, which facilitate the removal of cells without inducing inflammation (Elmore, 2007). Impairment of this native defense mechanism promotes aberrant cellular proliferation and the accumulation of genetic defects, ultimately resulting in tumorigenesis (Xiong, Kou, Yang, & Wu, 2007). Countless research findings have shown that fucoidan has both time- and dose-dependent cytotoxic effects on different types of cancer cells, including hepatocellular carcinomas, prostate cancers, breast cancers, lung cancers, acute leukemia, and (*in vitro*) colon cancers (Table 1). This seems to be indicative of the fact that fucoidan induces the characteristic changes associated with apoptosis and brings about drug-induced cell death.

Apoptosis is mediated through two major pathways, the extrinsic (cytoplasmic) pathway, whereby death receptors trigger the apoptotic or the intrinsic (mitochondrial) pathway in which changes in mitochondrial membrane potential (MMP) lead to cytochrome C release and death signal activation. Both pathways activate executive caspases that cleave regulatory and structural molecules (Ghobrial, Witzig, & Adjei, 2005).

In the extrinsic pathway, stimulation of death receptors, such as Fas and tumor necrosis factor receptor-1, leads to clustering and formation of a death-inducing signaling complex, which includes the adaptor protein Fas-Associated Death Domain (FADD) and initiator caspases, such as caspase-8. Activated caspase-8 directly activates downstream effector caspases, such as caspase-3 and -7 (Ashkenazi & Dixit, 1999). Also, caspase-8 can cleave Bid (Bcl-2 interacting protein) into tBid (truncated Bid), and interacts with the proapoptotic protein Bax, and the accumulation of Bax in mitochondria promotes cytochrome C being released into the cytosol (Eskes, Desagher, Antonsson, & Martinou, 2000; J. Li & Yuan, 2008).

In the intrinsic pathway, death receptors transmit death signals to the mitochondria, resulting in the release of several mitochondrial intermembrane space proteins, such as cytochrome C, which associate with Apaf-1 and procaspase-9 to form

the apoptosome. Activated caspase-9 can cleave and activate effector caspases, such as caspase-3 and -7 (Hengartner, 2000).

Several studies examining a variety of cancers such as hematopoietic, lung, breast and colon cancers have shown that fucoidan-mediated cell death occurs through triggering apoptosis (Table 1). Fucoidan from *F. vesiculosus* activated common caspases 3 and 7 in human colon cancer cells at very low concentration (20 µg/mL) (Kim et al., 2010), but induced the same activity in T-cell leukemia at a much higher concentration (3 mg/mL) (Haneji et al., 2005). Caspase 8 and 9, two of the best characterized molecules of the extrinsic and intrinsic pathways respectively, are activated by fucoidan (Yamasaki-Miyamoto, Yamasaki, Tachibana, & Yamada, 2009). As cytochrome C and Apoptosis-Inducing Factor (AIF) are increased in the cytosol, it is believed that fucoidan performs its activity through mechanisms altering mitochondrial function.

Fucoidan also affects other components of extrinsic and intrinsic pathways. Analyzing the extrinsic pathway, 20 µg/mL crude fucoidan from *F. vesiculosus* increased the levels of the death receptors Fas, DR5 and TRAIL but not FasL and DR4 in human colon cancer cell lines (Kim et al., 2010). Bcl-2 family members include anti-apoptotic, pro-apoptotic and regulatory proteins, which are mainly involved in the apoptosis intrinsic pathway, Contradictory results have been described in the expression of these regulatory molecules in response to fucoidan (Table 1). Treatment of MDA-MB231 breast cancer cells with 820 µg/mL of low molecular weight (LMW) fucoidan resulted in a significant decrease in anti-apoptotic proteins Bcl-2, Bcl-xl and Mcl-1 (Z. Zhang, Teruya, Eto, & Shirahata, 2013). In contrast, no changes in expression of Bcl-2, Bcl-xl, Bad, Bim and Bik were observed in colon cancer cells when they were treated with 20 µg/mL fucoidan from Fucus vesiculosus (Kim et al., 2010). Taken together, the results suggest that fucoidan may interact with several components of the apoptosis pathway.

2.3.3.3 Effect of Fucoidan on Angiogenesis

Angiogenesis is the formation of new blood vessels in physiological and pathological processes (Folkman, 1995). Highly regulated and transient angiogenesis is necessary for embryonic development and wound healing. However, uncontrolled and persistent angiogenesis occurs in several pathological states including tumor

progression. Tumor growth requires angiogenesis to supply nutrients and oxygen to highly metabolic tissues. Fucoidan inhibits the formation of new vessels by which tumor cells receive their oxygen and required nutrients. Anti-angiogenic therapy has become an effective strategy for inhibiting tumor growth, and in fact, thisinhibition of angiogenesis can lead to novel targets for cancer therapy (Nelson, 1998).

Fucoidan and oversulfated fucoidan significantly suppressed the mitogeneic and chemotactic actions of Vascular endothelial growth factor (VEGF), VEGF is an interesting inducer of angiogenesis and lymphangiogenesis, because it is a highly specific mitogen for endothelial cells (Koyanagi, Tanigawa, Nakagawa, Soeda, & Shimeno, 2003). Previous study examined the anti-angiogenic properties of fucoidan in 4T1 mouse breast cancer cells both in vitro and in vivo and observed a significant dose-dependent decrease in VEGF expression in cells treated with fucoidan. Further, in a mouse breast cancer model using 4T1 cells, intraperitoneal injections of 10 mg/kg body weight fucoidan from F. vesiculosus for 20 days markedly reduced the number of microvessels. Using immunohistochemistry, fucoidan was shown to reduce VEGF expression compared to the control group (Xue et al., 2012).

2.3.3.4 Effect of Fucoidan on Metastasis

Metastasis is a leading cause (up to 90%) of cancer-related deaths. The development of cancer metastasis consists of multiple processes, in which cancer cells first detach from the primary tumor, invade surrounding tissues and intravasate into blood and/or lymphatic systems and extravasate from the vasculature and subsequently settle and colonize at the target organs. Matrix Metallo Proteinases (MMPs) play a key role in tumor metastasis. The degradation of Extra Cellular Matrix (ECM) is crucial for cellular invasion, indicating the inevitable involvement of matrix degrading proteinases for the process.

Fucoidan suppressed MMP-2 activity and protein expression with increasing concentrations of fucoidan in A549 (lung) cancer cells (H. Lee, Kim, & Kim, 2012). Fucoidan demonstrated the ability to inhibit metastasis via MMP-2 suppression as well as regulate the expression and secretion of a vascular endothelial growth factor (VEGF) (Ye et al., 2005). The anti-metastatic activity of fucoidan was also proven in the animal model of experimental transplanted Lewis Lung Carcinoma (LLC) cells. Fucoidan was shown to influence the function of several cell surface proteins involved in migration

and cell adhesion, including integrins, VEGF 1 and 2, P-selectin and neuropilin-1 (Bachelet et al., 2009; Lake et al., 2006; Rouzet et al., 2011).

2.3.3.5 Effect of Fucoidan on Signaling Pathways

Published data indicates that various compounds exert chemopreventive and chemotherapeutic effects through the inhibition of phosphorylation of membrane receptors, including receptor tyrosine kinases (RTKs), EGFR and Platelet-Derived Growth Factor Receptor (PDGFR). Natural compunds are also involved in the transduction of mitogenic signals across the plasma membrane and in the regulation of cell growth and proliferation. Some marine compounds efficiently interrupt constitutive growth factor stimulated cell signaling pathways, typically triggering a pathway involving Ras → Raf → Extracellular Regulated Kinase (ERK) → mitogen activated kinase/ERK-kinase (MEK) → activator protein (AP)-1 pathway. Mitogen Activated Protein Kinase (MAPK) pathways are involved in cellular proliferation, differentiation, and apoptosis (Chang & Karin, 2001; Wada & Penninger, 2004).

The Extracellular signal-Regulated Kinase (ERK) pathway (or Ras/Raf/MAPK pathway) is often hyperphosphorylated and upregulated in a variety of human cancers. The potential for developing anticancer agents that cause ERK's dephosphorylation and pathway blockade have been explored. Various studies have shown that fucoidan inhibits tumor cell proliferation by decreasing ERKs activity through reduction of its phosphorylation (Patel, Mulloy, Gallagher, O'Brien, & Hughes, 2002).

JNK and p38 are other MAPK superfamily members whose activity is altered by fucoidan. As an example, fucoidan-induced cell death in breast cancer cells through phosphorylation and activation of JNK and p38 occurs after 30 min. The fucoidan-induced apoptosis significantly annulled in the presence of JNK inhibitor, thereby indicating a critical role of JNK in fucoidan-mediated apoptosis (Zhang et al., 2011).

Similarly, the PI3K/AKT, GSK and Wnt pathways have been shown to be triggered by fucoidan. The PI3K/AKT pathway generally inhibits apoptosis. AKT over-activation is also associated with drug resistance and tumor cell survival. As a result, deactivating this pathway could be another potential target for anti-cancer drug development (Lee et al., 2012). Noteably, upregulation of the Wnt signaling pathway is

believed to have a critical role in prostate cancer development, survival and progression (Boo et al., 2013).

2.3.3.6 Effect of Fucoidan on the Immune System

Immunomodulation refers to the action undertaken by medications on auto-regulating processes that steer the immunological defense system. Many polysaccharides obtained from natural sources are considered as biological response modifiers and have been shown to enhance various immune responses (Li, Lu, Wei, & Zhao, 2008). According to previous studies, oligosaccharides have been shown to have a variety of effects on the immune system, such as inhibition of cancer metastasis, antitumor activity, immunological activity, and complement activation, and may also be effective candidates for tumor immunotherapy (Yuan, Song, Li, Li, & Liu, 2011). Therefore, besides demonstrating roles in direct anticancer or antiproliferative properties, fucoidans can also suppress the development of tumor cells through enhancing the body's immunomodulatory activity.

The effects of fucoidan on molecules of the immune system have been studied both *in vitro* and *in vivo*, and effects on both cellular and humoral elements have been described. Of interest, fucoidan increases both activity and number of natural killer (NK) cells *in vivo* (Ale, Maruyama, et al., 2011).

2.3.4 Fucoidan as a Synergistic Anti-Cancer Agent

The ability of fucoidan to synergize with standard anti-cancer agents and/or reduce toxicity has recently been investigated. Fucoidan from Saccharina cichorioides has been reported to synergize with the anti-tumor activity of low dose resveratrol (a natural polyphenol extracted from foods and beverages) on the invasive and highly motile HCT 116 colon cancer cell line. In the colony formation assay, fucoidan plus resveratrol reduced the colony number by 60% compared to 34% and 27% in resveratrol alone or fucoidan alone, respectively (Vishchuk, Ermakova, & Zvyagintseva, 2013).

Zhang et al. studied the combinatory effect of fucoidan and three commonly used anti-cancer agents, cis-platin (CDDP), tamoxifen (TAM) and paclitaxel (Taxol), on signal transduction pathways. Fucoidan from Cladosiphon navae-caledoniae plus anti-cancer agents reduced the ERK phosphorylation in MDA-MB-231 breast cancer cells compared to untreated control or fucoidan alone (Zhang, Teruya, Yoshida, Eto, &

Shirahata, 2013). Dietary fucoidan synergistically reduced cell growth in the OE33 cell line when it was combined with lapatinib, a targeted therapy that acts as a tyrosine kinase inhibitor in advanced HER2-positive breast cancer cells (Oh et al., 2014).

In a xenograft transplantation study, the effect of fucoidan alone or in combination with cyclophosphamide was examined on tumor growth. Nine days after the injection of Lewis lung carcinoma cells into mice, fucoidan from Fucus evanescens was administered to animals either alone or combined with cyclophosphamide. The fucoidan group showed marked antitumor (33% tumor growth inhibition) and anti-metastatic (29% reduction of the number of metastases) activities. However, fucoidan did not exhibit a synergistic effect with cyclophosphamide on tumor growth, but significantly decreased the lung cancer cells metastasis (Alekseyenko et al., 2007).

2.3.5 Low-molecular-weight fucoidan (LMWF) as an anticancer agent

Molecular weight is another crucial factor in fucoidan activity. Yang et al. have studied the native and hydrolyzed fucoidan from Undaira pinnatifida, as anticancer agents. This studies have shown that the anticancer activity was enhanced from 37.6% to 75.9% with a concentration of 1.0 mg/mL of native and hydrolyzed fucoidan, respectively (Yang et al., 2008). This observed activity may be caused by the increase in molar concentration and the enhanced mobility and diffusivity of the partially hydrolyzed fucoidans. Yang et al. have suggested that the anticancer activity of fucoidans could be significantly improved by lowering their molecular weight when they are depolymerized by mild hydrolysis conditions without causing considerable desulfation (Yang et al., 2008). Recently, Cho et al. produced three fucoidan fractions with molecular weights of <5, 5–30 and >30 kDa and reported that the F5-30K showed the most tumor growth inhibitory effect despite the sulfate amount in F<5K being greater than in the two other fractions (Cho, Lee, & You, 2010).

2.4 Nucleolin inhibitor GroA

Guanosine-rich oligonucleotide (GRO，GroA) AS1411 has the sequence 5′-GGTGGTGGTGGTTGTGGTGGTGGTGG-3′ and is also known as GR026B and AGRO100, which is the first nucleic acid aptamer to be tested as a treatment for cancer. AS1411 is a 26-mer DNA aptamer which has attracted considerable attention as an anticancer agent, because the sequence exhibits cancer-selective antiproliferative activity and can form G-quadruplex structures with higher nuclease resistance and

cellular uptake, resulting in S-phase cell cycle arrest and apoptosis. In a Phase I dose-escalation trial, promising signs of anticancer activity were reported in renal cancer patients (9 of 12 patients showed clinical benefit (stable disease, partial or complete response)) and the drug produced no serious adverse events at any dose tested (up to 2 cycles of 7-day infusions of 40 mg/kg/day). Recently, a GRO, AS1411 preparation has reached phase II clinical trials for acute myeloid leukemia and renal cell carcinoma (Ritchie et al., 2007).

2.5 Fucoidan and Nucleolin inhibitor GroA: a possible combination

The three oncogenes, ErbB receptors, Ras proteins and nucleolin may contribute to malignant transformation. Previously, mutant Ras was shown to be capable of activating ErbB receptors in a ligand-independent manner. Moreover, nucleolin, a transcriptional regulator and ribosome biogenesis factor, could bind both K-Ras and the cytoplasmic tail of ErbB receptors to enhance ErbB receptor activation. Therefore, focus on ErbB receptors and nucleolin are important in improving cancer patient survival.

The epidermal growth factor receptor (EGFR; ErbB-1; HER1) is a cell surface protein that binds to epidermal growth factor (Herbst , 2004). ErbB-1 is a member of the ErbB family of receptors, a group whose mutated members have already been identified as affecting ErbB-1 expression or activity, and are associated with cancer (Zhang, et al., 2007). Moreover, activation of ErbB receptors induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation. The Ras/MAPK (mitogen activated protein kinase) cascade is one of the most prominent signaling pathways activated by ErbB receptors. Activated Ras/MAPK can induce the release of growth factors including EGF-like ligands, induce receptor phosphorylation via an autocrine or paracrine loop, and also induce ErbB phosphorylation in a ligand-independent manner. Therefore, targeting ErbB receptors activation can potentially reduce malignancy (Sebolt-Leopold & Herrera, 2004).

Fucoidan is a group of sulfated heteropolysaccharide molecules commonly found in brown seaweeds. Fucoidan is known to inhibit the growth of several types of carcinomas, but the underlying molecular mechanisms and molecular target(s) remain unclear. *In vivo* studies conducted using mouse xenograft models in different labs have

suggested that fucoidan suppresses the growth of several types of cancer cells: 4T1-derived breast cancer, A20-derived lymphoma and Ehrlich ascites carcinoma to name a few. Fucoidan inhibits metastasis of Lewis lung adenocarcinoma and 13762 MAT rat mammary adenocarcinoma, and have antiangiogenesis activity against B16 melanoma. In vitro studies have demonstrated that fucoidan inhibits the growth of non-small cell bronchopulmonary carcinoma NSCLCN6 cells and human lymphoma HS-Sultan cells. Fucoidan also induces apoptosis in cells derived from human lymphoma, promyelocytic leukemia, colon carcinoma, breast carcinoma, ovarian carcinoma, and hepatoma, including the prevention of angiogenesis by suppressing expression and secretion of the angiogenesis factor (VEGF). ErbB-1 (EGFR), one of the receptor tyrosine kinases, plays a pivotal role in regulating cell transformation, differentiation, and transformation. The effects of fucoidan on EGF-induced phosphorylation of EGFR (ErbB-1) was tested in mouse skin epidermal JB6 cells. Cells were treated with 1 ng/mL EGF for 15 min, in the absence or presence of various concentrations of fucoidan, and assayed by immunoblotting with the anti-phospho-EGFR and anti-EGFR antibodies. Results indicated that treatment with fucoidan significantly decreased the phosphorylation of EGFR (ErbB-1), but not the EGFR (ErbB-1) total protein level. One of the most important protein kinase cascades activated by tumor promoters such as EGF, are the Mitogen-Activated Protein Kinases (MAPKs), which occurs following the activation of EGFR (ErbB-1). Thus, the effect of fucoidan was tested on the MEK/ERK signalling pathway and found that fucoidan significantly suppressed EGF-induced phosphorylation of MEK, ERK1/2, and p90RSK, respectively in JB6 cells in a dose-dependent manner. Fucoidan also significantly inhibited the formation of EGF- promoted neoplastic cell transformation of JB6 cells in a dose-dependent manner (determined by using the soft agar assay). It was hypothesized that fucoidan may bind with EGF, resulting in the inhibition of EGF-induced cell transformation. Other studies here illustrated that fucoidan exerted a strong inhibitory activity on EGF-induced phosphorylation of EGFR (ErbB-1). Additionally, it was shown that fucoidan can directly bind with EGF in vitro to prevent the binding of EGF to its receptor. These results suggested that fucoidan has potent anticancer-promoting activity and mainly targets the EGFR-signaling pathway, which may contribute to its chemo-preventive potential.

Low-molecular-weight fucoidan (LMWF) from Undaria pinnatifida has been applied by researchers at Auckland University of Technology (AUT), New Zealand, to various cancer cell lines. High doses of fucoidan demonstrate a strong inhibitory effect

on several types of cells: A-549; MCF-7; WiDr; HDFb; HEK-293; HUVEC; LoVo and Malme-3M cells. The *Undaria* seaweed also contains fucoxanthin, an abundant marine xanthophyll which possesses growth inhibition effects on multiple types of cancer cells. In vitro studies have demonstrated that fucoxanthin induces apoptosis and enhances growth inhibition effects on several cancer cell lines.

Previous studies have shown that ErbB1 interacts with the multifunctional protein nucleolin; this interaction leads to receptor dimerization and activation as well as in increased colony growth in soft agar (Farin et al., 2011). Nucleolin is a ubiquitously expressed acidic phosphoprotein that is involved in important aspects of cell proliferation and cell growth. It is localized primarily to the nucleoli, but it undergoes nuclear cytoplasmic shuttling and is also found on the cell surface of some types of cells. Cell surface nucleolin is found in a wide range of tumor cells, and it is used as a marker for cancer diagnosis. Inhibition of cell-surface nucleolin and nucleolin activities suppresses cell and tumor growth and also results in expression of high levels of ErbB receptors or activated Ras protein (Schokoroy, Juster, Kloog, & Pinkas-Kramarski, 2013; Goldshmit, Trangle, Kloog, & Pinkas-Kramarski, 2014).

Nucleolin inhibitor GroA (AS1411) is a first-in-class anticancer agent, currently in Phase II clinical trials; it is a 26-base guanine-rich oligonucleotide (GRO) with an unmodified (phosphodiester) DNA backbone (Reyes-Reyes, Teng, & Bates, 2011). This molecule and its related analogs can inhibit proliferation and induce cell death in many types of cancer cells, but have little effect on normal cells (Hassan, Watari, AbuAlmaaty, Ohba, & Sakuragi, 2014). Previous experiment shows that antiproliferative GROs such as AS1411 can build stable G-quadruplex structures and produce an unusual resistance to cellular and serum nucleases. Moreover, GROs can bind directly and selectively to nucleolin, and the growth inhibitory activity of GROs is positively correlated with their ability to bind this protein (Hassan, Watari, AbuAlmaaty, Ohba, & Sakuragi, 2014). In addition to inhibitory effects, several biological effects of AS1411 have been shown to result from its ability to alter the subcellular localization of certain nucleolin-containing complexes, or to interfere with the molecular interactions of nucleolin (Reyes-Reyes, Teng, & Bates, 2011).

Ronit Pinkas-Kramarski's lab in Israel has identified non-nucleolar nucleolin as an ErbB receptor-interacting protein. This interaction leads to receptor dimerization and activation as well as to increased colony growth in soft agar. Previous studies have also

identified a crosstalk between nucleolin, ErbB1 and Ras proteins, and demonstrated that treatment with a combination of FTS (which inhibits the Ras proteins and GroA (AS1411)), and a drug that targets two out of the three proteins has a stronger inhibitory effect on several types of cancer cells: colon cancer cells (DLD-1 and HCT-116), prostate cancer cells (DU-145 and PC-3) and glioblastoma cells (U-87). Therefore, it seems likely that combining two drugs, Fucoidan and GroA, may have a better inhibitory effect on ErbB-1 receptor activation and from that activity, a stronger inhibitory effect on cancer cell growth and tumorogenicity.

Consider that, two human prostate cancer cell lines, PC-3 and DU-145, were utilized as a study model to examine the anticancer potential of LMWF and SF. It is worth mentioning here that among prostate cancer cell lines available, PC-3 and DU-145 were established for use as androgen-independent cancer cells. These cell lines were not derived from primary cancer tissue but were isolated from sites where the cancer had spread, metastatic lesions. DU-145 was originally isolated from a lesion in the brain of a patient with widespread prostate carcinoma in 1978 (Xu et al., 2001). A portion of tumour tissue was scraped with a scalpel to loosen the cells from the bulk of the tumour (spill out method) (Stone et al., 1978). The cells were then grown up in growth medium on collagen coated Petri dishes, characterised and were found not to be hormone sensitive or hormone dependent (Stone et al., 1978). PC-3 cell line was developed from a grade IV prostate adenocarcinoma in 1978 (Xu et al., 2001). The cell line is androgen independent and forms subcutaneous tumours in nude mice (Kaighn et al., 1979). The androgen-independent stage of prostate cancer is considered to be metastatically advanced cancer and therefore, to study drugs against these cell lines indicates how effective a potential agent may manifest its action against advanced prostate cancer.

Chapter 3 Methodology

3.1 Cell Lines used in this Study

Table 2: Cell line information

Cell Line Designation	Catalogue Number	Cell Line Description	Supplier
PC-3	CRL-1435	Human prostate; derived from metastatic site: bone	American type culture collection (ATCC)
DU-145	HTB-81	Human prostate; derived from metastatic site: brain	American type culture collection (ATCC)

PC-3 and DU-145 cell lines (Table 2) were stored at -80 °C or in liquid nitrogen. After thawing the cell lines, they were maintained in 25 or 75 cm^2 tissue culture flasks containing 5 mL or 15 mL of completed growth culture medium in a 37 °C incubator with 5% carbon dioxide, humidified air.

3.2 Cell Proliferation Assay

To study the effects of fucoidan and GRO A on cell proliferation, cell viability of cell lines incubated in a range of concentrations of drugs for various time periods was determined by using the methylthiazol-diphenyl-tetrazolium (MTT) assay, an indirect method to measure cell growth. The MTT assay is a colorimetric assay. This method is able to measure the decrease of yellow MTT by mitochondrial succinate dehydrogenase. When MTT enters the cells and passes into the mitochondria, it will be reduced to an insoluble and dark purple formazan product. Using an organic solvent such as Dimethyl sulfoxide (DMSO) to solubilise the cells, the released and solubilised formazan reagent can be measured spectrophotometrically. Since the reduction of MTT can only occur in metabolically active cells, this method can indirectly reflect the viability of cells (Hansen, Nielsen, & Berg, 1989).

3.2.1 Preparation of Cell Culture and Cell Viability Assay

Table 3: Main materials in cell culture and cell viability assay

Number	Material	Supplier
1	Cell culture medium (RPMI 1640, no phenol red)	Life technologies
2	Cell culture medium(Eagle's Minimum Essential Medium (EMEM))	ATCC
3	L-glutamine (200 mM; 100 mL)	Life technologies
4	Penicillin-Streptomycin (10,000 U/mL; 100 mL)	Life technologies
5	Trypan Blue Solution, 0.4%	Life technologies
6	TrypLE™ Express Enzyme (1X), no phenol red	Life technologies
7	PBS (Phosphate buffered saline), pH 7.2, no calcium magnesium and phenol red	Life technologies
8	Sterile filtered fetal bovine serum (FBS)	
9	MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (Cat No. M2128-1G)	Sigma- Aldrich (St Louis, USA)
10	DMSO (Dimethyl sulfoxide) (Cat No. 102952)	Merck-Chemicals
11	25 and 75 cm^2 cell culture flasks, 1, 5, 10 and 25 mL sterile disposable pipette tips, 15 and 50 mL centrifuge tubes, 96-well tissue culture plates, etc.	BD (Becton Dickinson) Bioscience (Auckland, NZ)

3.2.2 Preparation of Complete Growth Culture Medium

PC-3 was cultured in RPMI 1640 base medium with 1% Penicillin-Streptomycin, 1% L-glutamine and 10% fetal bovine serum.

DU-145 was cultured in Eagle's Minimum Essential Medium (EMEM) with 10% fetal bovine serum. EMEM was further modified to contain Earle's Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate.

3.2.3 Preparation of MTT Stock Solution

12 mM (5 mg/mL in PBS) MTT solution was prepared by weighing 5 mg MTT powder and dissolving it with 1mL phosphate buffered saline (PBS). The powder was

fully dissolved by vortexing, and the 12 mM MTT phosphate buffered saline was filtered through a sterile Millex GV 0.22 µm syringe filter to remove any pathogens, undissolved MTT and any spontaneously formed formazan crystals. Once prepared, the MTT solution was stored at 4°C in the dark or at -20°C for long term storage.

3.2.4 Preparation of Fucoidan Stock Solution

Table 4: General description of fucoidan used in this study

Type of Fucoidan	Quality	Supplier
Fucoidan from *Undaria pinnatifida*	≥ 95% pure	Sigma-Aldrich (USA) (CAS Number: 9072-19-9)
Low-molecular-weight fucoidan (LMWF) from *Undaria pinnatifida*	-	Extracted at Auckland University of Technology, New Zealand

Two types of fucoidan were used in this study.

The fucoidan from Undaria pinnatifida was bought from Sigma-Aldrich (CAS Number: 9072-19-9). The purity of the fucoidan was ≥ 95% as determined by HPLC. SF was dissolved in cell culture medium (RPMI 1640 base medium with 1% Penicillin-Streptomycin, 1% L-glutamine and 10% fetal bovine serum) to a final concentration of 2000 µg/ml as stock solution.

LMWF from Undaria pinnatifida was extracted at Auckland University of Technology, New Zealand, and dissolved in cell culture medium (RPMI 1640 base medium with 1% Penicillin-Streptomycin, 1% L-glutamine and 10% fetal bovine serum) to a final concentration of 3200 µg/ml and 600 µg/ml as stock solutions.

Aliquots of fucoidan stock solutions were separated in micro-tubes. The micro-tubes were wrapped up in aluminium foil and stored in the -80℃.

3.2.5 Preparation of Gro (GroA/ AS1411) and CRO Stock Solutions

Table 5: General description of GroA and Cro used in this study

Drugs	Quality	Supplier
Gro (GroA/ AS1411) (10umole DNA Oligo, 26 bases)	The GC Content was 65.4%, molecular weight was 8272.3.	Integration DNA Technology (Jerusalem, Israel)
Cro (10umole DNA Oligo, 26 bases)	The GC Content was 65.4%, molecular weight was 8272.3.	Integration DNA Technology (Jerusalem, Israel)

The aptamer Gro (GroA/AS1411) used in this study was purchased from Integration DNA Technology (10umole DNA Oligo, 26 bases) as unmodified desalted oligonucleotides. The GC Content was 65.4%, with a molecular weight of 8272.3.

The inactive oligomer Cro used in this study was bought from Integration DNA Technology (10umole DNA Oligo, 26 bases) as unmodified desalted oligonucleotides. The GC Content was 65.4%, with a molecular weight of 7591.9.

Both oligomers were dissolved in double distilled water (DDW) to a final concentration of 1000 μM to prepare stock solutions, and incubated at 65°C for 15 minutes. Aliquots of GroA stock solution were separated into micro-tubes and stored in a -20℃ freezer.

3.2.6 Cell Culture Protocols

3.2.6.1 Thawing Frozen Cells

The cryovial containing frozen cells was removed from liquid nitrogen storage and immediately place in a 37°C water bath. The cells were quickly thawed (< 1 minute) by gently swirling the vial in the 37°C water bath until there was just a small amount of ice left in the vial. Before opening, the outside of the vial was wiped with 70% ethanol. The desired amount of pre-warmed complete growth medium appropriate for each cell line was transferred dropwise into the centrifuge tube containing the thawed cells. The cell suspension was centrifuged. After the centrifugation, the clarity of supernatant and visibility of a complete pellet was checked. The supernatant was aseptically decanted

without disturbing the cell pellet. Finally, the cells were gently re-suspended in complete growth medium, and then transferred into a 25 cm^2 tissue culture flask and kept in a 37°C, 5% CO_2, humidified incubator.

3.2.6.2 Changing Medium

Cells that have been growing well for a few days but are not yet confluent require a medium change to replenish nutrients and maintain the correct pH. The spent cell culture medium was removed and discarded from the culture flask. PBS without calcium and magnesium (approximately 2 mL per 10 cm^2 culture surface area) was used to wash cells. The wash solution was removed and discarded. Finally, fresh pre-warmed culture medium (approximately 2 mL per 10 cm^2 culture surface area) was added and cells were returned to the 37°C incubator.

3.2.6.3 Passaging Adherent Cells

Cells were passaged when they were ~70-80% confluent. The cells were first washed using PBS (withoutcalcium and magnesium), and the wash was removed from the flask. The pre-warmed dissociation reagent TrypLE™ Express Enzyme was added to the side of the flask (approximately 0.5 mL per 10 cm^2). The culture flask was placed in the incubator for approximately 2 minutes (the actual incubation time varies with the cell line used). When more than 90% of the cells were detached, the equivalent of 2 volumes (twice the volume used for the dissociation reagent) of pre-warmed complete growth medium was added. The cells were transferred to a 15mL centrifuge tube and centrifuged for 5 to 10 minutes (the centrifuge speed and time vary based on the cell type). The cell pellet was resuspended in 1mL pre-warmed complete growth medium and a sample was removed for counting. An appropriate volume of cells was pipetted into a new cell culture flask, and returned to the incubator. Note that most cells must not be split more than 1:10 as the seeding density would be too low for the cells to survive. As a general guide, from a confluent flask of cells:

1:2 split should be 70~80% confluent and ready for an experiment in 1 to 2 days；

1:5 split should be 70~80% confluent and ready for an experiment in 2 to 4 days；

1:10 split should be 70~80% confluent and ready for sub-culturing or plating in 4 to 6 days.

3.2.6.4 Freezing Cells

Freezing medium was prepared and stored at 2 to 8°C until use (the appropriate freezing medium depended on the cell line). The freezing medium should contain a cryoprotective agent such as DMSO or glycerol. For adherent cells, cells were gently detached from the tissue culture vessel (following the procedure used during the subculture). The cells were then resuspended in complete medium and the total number of cells was determined by counting. According to the desired viable cell density, the required volume of freezing medium was calculated. After centrifugation and decanting the supernatant, the cell pellet was resuspended in cold freezing medium at the recommended viable cell density for the specific cell type. Aliquots of the cell suspension were dispensed into cryogenic storage vials. The cryovials containing the cells were placed in an isopropanol chamber and stored at -80°C overnight. The frozen cells were then transferred to liquid nitrogen, and stored in the gas phase above the liquid nitrogen.

3.2.7 MTT cell proliferation assay protocols

The MTT assay used in this study was performed according to the protocol first described by Mosmann (Mosmann, 1983).

3.2.7.1 Major Equipment used in Assays

Table 6: Major equipments and materials applied in MTT assay

Number	Equipment
1	Microtiter plate reader with 540 and 680 nm filters
2	Multi-functional orbital shaker
3	Inverted microscope
4	Multi-channel pipette
5	Haemocytometer
6	Centrifuge

3.2.7.2 Basic Steps for MTT cell proliferation assay

Table 7: Basic steps for MTT assay

Step	Action
1	Basic cell culture and detaching cells from culture flask.
2	Determining the cell concentration.
3	Seeding cells on 96-well plates, 100 μL each well.
4	Adding treatments, 100 μL each well.
5	Replacing the original medium with 100 μL of fresh culture medium.
6	Adding MTT stock solution and incubating.
7	Adding DMSO and recording absorbance.

Step 1: Cell preparation

PBS and TrypLE™ Express Enzyme were used to wash and detach cells. The cells were collected in a 15 mL centrifuge tube to centrifuge. After centrifugation, the supernatant was carefully removed and 1 mL of new completed culture medium was added into the tube to resuspend cells gently but thoroughly.

Step 2: Cell counting

10 μL of the cell suspension was removed onto a piece of parafilm and mixed thoroughly with 10 μL of Trypan Blue. 10 μL of this mixture was placed to one side of the hemocytometer and the number of cells was determined under the microscope. The suspension was diluted enough so that the cells do not overlap each other on the grid, and was uniformly distributed on the hemocytometer. The total number of cells counted (at least four squares) was recorded. The total number of cells in 1 mL culture medium was calculated by using the formula below:

Total cells/mL= (Total cells counted / Number of squares) × dilution factor ×10,000 cells/mL

Because the sample was diluted 1:1 with Trypan blue, the dilution factor is 2.

Step 3: Seeding cells

The cell density for all of the cell lines in this study was 50,000 cells/mL. Based on this concentration, complete culture medium was applied to dilute the cell stock solution. After dilution, 100 μL of cells was seeded into each well of a 96-well plate. A column of blank wells containing the medium only was included for each plate. The plates were kept in the incubator for 6 to 24 hours.

Step 4: Adding treatment

After incubation, all of the cells were attached to the walls of the wells. Different concentrations of drugs were prepared with pre-warmed complete culture medium before adding to each well. 100 μL of fresh complete culture medium containing various concentrations of drugs was added slowly to corresponding wells. A column of wells were set as a negative control group containing cells with no treatment. For the Day 0 plate, no treatment was added into the wells. The plates were kept in the incubator.

Step 5: Changing medium (Optional step)

After incubation for a set time (normally 0, 24, 48, 72 and 96 hours), the old medium containing treatment was carefully removed and replaced with 100 μL of fresh complete culture medium. This step depends on the treatment. If the treatment was

known to impact MTT assay results, then the treatment was removed before adding MTT solution.

Step 6: Adding MTT stock solution

An aliquot of 10 μL of MTT stock solution was added to each well and the plates were placed back in the 37°C incubator. After incubation for ~2-4 hours, purple precipitate was clearly visible under an inverted microscope. The supernatant was gently removed from the wells.

Step 7: Quantification

An aliquot of 100 μL of DMSO was added to each well and mixed thoroughly using an orbital plate shaker. After incubating at 37°C for 20 to 30 minutes, the plate was shaken briefly and absorbance was measured by a plate reader (FLUOstar Omega, Alphatech) at a wavelength of 540 nm, with the reference wavelength at 680nm. The average absorbance value (OD value) was determined from sextuplicate readings, and the average values were subtracted from the average value from the blank readings in order to yield the final absorbance value.

3.2.8 Determination of MTT Assay Linearity Range

Suitable cell density and culture times are important to ensure that a very good linear relationship can be shown between the MTT formazan assay results and cell numbers. The cell number vs. absorbance standard curve is used to determine the linearity of the MTT assay and the cell number used in cell viability studies should fall within the linear portion of the curve.

The highest cell concentrations set in this study were 250,000. Cells were kept in 1.5 mL micro-tubes and after a series of one in two (1:2) dilutions by cell culture medium (Appendix 7), an aliquot of 100 μL well mixed cells in each 1.5 mL micro-tubes were seeded in each well of a 96-well plate. Each concentration was repeated six times. After seeding for 18 hours, step 7 (in section 3.2.7.1) was undertaken to measure absorbance at 540 nm. A linear relationship between absorbance value (OD value) and cell number was generated by plotting absorbance on the Y-axis against cell numbers on the X-axis.

3.2.9 Determination of Cells' Population Doubling Time

Cells Population Doubling Time (PDT), or more accurately, cells doubling time in a logarithmic growth period, is the period of time required for cell numbers to double. Two methods were used to calculate the doubling time of cells in this study. One was obtained directly through the formula below and the second was calculated from the cell growth curve.

The cell doubling time calculation formula is shown below:

$$T_d = T \times Lg\ 2 / Lg\ (N / N_0)$$

Td: Doubling time; T: Time interval; N: Final cell number, N_0: Initial cell number

To make a cell growth curve, 200 µL of cells from each well (density 5,000 cells/mL), was seeded onto six 96-well plates. The cell culture time points set were 0, 24, 48, 72, 96 and 120 hours. An MTT assay was conducted every day at the same time. Based on the experimental data, the relationship between absorbance and time was determined. According to the cell number vs. absorbance standard curve gotten previously, cell numbers at different time points were calculated and then the cell growth curve was drawn. Finally, the cell doubling time was calculated by cell growth curve fitting. According to the results cells doubling time in logarithmic growth period for PC-3 is 23.86 hours and for DU-145 is 29 hours.

3.2.10 Determination of the Inhibitory Effect of different types of Fucoidan

3.2.10.1 Determining the concentration range

Step 1：Cell preparation

The cell density chosen in this study for PC-3 and DU-145 cell lines was 5, 000 cells/well. After proper dilution, a multi-channel pipette was used to seed the well-mixed cells into the 96-well plate. Each well contained 100 µL of cells.

Step 2: Fucoidan solution preparation

The concentration of SF stock solution was 2000 µg/ml.

The concentration of LMWF stock solution was 3200 μg/ml and 600 μg/ml.

Prior to addition to experimental wells, all of the diluted treatments were kept in 1.5 mL micro-tubes. Fucoidan was diluted with cell culture medium (RPMI 1640 base medium with 1% Penicillin-Streptomycin, 1% L-glutamine and 10% fetal bovine serum). For different cancer cell lines, the fucoidan concentration ranges were kept the same. The dilution plans are shown in Appendix 8, 9, 10.

Since fucoidan stock solutions were prepared by using cell culture medium, determination of the toxicity of the dilution solvent was not required.

Step 4: Adding treatment

Once various fucoidan treatments were prepared, 100 μL of each diluted fucoidan solution were added immediately to their target wells. Each fucoidan concentration was repeated six times. The blank (200 μL cell culture medium only) and control (100 μL cell with 100 μL cell culture medium) groups were set in the same plate. Using PC-3 treated with LMWF as a sample, the design of the plate is shown in Figure 1. The next steps are the same in section 3.2.7.1 steps 5, 6 and 7.

	Blank	Control	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9	
200 μL of **PBS** each well around the plate											

Figure 1: Plate design for PC-3 treated with LMWF

3.2.11 Determination of Fucoidan's Colour Effect on Absorbance Value (OD Value)

Different concentrations of fucoidan solutions were prepared to test their respective absorbance values. The test methods were used in this study was MTT assay, remove 100µl solution from each well and 10µL MTT solution was added to each well, (after 2-4 hours) and then 100 µL DMSO was added and finally the absorbance was read.

The concentration of SF versus LMWF in this study were determined by 2.6.1 results.

- SF: 500, 750, 1000 µg/ml
- LMWF: 100, 200, 300 µg/ml

3.2.12 The Optimum Concentration of GroA (AS1411)/Cro

The optimum concentration of GroA/Cro in this study was 10 µM, which is based on previous studies (Yona Goldshmit, Schokoroy Trangle, Kloog, & Pinkas Kramarski, 2014).

The aptamer Gro (GroA/ AS1411) and the inactive oligomer Cro, were purchased from IDT (Jerusalem, Israel) as unmodified desalted oligonucleotides. The oligonucleotides were reconstituted in DDW to 1000µM concentration and incubated at 65°C for 15 minutes. Aliquots of GroA and Cro stock solutions were separated in micro-tubes and stored in the -20℃ freezer.

Since the fucoidan stock solutions were prepared by DDW, determination of the toxicity of the dilution solvent was not required.

3.2.13 Study on combined treatment of fucoidan and GroA (AS1411)/Cro

According to previous single treatment experimental results, the optimum concentrations of fucoxanthin and gemcitabine were established for this study.

In the combination treatment portion of the experiment, SF at concentrations of 500, 750, and 1000 µg/ml, LMWF at concentrations of 100, 200, and 300 µg/ml, and GroA (AS1411)/Cro at a concentration of 10 µM, were concurrently combined with

each other to treat PC-3 and DU-145 cell lines. The concentrations of stock solutions have been described.

The combination plan and dilution plan for different fucoidan and GroA/Cro preparations are shown in Tables 12, 13, 14 and 15. Using PC-3 as a sample, the design of the plate is shown in Figure 2. Other experimental steps were the same as described in the single treatment study.

Table 8: Combination plan of SF, LMWF and GroA/Cro for PC-3 and DU-145.

SF (µg/ml)	**GroA/Cro**		
	0	**GroA 10µM**	**Cro 10µM**
0	Control	GroA 10 µM only	Cro 10 µM
500	SF 500 µg/ml only	SF 500 µg/ml+ GroA 10 µM	SF 500 µg/ml+ Cro 10 µM
750	SF 750 µg/ml only	SF 750 µg/ml+ GroA 10 µM	SF 750 µg/ml+ Cro 10 µM
1000	SF 1000 µg/ml only	SF 1000 µg/ml+ GroA 10 µM	SF 1000 µg/ml+ Cro 10 µM
LMWF (µg/ml)			
100	LMWF 100 µg/ml only	LMWF 100 µg/ml+ GroA 10 µM	LMWF 100 µg/ml+ Cro 10 µM
200	LMWF 200 µg/ml only	LMWF 200 µg/ml+ GroA 10 µM	LMWF 200 µg/ml+ Cro 10 µM
300	LMWF 300 µg/ml only	LMWF 300 µg/ml+ GroA 10 µM	LMWF 300 µg/ml+ Cro 10 µM

Table 9: Dilution plan of single treatment (SF vs LMWF) for PC-3 and DU-145.

Tube Number	**Actual fucoidan concentration (µg/ml)**	**Prepared fucoidan concentration (µg/ml)**	**Dilution plan (Each 1.5 mL micro-tube has 1000 uL Fucoidan and complete culture medium mixture)**
1	0	0	1000 µL medium
2	500	1000	500 µL (**2000µg/ml SF stock solution**) + 500 µL medium
3	750	1500	750 µL (**2000µg/ml SF stock solution**) + 250 µL medium
4	1000	2000	1000 µL (**2000µg/ml SF stock solution**) + 0 µL medium
5	100	200	333.3 µL (**600µg/ml LMWF stock solution**) + 666.7 µL medium
6	200	400	666.7 µL (**600µg/ml LMWF stock solution**) + 333.3 µL medium
7	300	600	1000 µL (**600µg/ml LMWF stock solution**) + 0 µL medium

Table 10: Dilution plan of combination treatment (SF and GroA) for PC-3 and DU-145.

Tube Number	Actual drug concentration		Prepared drug concentration		Dilution plan (Each 1.5 mL micro-tube has 1000 uL drug and complete culture medium mixture)		
	Sigma Fucoidan (SF) (µg/ml)	GroA/Cro (µM)	Sigma Fucoidan (SF) (µg/ml)	GroA/Cro (µM)	Sigma Fucoidan (SF) (µl)	GroA/Cro (µl)	Medium (µl)
1	0	0	0	0	0	0	1000
2	0	Cro 10 µM	0	Cro 20 µM	0	Cro 20 µl	880
3	0	GroA 10 µM	0	GroA 20 µM	0	GroA 20 µl	880
4	500	Cro 10 µM	1000	Cro 20 µM	500	Cro 20 µl	480
5	750	Cro 10 µM	1500	Cro 20 µM	750	Cro 20 µl	230
6	1000	Cro 10 µM	2000	Cro 20 µM	1000	Cro 20.4 µl	0
7	500	GroA 10 µM	1000	GroA 20 µM	500	GroA 20 µl	480
8	750	GroA 10 µM	1500	GroA 20 µM	750	GroA 20 µl	230
9	1000	GroA 10 µM	2000	GroA 20 µM	1000	GroA 20.4 µl	0

Table 11: Dilution plan of combination treatment (LMWF and GroA) for PC-3 and DU-145.

Tube Number	Actual drug concentration		Prepared drug concentration		Dilution plan (Each 1.5 mL micro-tube has 1000 uL drug and complete culture medium mixture)		
	LMWF (µg/ml)	GroA/Cro (µM)	LMWF (µg/ml)	GroA/Cro (µM)	LMWF (µl)	GroA/Cro (µl)	Medium (µl)
1	0	0	0	0	0	0	1000
2	0	Cro 10 µM	0	Cro 20 µM	0	Cro 20 µl	880
3	0	GroA 10 µM	0	GroA 20 µM	0	GroA 20 µl	880
4	100	Cro 10 µM	200	Cro 20 µM	333.3	Cro 20 µl	646.7
5	200	Cro 10 µM	400	Cro 20 µM	666.7	Cro 20 µl	313.3
6	300	Cro 10 µM	600	Cro 20 µM	1000	Cro 20.4 µl	0
7	100	GroA 10 µM	200	GroA 20 µM	333.3	GroA 20 µl	646.7
8	200	GroA 10 µM	400	GroA 20 µM	666.7	GroA 20 µl	313.3
9	300	GroA 10 µM	600	GroA 20 µM	1000	GroA 20.4 µl	0

	Blank	Control	Cro	GroA	100F+Cro	200F+Cro	300F+Cro	100F+GroA	200F+GroA	300F+GroA	
200 μL of PBS each well around the plate											

Figure 2: Plate design for PC-3 cells treated with single and combination treatments (LMWF and GroA).

In each well of the 96-well plate 200 μL of solution was aliquotted according to the following assignments. All groups other than the blank group had 100 μL of medium and 100 μL of treatment as illustrated in Figure 1. The treatments were LMWF, GroA, and combination groups.

3.3 Cell Apoptosis Assay

Apoptosis is a carefully regulated process of cell death that occurs as a normal part of development. Inappropriately regulated apoptosis is implicated in disease states, such as Alzheimer's disease and cancer. Apoptosis is distinguished from necrosis, or accidental cell death, by characteristic morphological and biochemical changes, including compaction and fragmentation of the nuclear chromatin, shrinkage of the cytoplasm, and loss of membrane asymmetry (LINCZ, 1988) (Darzynkiewicz et al., 1997) (Allen, Hunter, & Agrawal, 1997). In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment (Van Engeland, Nieland, Ramaekers, Schutte, & Reutelingsperger, 1998). In leukocyte apoptosis, PS on the outer surface of the cell marks the cell for recognition and phagocytosis by macrophages (Fadok et al., 1992) (Fadok et al., 1993). The human anticoagulant, annexin V, is a 35–36 kDa Ca2+-dependent phospholipid-binding protein that has a high affinity for PS. Annexin V

labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet (Koopman et al., 1994).

The FITC Annexin V/Dead Cell Apoptosis Kit with FITC annexin V and PI for flow cytometry provides a rapid and convenient assay for apoptosis. The kit contains recombinant annexin V conjugated to fluorescein (FITC annexin V), as well as a ready-to-use solution of the red-fluorescent propidium iodide (PI), a nucleic acid binding dye. PI is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with FITC annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. These populations can easily be distinguished using a flow cytometer with the 488 nm line of an argon-ion laser for excitation.

To explore the possible mechanisms of single and combined effects of different fucoidan and GroA preparations in PC-3 and DU-145 cells, flow cytometry allowed the measurement of several apoptotic traits in a single sample, making it a powerful tool to study the complexity of cell death.

3.3.1 Major Equipment and Materials used in Assays

Table 12: Major equipment and materials used in cell apoptosis assay

Number	Material / Equipment	Supplier
1	Dead Cell Apoptosis Kit with Annexin V FITC and PI, for flow cytometry. Catalog number: V13242 Alexa Fluor® 488 annexin V (Component A) Propidium iodide (PI, Component B) 5X annexin-binding buffer (Component C)	Thermo Fisher Scientific
2	Samples (appropriate sample concentrations range from 2×10^5 to 1×10^6 cells/mL)	Prepared
3	PBS (Phosphate buffered saline), pH 7.2	Life technologies
4	Deionized water	
5	Flow cytometer	Beckman coulter
6	Six well plate	BD (Becton Dickinson) Bioscience (Auckland, NZ)
7	Test Tube, 12 x 75 mm, Polypropylene Blue (250/ pack) (Cat No.63058857)	Beckman coulter

3.3.2 Protocols for Cell Apoptosis Assay

3.3.2.1 1X Annexin-binding Buffer Preparation

5X annexin-binding buffer (Component C) was diluted with deionized water to a final concentration of 1X Annexin-binding Buffer. For example, for ~10 assays, add 1 mL 5X annexin-binding buffer (Component C) to 4 mL deionized water. The total solution was kept in 15 mL centrifuge tubes and stored at 2-6℃.

3.3.2.2 PI Stock Solution Preparation

The 100 μg/mL working solution of PI was prepared by diluting 5 μL of the 1mg/mL PI stock solution (Component B) in 45 μL 1X annexin-binding buffer. The total solution was kept in 1.5 mL micro-tubes wrapped with aluminium-foil and stored

at 4℃. The unused portion of this working solution was stored under the same conditions for use in future experiments.

3.3.2.3 Cell Preparation and Drug Treatment

Step 1: Cell Seeding

After detaching, counting and diluting cells, cells were seeded onto 6-well plates. The seeding density for all cell lines in this study was 40,000 cell/mL and a 2 mL cell solution was contained in each well. The plates were kept in a 37 ℃ incubator for 6 to 24 hours to ensure almost all of the cells attached to the walls of the wells.

Step 2: Addition of treatments

All treatments were kept in 15 mL centrifuge tubes which were prepared with complete culture (10% FBS) medium. The spent complete culture medium was removed gently and then 2mL well-mixed treatment solution was carefully added into designated wells. The control well was set in the plate of treatment group with 2 mL complete culture medium. All of the plates were kept in the incubator for 72 hours (day 3). Cell collection was needed for plate 1 at day 0 samples. The design of the 6-well plate is shown in Table 17-Plate 1,2,3,4 and 5. All of the concentrations determined for this part of the study were the same as described in Section 3.2.12. Namely, fucoidan (Sigma-Aldrich), 500, 750, and 1000 μg/ml, LMWF 100, 200, and 300 μg/ml and GroA (AS1411)/Cro 10μM were used to treat PC-3 and DU-145 jointly.

The combination plan and dilution plan for different fucoidan and GroA/Cro preparations were the same as described in the combination treatment study of 3.2.12 Tables10 and 11.

Table 13: Plate design for cell apoptosis assay

A	B	C
control with 2 mL complete culture medium	control with 2 mL complete culture medium	control with 2 mL complete culture medium

Plate 1 (Blank treatment group Day 0)

1	2	3
Control	Cro	GroA
4	5	6
500 µg/ml SF +10 µM Cro	750 µg/ml SF +10 µM Cro	1000 µg/ml SF +10 µM Cro

Plate 2 (Treatment group: SF and GroA for PC-3 and DU-145.)

7	8	9
500 µg/ml SF +10 µM GroA	750 µg/ml SF +10 µM GroA	1000 µg/ml SF +10 µM GroA

Plate 3 (Treatment group: SF and GroA for PC-3 and DU-145.)

1 Control	2 Cro	3 GroA
4 100 µg/ml LMWF +10 µM Cro	5 200 µg/ml LMWF +10 µM Cro	6 300 µg/ml LMWF +10 µM Cro

Plate 4 (Treatment group: LMWF and GroA for PC-3 and DU-145.)

7 100 µg/ml LMWF +10 µM GroA	8 200 µg/ml LMWF +10 µM GroA	9 300 µg/ml LMWF +10 µM GroA

Plate 5 (Treatment group: LMWF and GroA for PC-3 and DU-145.)

3.3.2.4 Cell Harvesting

After completing their timecourse, cells were harvested and stored for the following analysis. The whole experimental process was conducted on ice.

Step 1: Cell detaching

In order to detach the cells from each of their wells, the entire medium in each well was removed into 15 mL marked centrifuge tubes. 500 µL PBS was added to wash cells and then transferred them to relevant tubes. 500 µL TrypLE™ Express Enzyme was used to detach cells and the plate was kept in the incubator for no more than 5 minutes. After detaching, the cell solution was transferred to relevant 15 mL tubes, and 500 µL PBS was added to wash each well, then collected.

Step 2: Cell centrifuging

The cells were centrifuged at 1200 RPM at 4°C for 5 minutes. After centrifuging, the supernatant in each tube was discarded. 1mL PBS was added and then cells were centrifuged again for 5 minutes, followed by discarding the supernatant.

Step 3: Dead Cell Apoptosis Kit

Cells were resuspended in 1X annexin-binding buffer. Cell density was determined and diluted in 1X annexin-binding buffer to ~1 × 10^6 cells/mL, preparing a sufficient volume to have 100 µL per assay. Add 5 µL of FITC annexin V (Component A) and 1 µL of the 100 µg/mL PI working solution (prepared in 3.3.2.2) to each 100 µL of cell suspension. Incubate the cells at room temperature for 15 minutes. After the incubation period, add 400 µL of 1X annexin-binding buffer, mix gently and keep the samples on ice.

3.3.2.5 Analysis of stained cells

As soon as possible, analyze the stained cells by flow cytometry, measuring the fluorescence emission at 530 nm (e.g., FL1) and >575 nm (e.g., FL3). The population should separate into three groups: live cells will show only a low level of fluorescence, apoptotic cells show green fluorescence, and dead cells show both red and green fluorescence. Confirm the flow cytometry results by viewing the cells under a fluorescence microscope, using filters appropriate for fluorescein (FITC) and rhodamine (TRITC) or Texas Red® dye.

3.4 Cell Cycle Assay

The cell cycle or cell-division cycle is a set of events that result in cell growth and division into two daughter cells. The cell cycle is a very orderly progression strictly following the sequence of G1-S-G2-M. The G1 phase stands for “GAP 1”, in which the cell size increases. The S phase represents “Synthesis” which is the stage for DNA replication. The G2 phase stands for “GAP 2”. Cells keep on growing in this phase. The M phase is an abbreviation of “Mitosis”, where chromosomes separate and cytokinesis occurs. Moreover, there is a G0 phase, which is a resting phase for the cells which are not in the cycle and stop dividing (Vermeulen, Van Bockstaele, & Berneman, 2003).

Flow cytometry is an important technique applied in cell cycle studies. Cellular DNA content is often the single parameter measured by flow cytometry for cell cycle studies. Once fluorescent molecules are specifically and stoichiometrically used to bind DNA, a linear relationship between cellular fluorescence intensity and DNA amount can be measured. The emitted fluorescence of the DNA-specific dyes is proportional to DNA content present in different phases of the cell cycle. PI is the most commonly used dye to quantitatively assess DNA content. PI binds to DNA by intercalating between the

bases with little or no sequence preference. Because PI also binds to RNA, nucleases such as Ribonuclease (RNAse), are necessary to distinguish between RNA and DNA (Hansen, Nielsen, & Berg, 1989).

To explore the possible mechanisms of single and combined effects of different fucoidan and GroA, PC-3 and DU-145 cells were analysed for cell cycle alterations by staining with propidium iodide (PI).

3.4.1 Major Equipment and Materials used in Assays

Table 14: Major equipment and materials used in cell cycle analysis

Number	Material / Equipment	Supplier
1	Ribonuclease A from bovine pancreas (Cat No. R4875- 100mg)	Sigma-Aldrich
2	Triton™ X-100 for molecular biology (Cat No.T8787-250ML)	Sigma-Aldrich
3	Ethanol, Anhydrous (Histological grade) (Cat No. 64-17-5)	Sigma-Aldrich
4	PBS (Phosphate buffered saline), pH 7.2	Life technologies
5	Flow cytometer	Beckman coulter
6	Six well plate	BD (Becton Dickinson) Bioscience (Auckland, NZ)
7	Test Tube, 12 x 75 mm, Polypropylene Blue (250/ pack) (Cat No.63058857)	Beckman coulter

3.4.2 Protocols for Cell Cycle Analysis

3.4.2.1 80% Ethanol Preparation

Absolute ethanol was diluted with distilled water to a final value of 80%. The 80% ethanol solution was kept in a parafilm-sealed tube at -20℃.

3.4.2.2 RNAse A Solution Preparation

The amount of RNAse A (Ribonuclease A) powder in the manufacturer’s vial was 100mg. RNAse A was dissolved in distilled water (DDW) at 1mg/mL as a stock solution. The total solution was separated into 1.5mL micro-tubes and kept at -20℃.

3.4.2.3 PI Stock Solution Preparation

The amount of PI powder in the vial was 10 mg. 10mL distilled water (DDW) was used to dissolve PI powder and prepare a 1mg/ml stock solution. The total solution was separated in 1.5 mL micro-tubes wrapped with aluminium-foil and then stored at 4℃.

3.4.2.4 Cell Preparation and Drug Treatment

Step 1: Cell Seeding

After detaching, counting and diluting cells, cells were seeded onto 6-well plates. The seeding density for all cell lines in this study was 40,000 cells/mL and a 2 mL cell solution was contained in each well. The plates were kept in a 37 ℃ incubator for 6 to 24 hours to ensure almost all of the cells attached to the walls of the wells.

Step 2: Cell (0% serum) starvation

When the cell attachment rate was the highest, cells were treated with 2 mL of 0% FBS medium (with 1% Penicillin and 1% L-glutamine) in each well, for synchronizing cell proliferation. One plate set in this study was a control group and other plates were designed as treatment groups (see Table19). In Plate 1(no treatment group), Well Nos.A, B and C were treated with 2 mL of 0% FBS medium, and Well Nos. D, E and F were filled with 2 mL of complete culture (10%) medium. Before adding the fresh medium (0% or 10% FBS medium), the entire old medium was removed. The medium was added slowly and gently in circles along the wall of the well. For Plate 2, 3, 4, and 5 (treatment groups), all wells received 2 mL of 0% FBS medium. The plates were kept in the 37 ℃ incubator for 24 hours.

Table 15: Plate design for cell cycle assay

A starvation	B starvation	C starvation
D control with 2 mL complete culture medium	E control with 2 mL complete culture medium	F control with 2 mL complete culture medium

Plate 1 (Blank treatment group)

1 Control	2 Cro	3 GroA
4 500 µg/ml SF +10 µM Cro	5 750 µg/ml SF +10 µM Cro	6 1000 µg/ml SF +10 µM Cro

Plate 2 (Treatment group: SF and GroA for PC-3 and DU-145.)

7 500 µg/ml SF +10 µM GroA	8 750 µg/ml SF +10 µM GroA	9 1000 µg/ml SF +10 µM GroA

Plate 3 (Treatment group: SF and GroA for PC-3 and DU-145.)

1 Control	2 Cro	3 GroA
4 100 µg/ml LMWF +10 µM Cro	5 200 µg/ml LMWF +10 µM Cro	6 300 µg/ml LMWF +10 µM Cro

Plate 4 (Treatment group: LMWF and GroA for PC-3 and DU-145.

7 100 µg/ml LMWF +10 µM GroA	8 200 µg/ml LMWF +10 µM GroA	9 300 µg/ml LMWF +10 µM GroA

Plate 5 (Treatment group: LMWF and GroA for PC-3 and DU-145.)

All treatments were kept in 15 mL centrifuge tubes which were prepared with complete culture (10% FBS) medium. The old 0% medium was removed gently first and then 2 mL well-mixed treatment solution was carefully added into designated wells. The control well was set in the treatment group plate with 2 mL complete culture medium. All of the plates were kept in the incubator for 72 hours (day 3) (Table19). Cell collection was needed for plate 1 at day 0 samples. The design of the 6-well plate is shown in Table 19-Plate 2, 3 ,4 and 5. All the concentrations decided for this part of the study were the same as Section 3.2.12. Namely, SF 500, 750, and 1000 µg/ml, LMWF 100, 200, and 300 µg/ml and GroA 10 µM, were used to treat PC-3 and DU-145 jointly.

The combination plan and dilution plan for different fucoidan and GroA/Cro preparations were the same as described in combination treatment study 3.2.12 table14, 15.

3.4.2.5 Cell Harvesting

After cells were cultured for their determined time, they were harvested and stored for the following analysis. The whole experiment process was conducted on ice.

Step 1: Cell detaching

In order to detach the cells from each of their wells, the entire medium in each well was removed into 15 mL marked centrifuge tubes. 500 µL PBS was added to wash cells and then transferred into relevant tubes. 500 µL TrypLE™ Express Enzyme was used to detach cells and the plate was kept in the incubator for no more than 5 minutes. After detaching, the cell solution was transferred to relevant 15mL tubes, and 500 µL PBS was added to wash each well, then collected.

Step 2: Cell centrifuging

The cells were centrifuged at 1200 RPM at 4°C for 5 minutes. After centrifuging, the supernatant in each tube was discarded. 1 mL PBS was added and then cells were centrifuged again for 5 minutes.

Step 3: Cell mixing

After centrifugation, most of the supernatant in each tube was discarded and a little of PBS was left inside. The tube rack was used to mix the cells with the rest of PBS. Tubes were swiped left and right 3 times to re-suspend and mix cells.

Step 4: Cell fixing and storing

1 mL ice cold 80% ethanol was added into each tube. (Each tube was previously stored in the -20℃ freezer). The vortex was set at low speed and ethanol was added slowly. The tube was tilted diagonally so the ethanol was added to the sides and not directly onto the cells, thereby avoiding formation of aggregates. Each tube was sealed with parafilm and kept at -20℃ for at least overnight and not more than 10-14 days.

3.4.2.6 Cell Cycle Analysis

Step 1: Permeabilizing solution preparation

The permeabilizing solution consisted of a total of 1ml per test tube: 0.1% Triton X100 (1μL for each 1 mL) + RNAse A (50 μg/mL from stock solution of 1 mg/mL). For example for 20 tubes, 20 μL Triton-X100 + 1000 μL RNAse A + 19,980 μL PBS was prepared.

Step 2: Ethanol removal

All tubes were centrifuged first at 1200RPM for 2 minutes after removing the parafilm from the outside of the tubes. The ethanol was gently removed and 3mL ice cold PBS was added to each tube, followed by a second centrifugation step. After the second centrifugation, another 3 mL ice cold PBS was added to each tube to replace the initial wash. In total, the cells were washed twice, each time with 3 mL ice cold PBS.

Step 3: PI staining

In the process of PI staining, the supernatant in each well was gently removed and then 1mL well-mixed permeabilizing solution was added to each tube. All cells in tubes were carefully mixed before being transferred to test tubes and then incubated at

37℃ for 30-45 minutes. After permeabilization, 5 μg/mL of PI (5 μL to 1ml in each tube) was added to each test tube and kept for 5 minutes. Finally all tubes were run under the flow cytometer. The optimal voltage for each sample running is attached in Appendix A6.

3.5 Data Analysis

3.5.1 Analysis of MTT Assay Results

IC50, the half maximal inhibitory concentration, is commonly used as a measure of drug effectiveness. IC50 was an important reference to measure the inhibitory effect of gemcitabine and fucoidan in this study. It was calculated by PRISM® software (Graphpad, Version 7.0) and the IC50 values were obtained by using dose-response inhibition, nonlinear regression (curve fit): Log (inhibitor) vs. Response-Variable slope (four parameters).

3.5.2 Analysis of Cell Apoptosis

Kaluza® Flow Cytometry Analysis Software (Version 1.3) bought from Beckman Coulter was the software applied in cell apoptosis results analysis to measure the cell apotosis values determined in this study.

3.5.3 Analysis of Cell Cycle Assay Results

Kaluza® Flow Cytometry Analysis Software (Version 1.3) bought from Beckman Coulter was the software applied in cell cycle results analysis to measure the cell cycle distribution in this study.

3.6 Statistical Analysis

All experiments in this study were performed at least three times. Statistical differences in multiple groups were determined by one-way and two-way ANOVA (Analysis of Variance) with PRISM® software (Graphpad, Version 7.0) and SPSS (Version 22.0). Statistical comparisons were made by Post Hoc (Turkey's test). Analysis between two groups was determined by using unpaired Student's t test Data, and are expressed as means ± S.D. (standard deviation). Differences with $p < 0.05$ were considered significant and $p < 0.01$ were considered very significant.

Chapter 4 Results

4.1 Single Inhibitory Effect of LMWF and SF on PC-3 and DU-145 Prostate Cancer Cell Lines

LMWF and SF from *Undaria pinnatifida* were tested for their inhibitory effect on PC-3 and DU-145 cell lines using the MTT cell proliferation assay. The experimental results for the assay for each cell line are presented in the figures below. For each cell line the linearity was established first, followed by determine the inhibitory effect of two types of fucoidan against each cell line tested in this study.

4.1.1 Linearity of MTT Assay for PC-3 and DU-135 Cell Lines

A standard (linearity) curve, comparing cell number vs. absorbance was important in establishing a starting point in the MTT assay portion of the study. A good linearity curve reflects a good growing status of the cells. Cell number used in cell viability studies should fall within the linear portion of the curve. Cell seeding concentrations can neither be too high nor too low, so that a good growing condition for cells can be guaranteed.

4.1.1.1 PC-3 cell line

Figure 3 shows the linearity curve of PC-3 cells. It shows a good linear relationship (R^2 = 0.9919) between the absorbance, determined at 540nm wavelength, and different cell densities (from 250,000 cells/mL to 976 cells/mL).

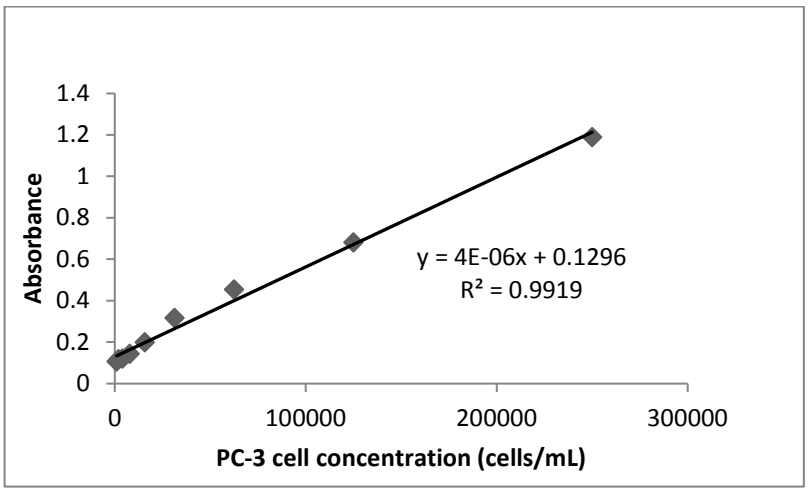


Figure 3: Linearity between PC-3 cell numbers and absorbance values.

Data are presented as means ± S.D., n=6.

4.1.1.2 DU-145 cell line

The cell number vs. absorbance linearity curve of DU-145 is given in Figure 4. The linear relationship between the absorbance determined at 540 nm wavelength and different cell densities (from 250,000 cells/mL to 976 cells/mL) is very good (R^2 = 0.9968). This result indicates the DU-145 cells grew very well in the culture conditions and they were ready for the subsequent experiments. This curve shows that cell concentration around 50,000 cells/mL fits the curve very well, which is neither too crowed nor too sporadic and falls within the linear portion of the curve. Thus, a cell density of 40,000 cells/mL used in MTT and cell cycle assays was determined to be reasonable.

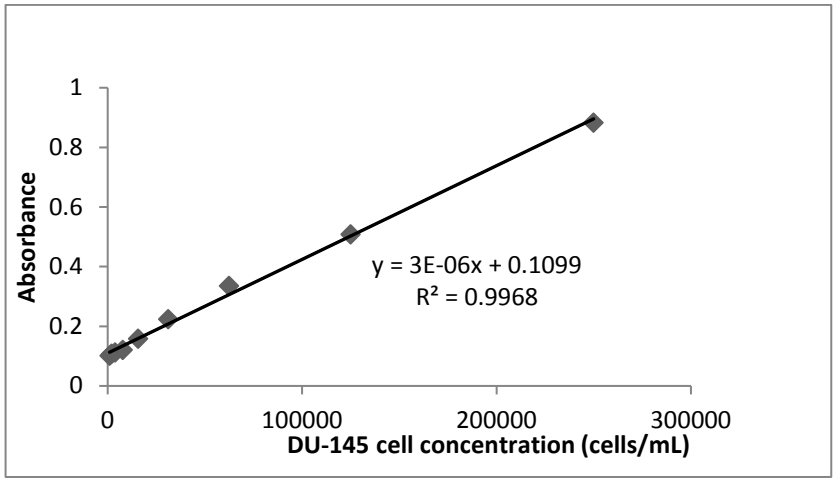


Figure 4: Linearity between DU-145 cell numbers and absorbance values.

Data are presented as means ± S.D., n=6.

4.1.2 Single Inhibitory Effect of LMWF on PC-3 and DU-145 Cell Lines

In this study, LMWF was first examined in order to explore its effect on the cell viability of PC-3 and DU-145 cell lines.

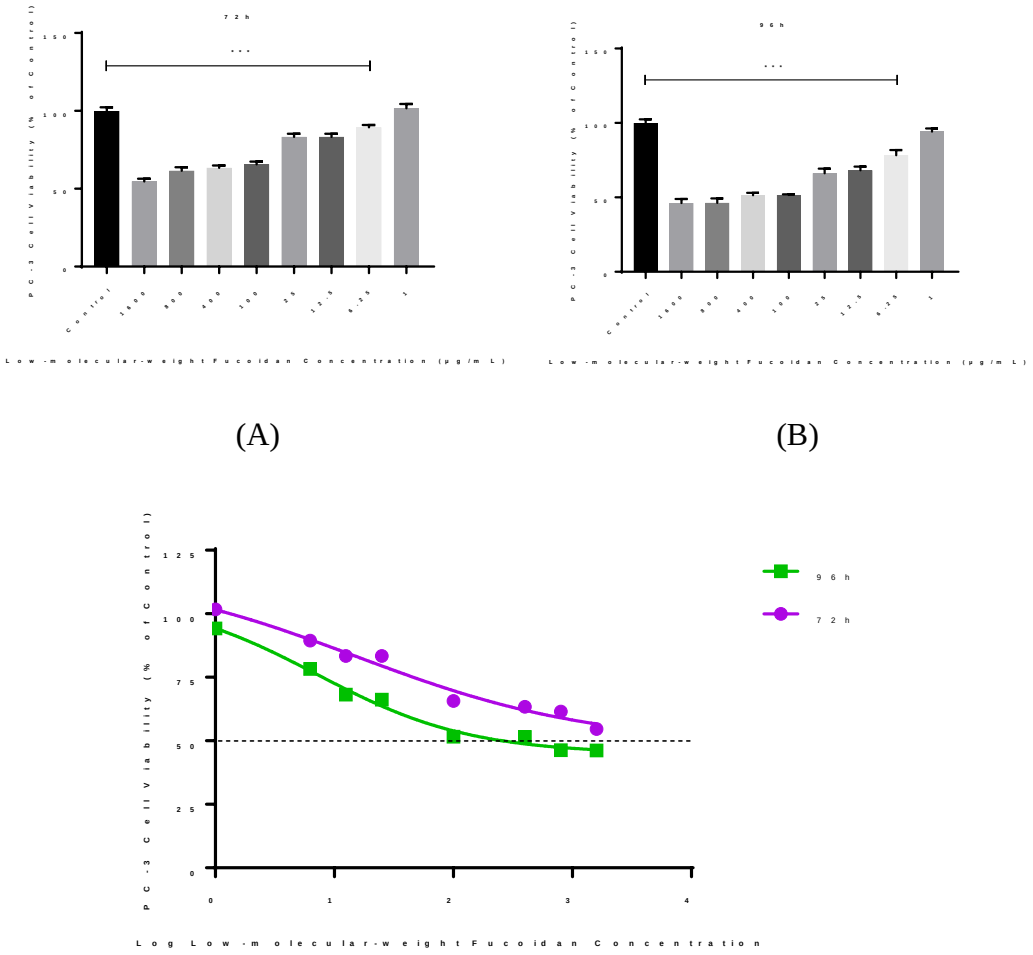
Culture of PC-3 and DU-145 cells with different concentrations of LMWF for 72 and 96 hours resulted in very significant suppression of cell viability in a dose-dependent manner ($P < 0.001$) and the IC_{50} of 96 hours was around 400 µg/ml in PC-3 and 420 µg/ml in DU-145. The IC_{50} was more than 1600 µg/ml in both cell lines after 72 hours treatment. In addition, observations under an inverted microscope showed that

PC-3 and DU-145 cells in the previous two days (culturing for 24 and 48 hours) presented no remarkable morphological changes. However, after 72 hours and 96 hours cell density testing, the cell attachment rate reduced with the increase of LMWF concentration, compared with control groups.

Figure 5(C) and 6(C) show that the cell viability vs. Log LMWF concentration curve fits well with a sigmoidal model of negative exponential distribution in two cell lines. According to one-way ANOVA testing, both cell lines had no significant difference ($p > 0.05$) of cell viability between LMWF 1 µg/ml and control, or 25 µg/ml and 12.5 µg/ml in 72 hour and 96 hour groups. Additionally, LMWF concentration from 1600-100 µg/ml showed about 54-65% cell death at 72 hours, and 56-51% in 96 hour samples of PC-3 and DU-145 cells (Figure 5 (A)(B) and 6(A)(B)), indicating that the concentrations of LMWF from 1600 µg/ml to 100 µg/ml were responsible for approximately 50-60% of observed cell death.

LMWF was decreased by 16-fold from 1,600 to 100 µg/ml, while the cell viability was only about 11% lower than that observed in the highest drug concentration at the 72 hour time point, and around 5% in 96 hour samples, across both cell lines. In the higher concentration groups, numerous morphological changes were observed. Based on these findings, LMWF concentrations from 300 µg/ml to 100 µg/ml were chosen in the following combination study for PC-3 and DU-145 cell lines.

4.1.2.1 PC-3 cell line



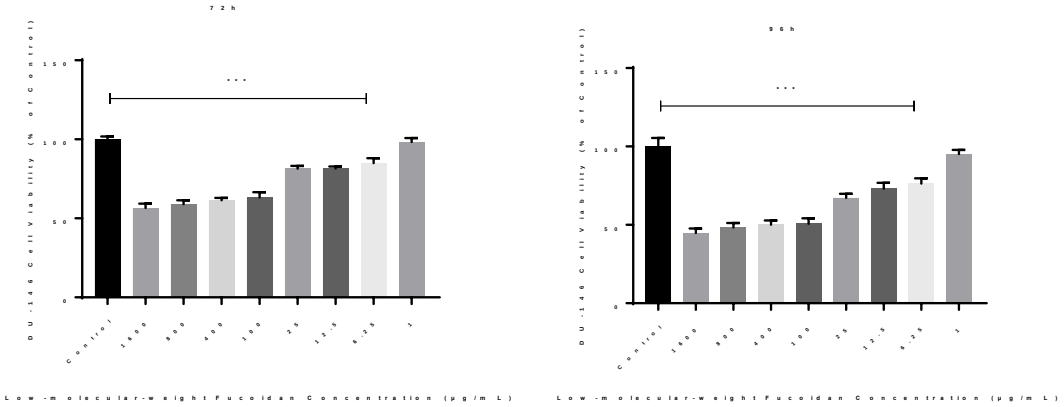
(A) (B)

(C)

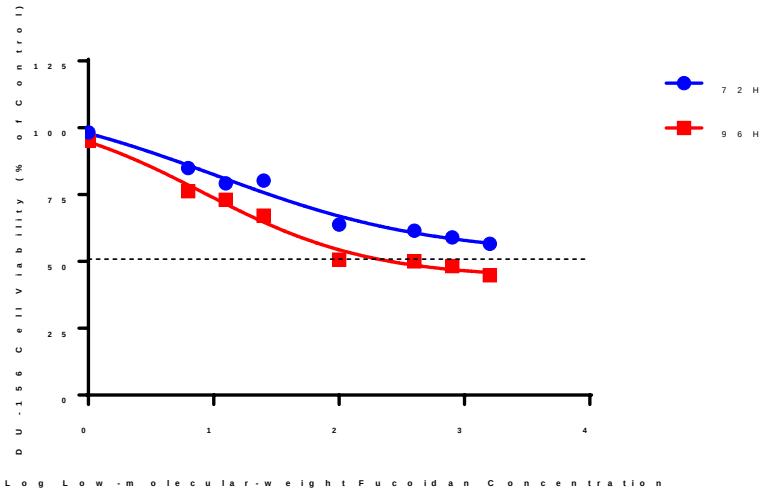
Figure 5: Inhibitory effect of LMWF on the growth of PC-3 cells at 72 hours and 96 hours.

Cells were incubated in the presence of various concentrations of LMWF (1, 6.25, 12.5, 25, 100, 400, 800 and 1600 µg/ml). A relative viability of 100% was designated as the total number of cells that grew in 72 hour and 96 hour cultures in the absence of LMWF. Data are presented as means ± S.D., n=6. Asterisks indicate a value significantly different from the control value, ***$p < 0.001$ (Student's t test).

4.1.2.2 DU-145 cell line



(A) (B)



(C)

Figure 6: Inhibitory effect of LMWF on the growth of DU-145 cells at 72 hours and 96 hours.

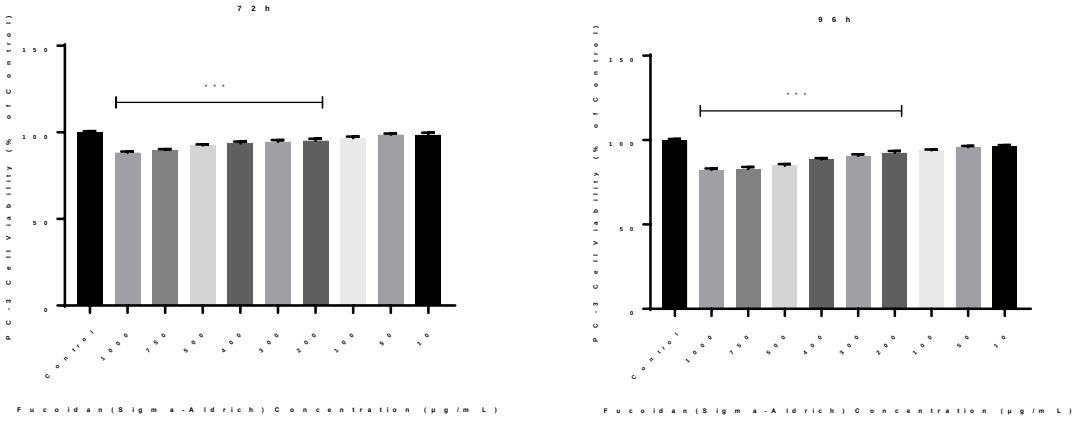
Cells were incubated in the presence of various concentrations of LMWF (1, 6.25, 12.5, 25, 100, 400, 800 and 1600 µg/ml). A relative viability of 100% was designated as the total number of cells that grew in 72 hour and 96 hour cultures in the absence of LMWF. Data are presented as means ± S.D., n=6. Asterisks indicate a value significantly different from the control value, ***$p < 0.001$ (Student's t test).

4.1.3 Single Inhibitory Testing of SF on PC-3 and DU-145 Cell Lines

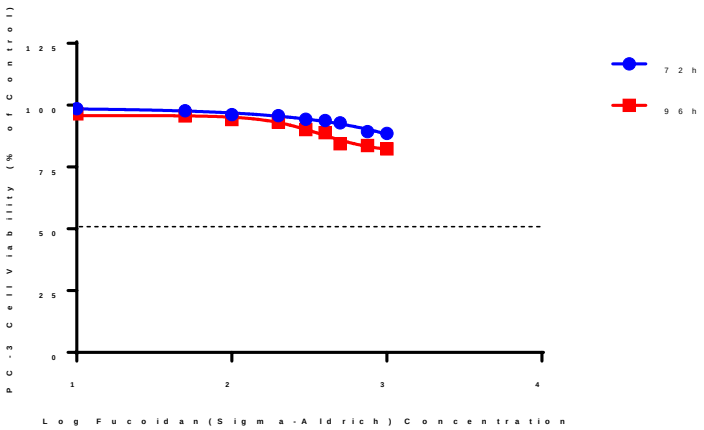
SF inhibited the proliferation of PC-3 and DU-145 cells depending upon its treatment time and concentration. Even though the anti-cancer ability of fucoidan has been reported in different cancer cell lines, this study's highest concentration of 1,000 µg/ml SF could only induce 17 % cell death in PC-3 cells and 16 % cell death in DU-145 cells compared to control, under 72 hour and 96 hour treatment schedules (Figure 7)(A)(B) (Figure 8)(A)(B). In the higher concentration groups, numerous morphological changes were observed. SF was decreased by 100 fold from 1,000 to 10 µg/ml, while the PC-3 and DU-145 cell viability was only about 8% lower than that observed in the highest drug concentration. In the previous 24 and 48 hour samples, there were no substantial morphological changes observed under the microscope.

Based on this result, a higher Sigma SF concentration range (1000, 750, and 500 µg/ml) was instituted, while keeping the incubation times at 72 hours and 96 hours.

4.1.3.1 PC-3 cell line



(A) (B)

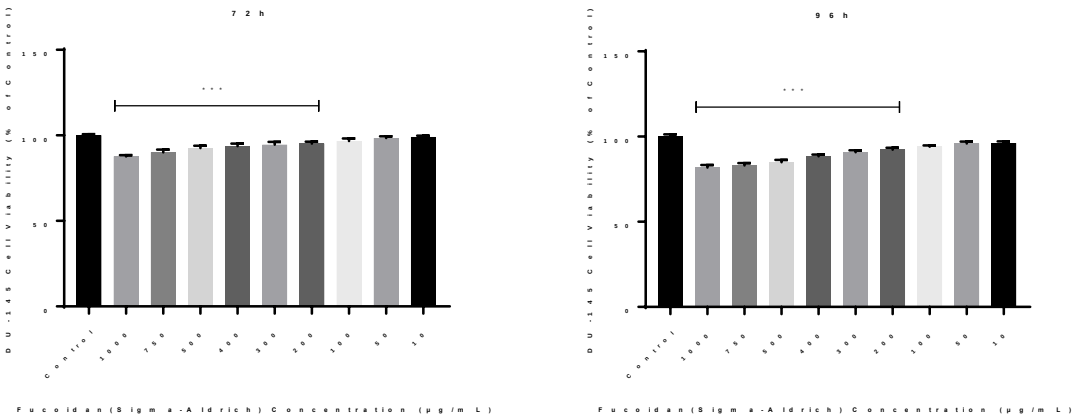


(C)

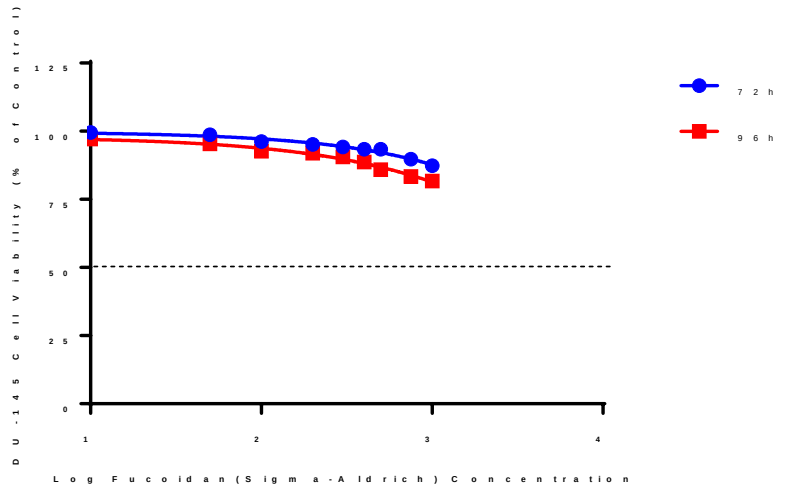
Figure 7: Inhibitory effect of SF on the growth of PC-3 cells at 72 hours and 96 hours.

Cells were incubated in the presence of various concentrations of SF (10, 50, 100, 200, 300, 400, 500, 750 and 1000 µg/ml). A relative viability of 100% was designated as the total number of cells that grew in the 72 hour and 96 hour cultures in the absence of SF. Data are presented as means ± S.D., n=6. Asterisks indicate a value significantly different from the control value, ***$p < 0.001$ (Student's t test).

4.1.3.2 DU-145 cell line



(A) (B)



(C)

Figure 8: Inhibitory effect of SF on the growth of DU-145 cells at 72 hours and 96 hours.

Cells were incubated in the presence of various concentrations of SF (10, 50, 100, 200, 300, 400, 500, 750 and 1000 µg/ml). A relative viability of 100% was designated as the total number of cells that grew in the 72 hour and 96 hour cultures in the absence of SF. Data are presented as means ± S.D., n=6. Asterisks indicate a value significantly different from the control value, ***$p < 0.001$ (Student's t test).

4.1.4 Single Inhibitory Effect of FS compared with LMWF on PC-3 and DU-145 Cell Lines

Based on previous studies, different LMWF concentration ranges (100, 200 and 300 µg/ml) and different FS concentration ranges (500, 750 and 1000 µg/ml) were prepared to treat PC-3 and DU-145 cells in this portion of the study. Fucoidan reduced cell viability in a dose- dependent manner (Figure 9, 10).

Figures 9 and 10 present the PC-3 and DU-145 cell viabilities at 500, 750 and 1000 µg/ml FS concentrations, at 72 hour and 96 hour timepoints. Viability is higher with FS compared to LMWF concentrations at 100, 200 and 300 µg/ml. In the PC-3 cell line, the cell viabilities have no significant differences ($P>0.05$) between three different FS concentrations. Viability at 72 hours was 85-90%, and at 96 hours was approximately 82%. DU-145 cell viability showed the same tendency. Alternatively, LMWF (300 µg/ml) treatments showed that the cell viability was remarkably decreased after 72 hours and 96 hours, in both the PC-3 and DU-145 cell lines.

4.1.4.1 PC-3 cell line

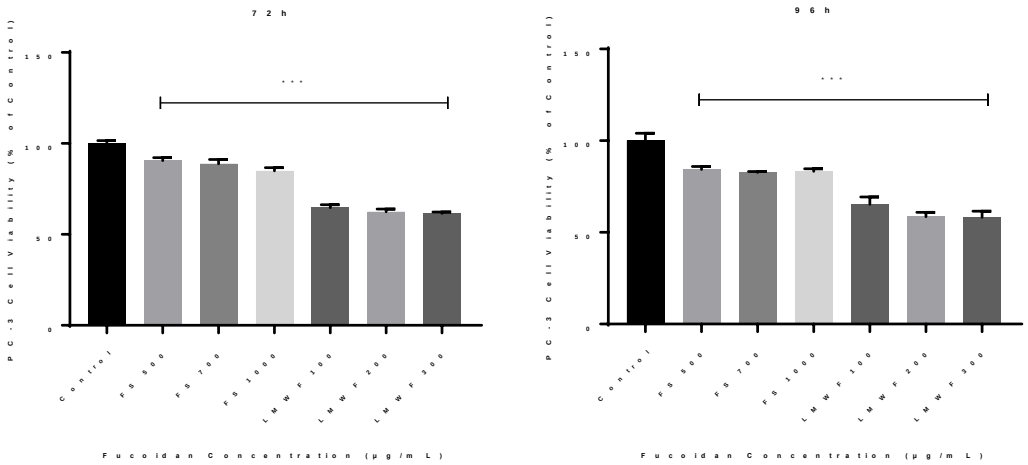


Figure 9: Inhibitory effect of SF compared with LMWF on the growth of PC-3 cells at 72 hours and 96 hours.

Cells were incubated in the presence of various concentrations of LMWF (100, 200 and 300 µg/ml), SF (500, 750 and 1000 µg/ml). A relative viability of 100% was designated as the total number of cells that grew in 72 hour and 96 hour cultures in the absence of LMWF and SF. Data are presented as means ± S.D., n=6. Asterisks indicate a value significantly different from the control value, ***$p < 0.001$ (Student's t test).

4.1.4.2 DU-145 cell line

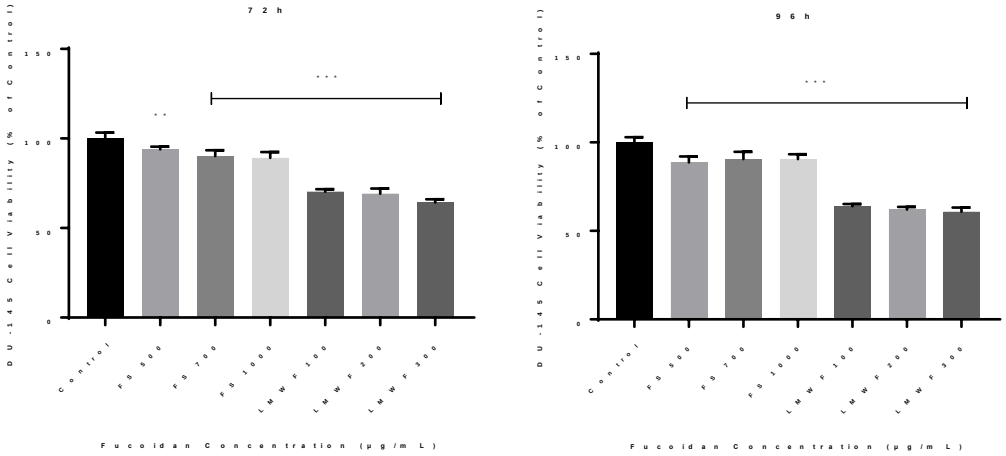


Figure 10: Inhibitory effect of SF compared with LMWF on the growth of DU-145 cells at 72 hours and 96 hours.

Cells were incubated in the presence of various concentrations of LMWF (100, 200 and 300 µg/ml), SF (500, 750 and 1000 µg/ml). A relative viability of 100% was designated as the total number of cells that grew in 72 hour and 96 hour cultures in the absence of LMWF and SF. Data are presented as means ± S.D., n=6. Asterisks indicate a value significantly different from the control value, **p < 0.01, ***p < 0.001 (Student's t test).

4.2 Joint Inhibitory Effect of LMWF and GroA compared with SF and GroA on PC-3 and DU-145 Prostate Cancer Cell Lines

From previous single treatment studies, it's known that both LMWF and SF showed inhibitory abilities, particularly LMWF, but only for the high concentration treatment. For SF, the result was not as satisfactory. Hence, a joint inhibitory effect study of these drugs was conducted in order to investigate whether LMWF and GroA/Cro, or SF and GroA/Cro can make a stronger inhibitory effect at lower concentration ranges when combined together.

The optimum concentration of GroA/Cro in this study was 10µM which is based on a previous inhibitory study (Goldshmit et al., 2014).

Some reasonable GroA/Cro and fucoidan concentrations were chosen to treat prostate cancer cell lines PC-3 and DU-145, based on previous single treatment results.

For both of cell lines, the chosen GroA/Cro concentration was 10µM, whereas, LMWF concentrations were 100, 220, and 300 µg/ml. For the other group GroA and Cro concentrations were also 10 µM, however SF concentrations were 500, 750 and 1000 µg/ml. Combinations of the selected drugs at their different concentrations were used to treat the cancer cells and the controls.Single drug treatment groups were also set up at the same time. Thus, there were eight treatment groups and one control group in the PC-3 and DU-145 cell line cytotoxicity assays.

4.2.1 Joint Inhibitory Effect of LMWF and GroA on PC-3 and DU-145 Cell Lines

Based on single drug experiments, the incubation time determined for combination drug treatments of PC-3 cells was 72 hours and 96 hours. Figure 11 shows the joint inhibitory effect of LMWF with the aptamer GroA and the inactive oligomer Cro on PC-3 cells. Cells incubated with LMWF (100, 200 and 300 µg/ml) and with Cro for 72 hours, show an LMWF inhibitory effect on the cells, with viability at 53-63%. As for cells treated with GroA (10µM) alone for 72 hours incubation, the cell viability was about 60%. The cell viability in groups treated with a combination of GroA and fucoidan for 72 hours, is shown in Figure 11. GroA 10 µM combined with 100, 200 and 300µg/ml fucoidan significantly reduced the cell viability by 8%, 18% and 16% respectively, as compared with GroA 10µM applied to treat cells alone. Likewise, the joint enhanced inhibitory effect of the 96 hour treatment group was slightly higher than the 72 hour group. Compared with GroA 10µM applied to treat cells alone, the cell viability reduced around 13%, 19% and 21% respectively, and the proliferation rates of PC-3 cells were all under 50%. As observed with the PC-3 treatment trends, the DU-145 cell line shows similar inhibition rates in response to treatments. According to two-way ANOVA statistical analysis, the interaction between LMWF plus Cro and LMWF combined with GroA was very significant ($p < 0.001$) (Figure 12). In all groups, increasing the concentration of LMWF combined with GroA gradually reduced the cell viability of PC-3 and DU-145 cells. Hence, fucoidan was able to enhance the inhibitory effect of GroA on PC-3 and DU-145 cell proliferation in a concentration dependent manner, even at small concentrations.

4.2.1.1 PC-3 cell line

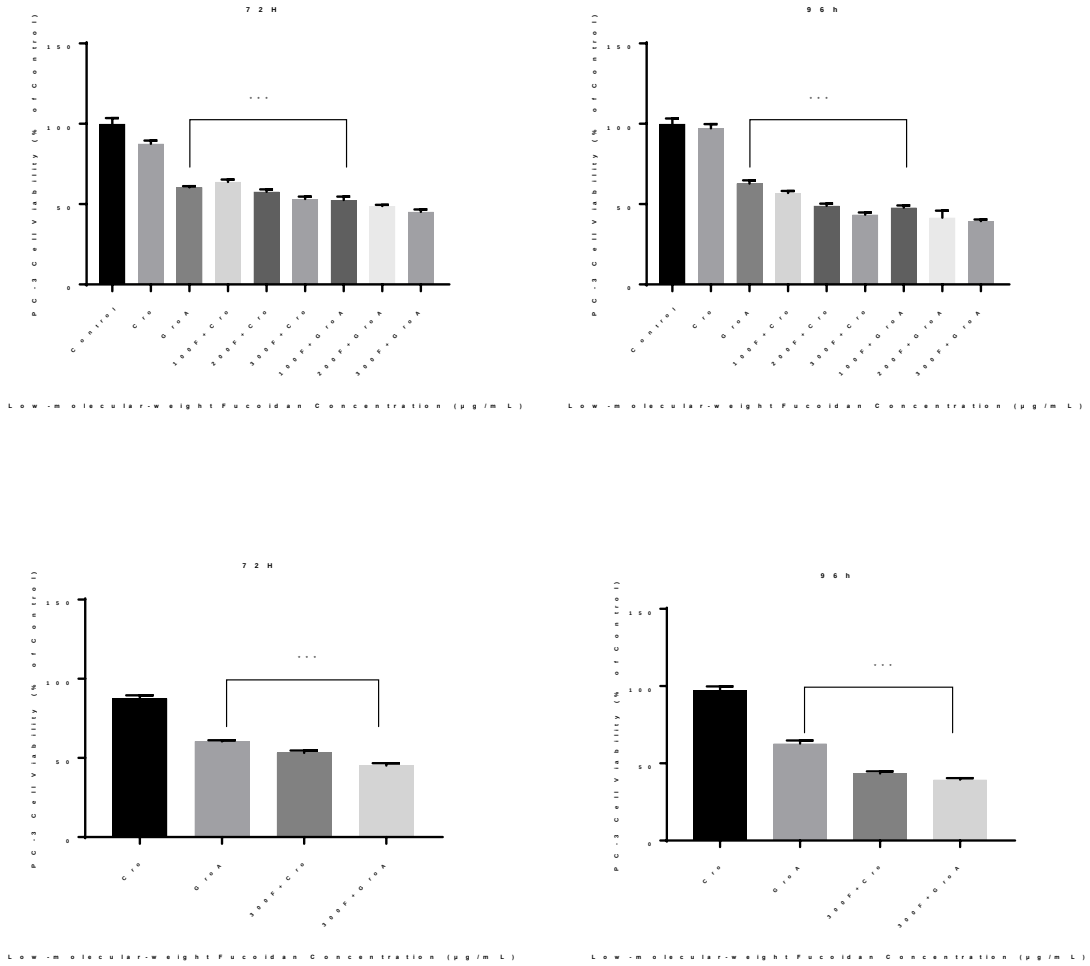


Figure 11: Joint inhibitory effect of GroA/Cro and LMWF on the growth of PC-3 cells at 72 and 96 hours.

Data are presented as means ± S.D., n=6. Asterisks indicate a value significantly different, ***$p < 0.001$ (Student's t test).

4.2.1.2 DU-145 cell line

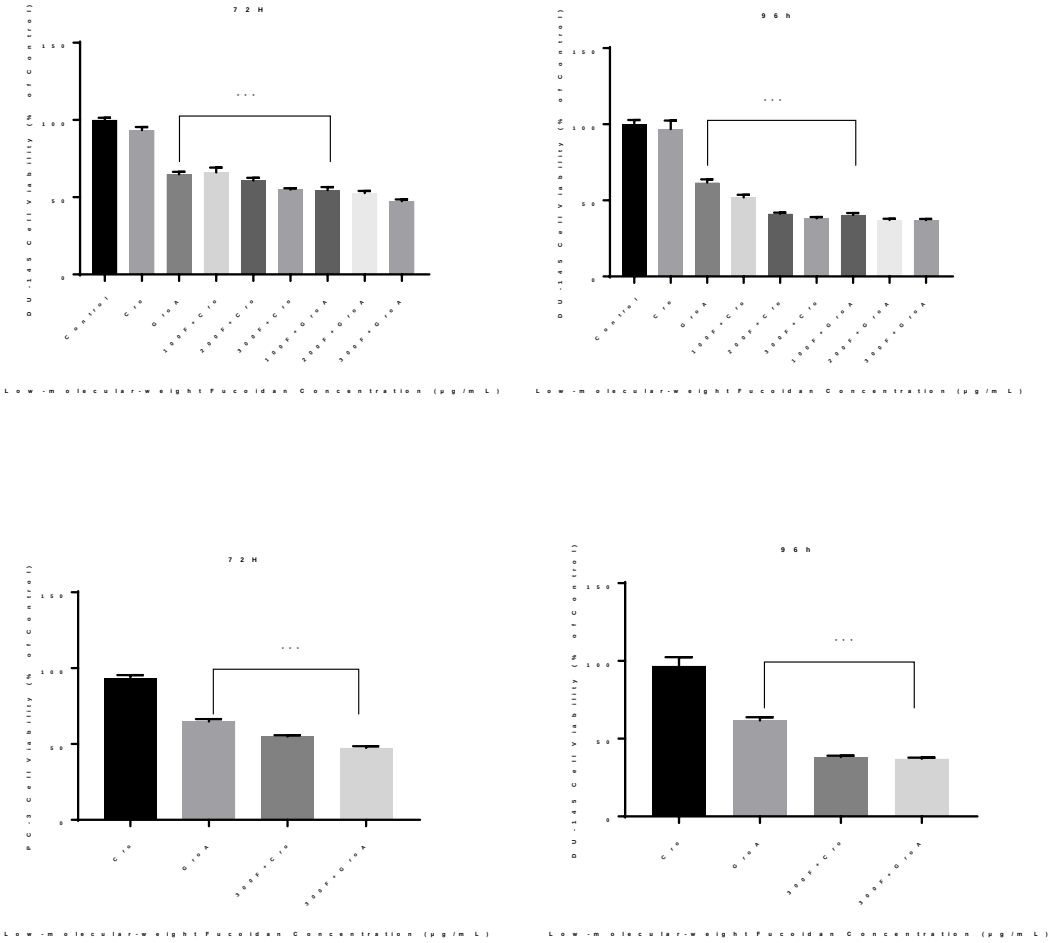


Figure 12: Joint inhibitory effect of GroA/Cro and LMWF on the growth of DU-145 cells at 72 and 96 hours.

Data are presented as means ± S.D., n=6. Asterisks indicate a value significantly different, ***$p < 0.001$ (Student's t test).

4.2.2 Joint Inhibitory Effect of SF and GroA on PC-3 and DU-145 Cell Lines

Based on this study's single treatment results, a conclusion was made that the SF lower concentration preparations were not effective against PC-3 and DU-145 cell lines compared to LMWF effectiveness against the same cell lines. Therefore, higher concentrations of SF (500, 750 and 1000 µg/ml) and GroA (10µM) were prepared to test against PC-3 and DU-145 cell lines. The treatment times for these drugs were 72 hours and 96 hours.

The joint inhibitory effect of SF and GroA on PC-3 cells is shown in Figures 13 and 14, and the specific cell viability values under different concentrations of drugs are listed in Appendixes 2 and 3. After 72 hours of incubation with either 500, 750 or 1000 µg/ml SF and Cro, the number of viable cells did not show remarkable inhibitory effect (10%), as compared to the control. As for cells treated with GroA (10µM) alone for 72 hours incubation, the cell viability was about 60%. When PC-3 cells were cultured in GroA and SF for the same amount of time, there was no significant difference in viability compared with GroA alone ($p > 0.5$) according to two-way ANOVA statistical analysis. This is also the result for DU-145 cell line groups.

In all groups, an increasing concentration of FS combined with GroA yielded no difference in the cell viability of PC-3 and DU-145 cell line. Hence, FS was not able to enhance the inhibitory effect of GroA on proliferation of PC-3 and DU-145 cells in a concentration dependent manner, even at a very high concentration range.

4.2.2.1 PC-3 cell line

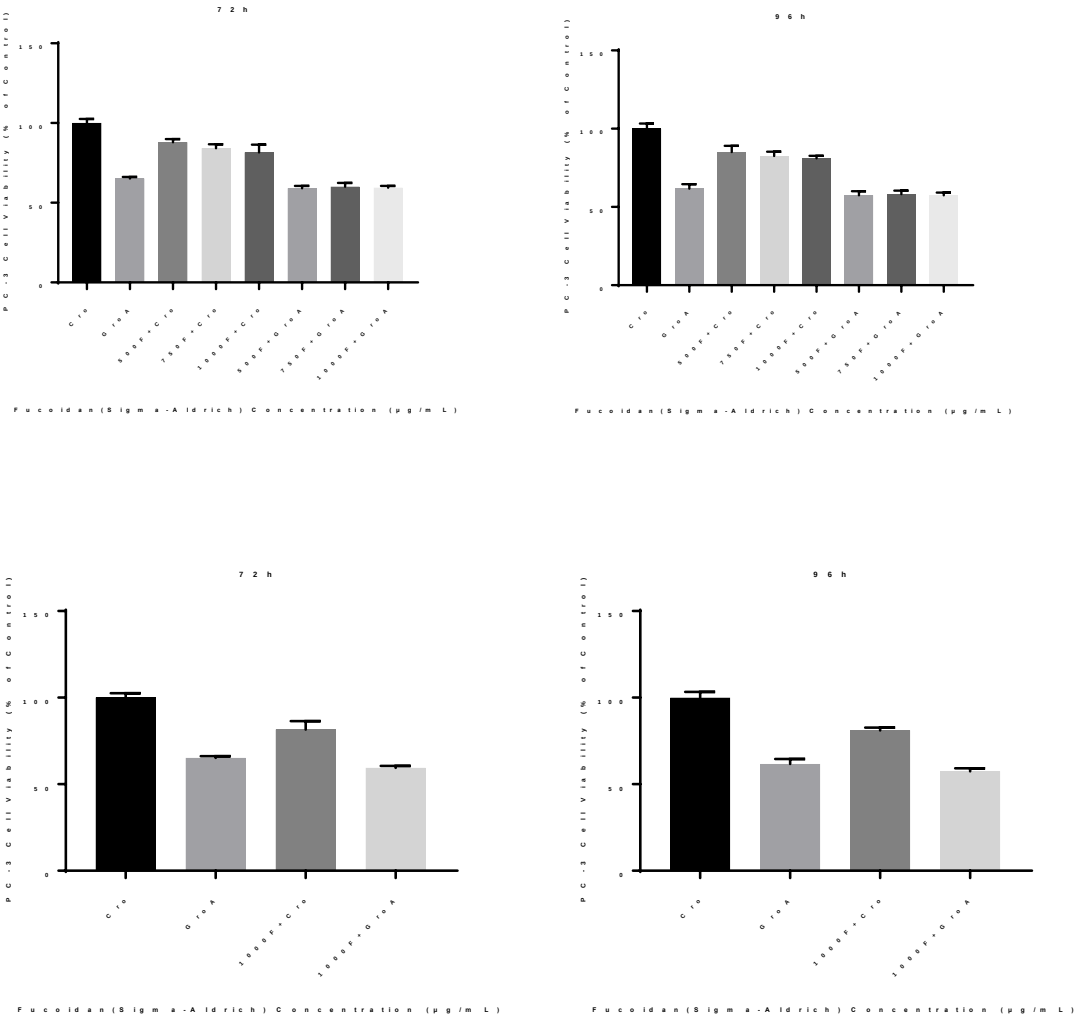


Figure 13: Joint inhibitory effect of GroA/Cro and FS on the growth of PC-3 cells at 72 and 96 hours.

Data are presented as means ± S.D., n=6.

4.2.2.2 DU-145 cell line

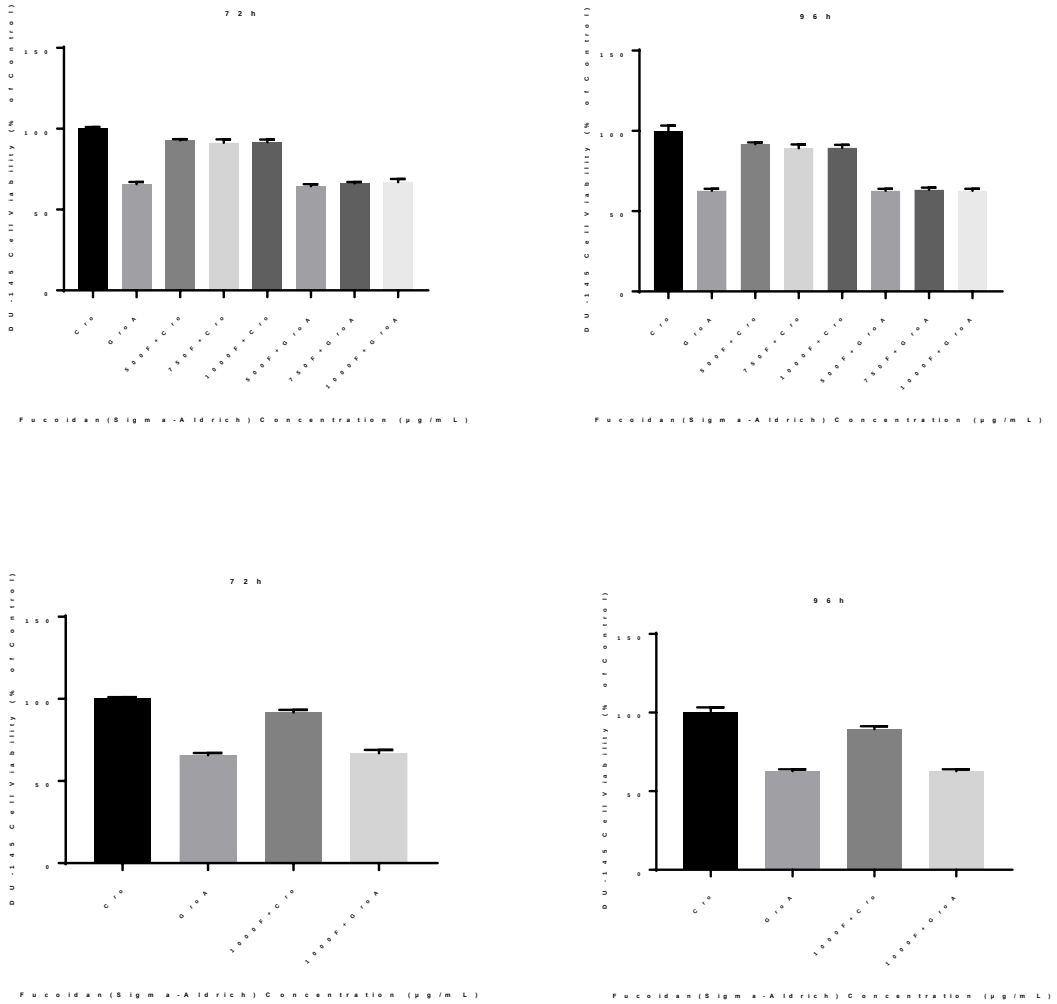


Figure 14: Joint inhibitory effect of GroA/Cro and FS on the growth of DU-145 cells at 72 and 96 hours.

Data are presented as means ± S.D., n=6.

4.3 Joint Effects of LMWF and GroA compared with SF and GroA in cell apoptosis assays.

Single or combination effects of GroA and LMWF, or GroA and SF in cell apoptosis assays of human prostate cancer cell lines PC-3 and DU-145 were studied. The cells were treated with either GroA or LMWF/SF alone, or both combined, for 72 hours. Flow cytometry allowed the measurement of several apoptotic traits in a single sample, making it a powerful tool to study the complexity of cell death. Each experiment was performed in triplicate.

4.3.1 Effects of LMWF and GroA on cell apoptosis in 72 hour treatment samples.

4.3.1.1 DU-134 cell line

Figure 15 (A) indicates that the blank treatment group (72 hours culture time in complete medium) of DU-145 cells has a percentage of viable cells at 97.3%, and the total apoptotic cells was around 2%.

The DU-145control group (Fig. 15 (B)) indicates that the distribution of cell apoptosis was not changed by incubation with 10 μM Cro. GroA 10μM did have some effect on the cell line: the percentage of viable cells decreased about 30% whereas early apoptotic cells and late apoptotic cells increased 23% and 5% respectively compared to the blank treatment group.

Combination treatments using Cro 10 μM with different concentrations of LMWF (100, 200 and 300 μg/ml) for 72 hours, resulted in enrichment of DU-145 cells in viable cells (from 97.08% to 77.76%, 68.32% and 51.43%), compared with Cro 10 μM alone (Fig. 15 (C)). In addition, the percentage of late apoptotic cells was increased with the concentration of LMWF, which was 7.07%, 7.05% and 11.07%, and the same trend was observed with the early apoptotic cells (11.91%, 16.49% and 18.16%). Comparison of GroA 10 μM treated cells alone, with LMWF (100, 200 and 300 μg/ml) combined with GroA 10 μM shows an induction of cellular apoptosis, with the percentage of total apoptotic cells higher (from 28% to 31%, 50% and 58%), and the percentage of viable cells reduced with the increase of LMWF concentrations.

Therefore, combination of LMWF with GroA caused a significant increase in apoptosis of DU-145 cells in 72 hour treatment samples.

Fig. 15 (A) Blank treatment group

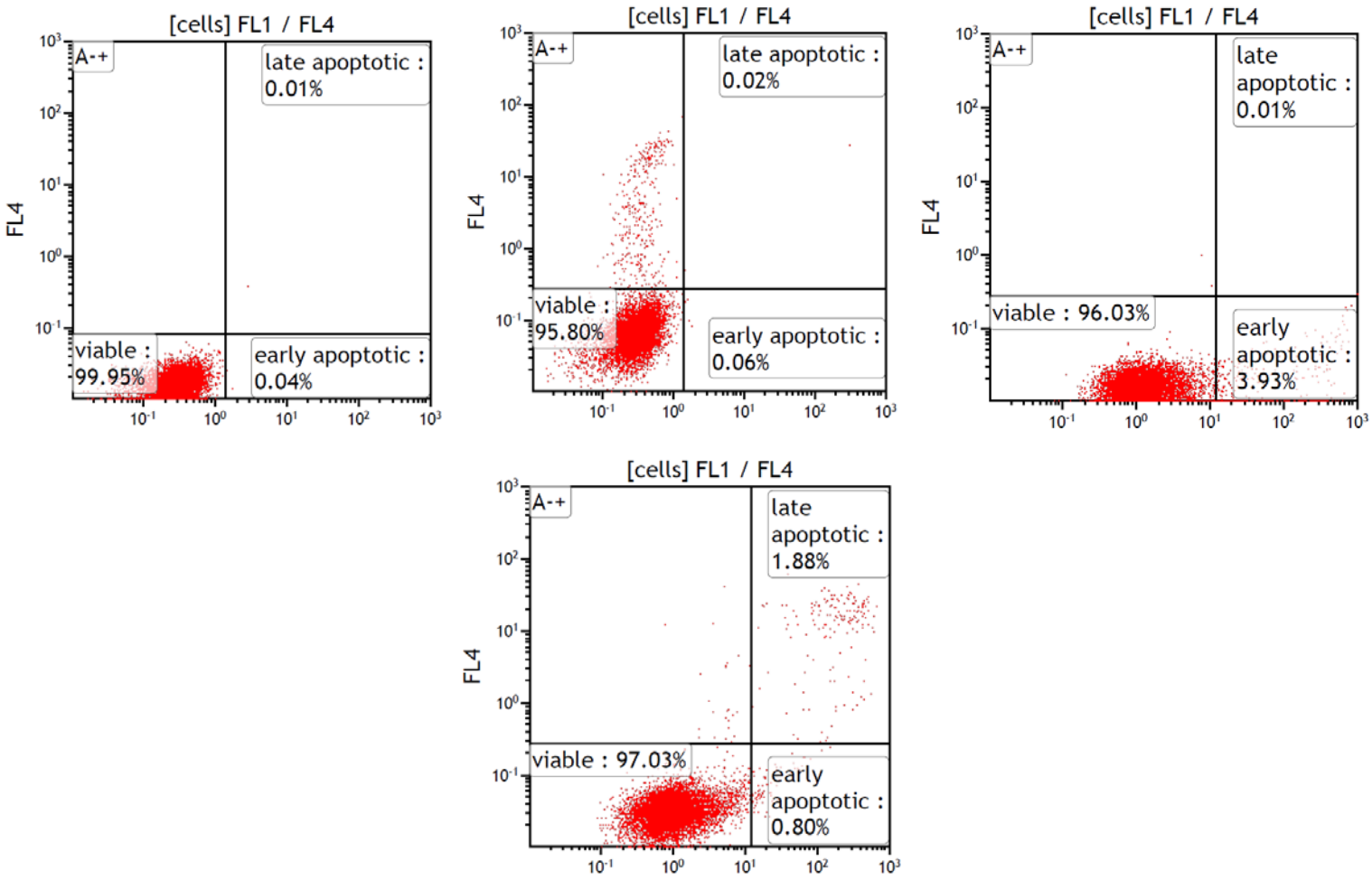


Fig. 15 (B) Control group

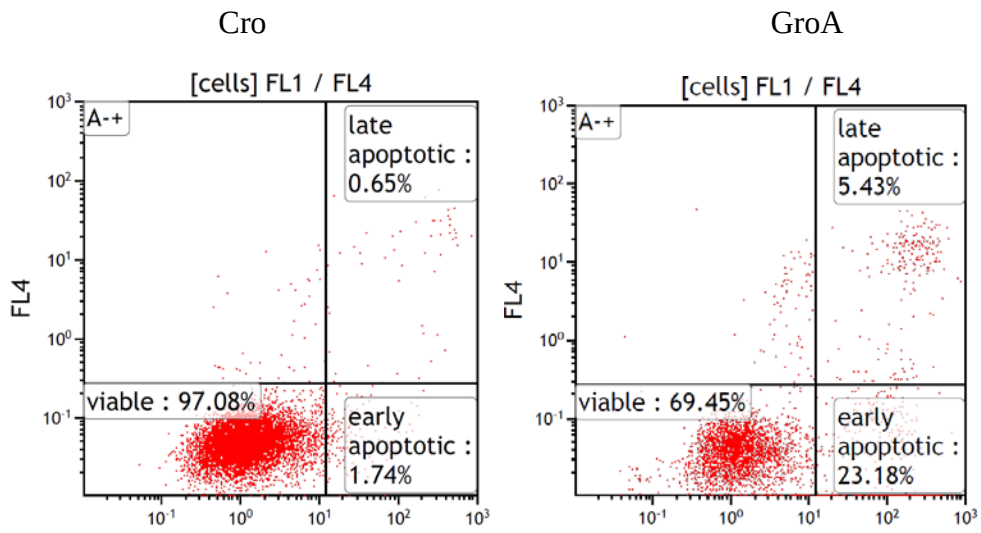


Fig. 15 (C) Joint treatment group 100F+Cro 200F+Cro 300F+Cro

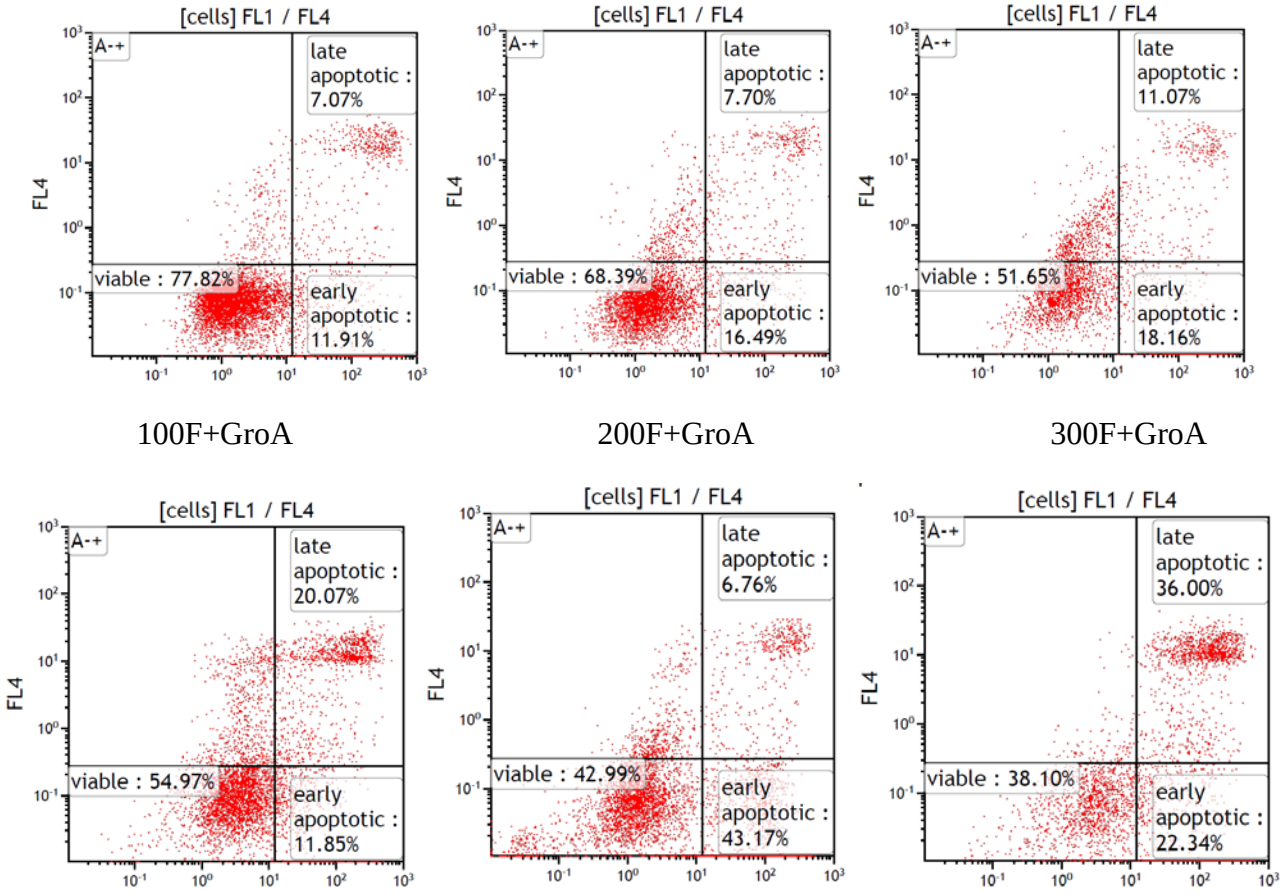


Figure 15: Apoptosis inducing effect on DU-145 cells after treatment with either GroA/Cro or LMWF alone, or both combined, for 72 hours. (A) Blank treatment group. (B) Control group, treated with Cro and GroA in the presence and absence of fucoidan. (C) Joint treatment group, LMWF (100, 200 and 300 µg/ml) combined with GroA/Cro (10 µM).

4.3.1.2 PC-3 cell line

Results show that the percentage of viable cells in the blank treatment group was 96.2% and the total apoptotic cells were around 2% of the population assayed. (Fig. 16(A)).

The apoptosis results of PC-3 cells are shown below. In control treatment groups, the apoptosis of cells in Cro and GroA groups was similar to that observed in DU-145 cells 72 hours after treatment. There was no change in Cro alone apoptosis values, but some effect was observed in the GroA group, which induced about 23% higher cell apoptosis values than the blank treatment group. The percentage of viable cells was lower than that of the blank treatment group.

The combination treatment groups (Fig. 16 (C)) of LMWF (100, 200 and 300 μg/ml) combined with Cro10μM for 72 hours showed a similar trend to that observed with DU-145 cells, namely that the total apoptotic percentage decreased with the increasing concentration of LMWF (about 13%, 20% and 21% respectively), and LMWF induced cellular apoptosis up to 20% compared with Cro alone.

Moreover, the comparison of GroA 10 μM (alone) treated cells for 72 hours with LMWF (300 μg/ml) combined with GroA 10 μM resulted in the combination's induction of more cells to undergo apoptosis -- 35% higher than GroA alone. Other GroA and LMWF combine groups also show remarkable changes, but not higher than those resulting from LMWF (300 μg/ml).

Therefore, LMWF (100, 200 and 300 μg/ml) did cause about 20% of PC-3 cells to undergo apoptosis. In addition, LMWF (100, 200 and 300 μg/ml) combined with GroA 10 μM showed remarkable changes, compared with GroA alone and LMWF alone.

Fig. 16 (A) Blank treatment group

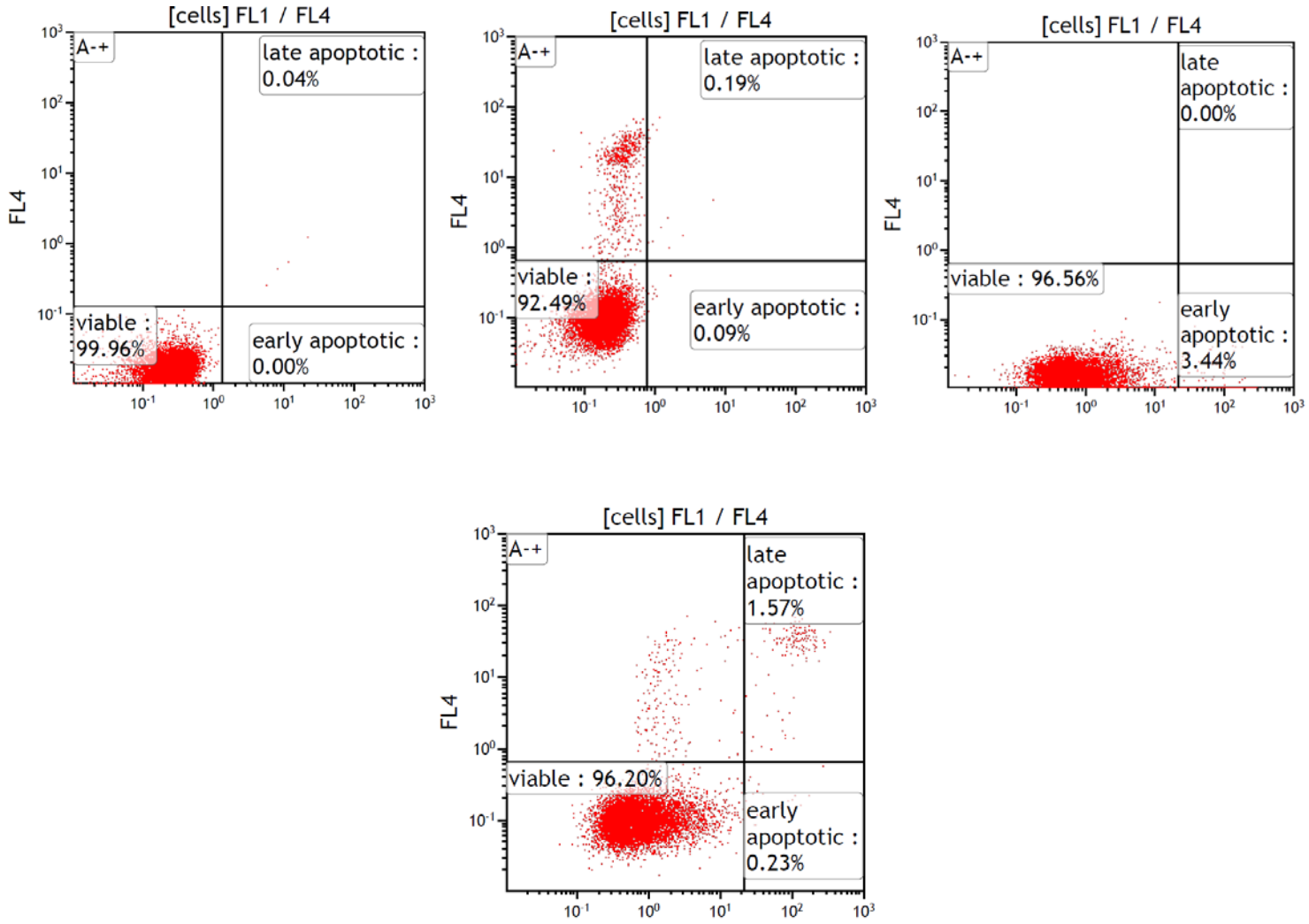


Fig. 16 (A) Control group

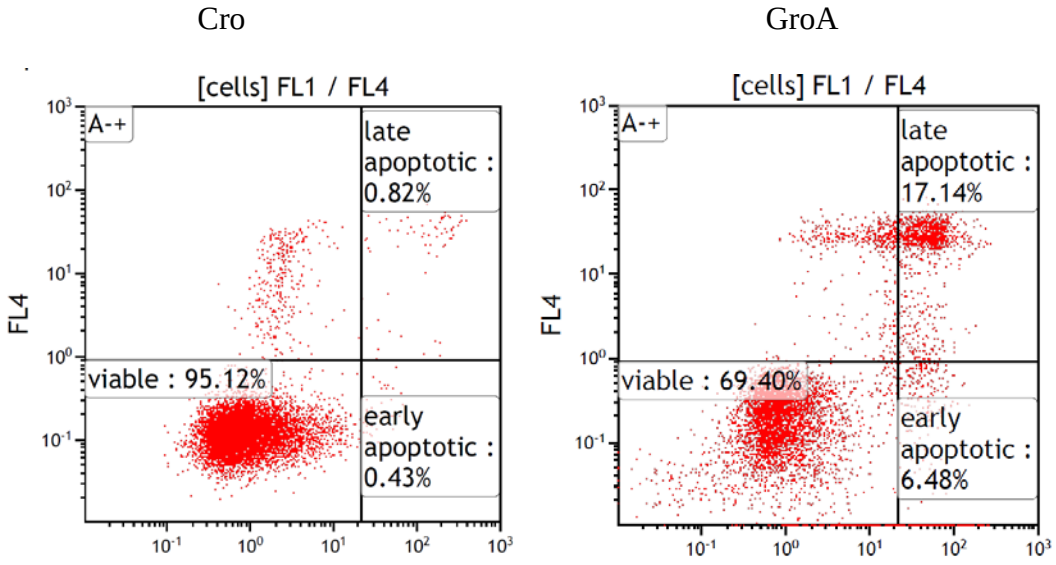


Fig. 16 (A) Joint treatment group 100F+Cro 200F+Cro 300F+Cro

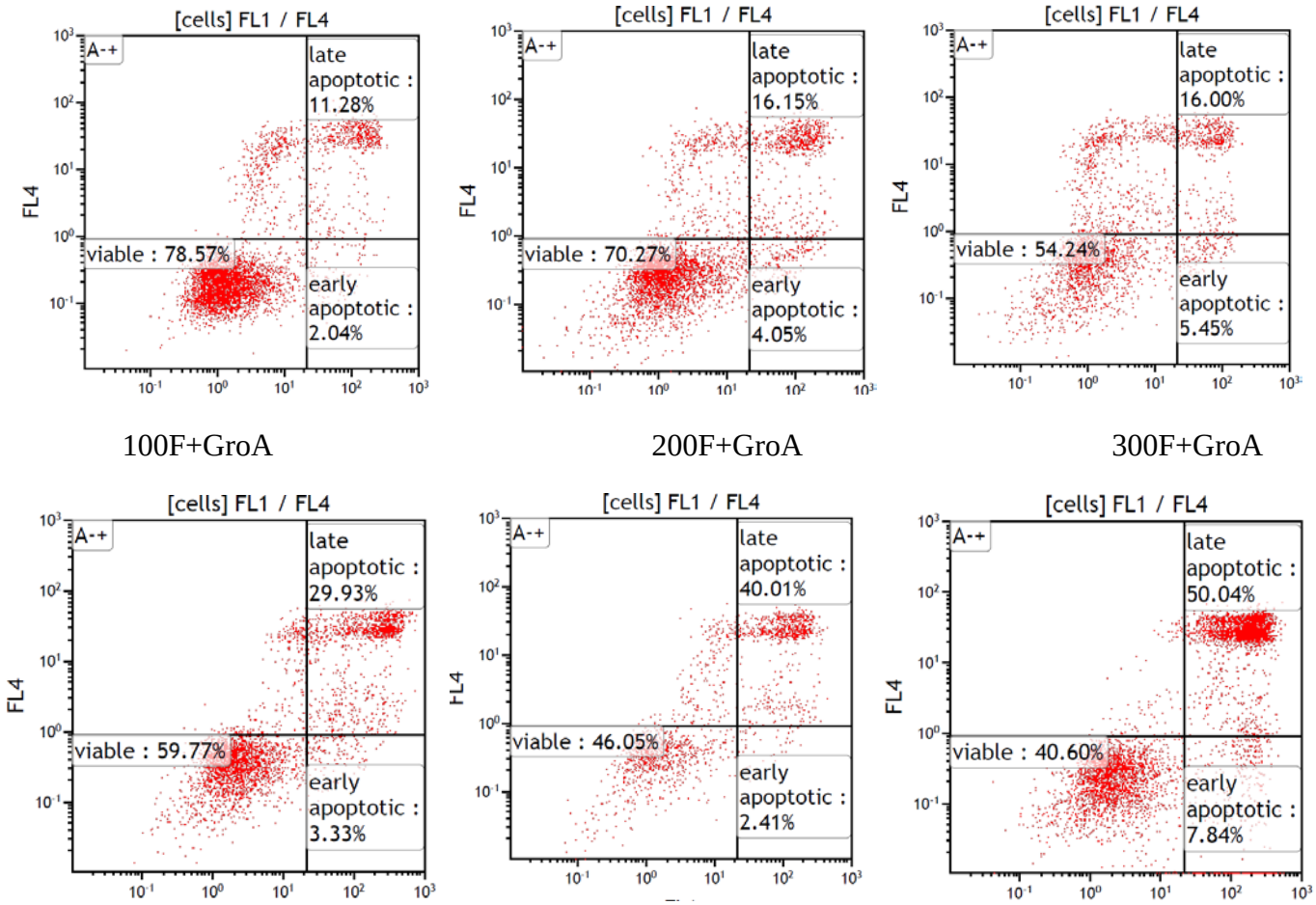


Figure 16: Apoptosis inducing effect on PC-3 cells after treatment with either GroA/Cro or LMWF alone, or both combined, for 72 hours. (A) Blank treatment group. (B) Control group, treated with Cro and GroA in the presence and absence of fucoidan. (C) Joint treatment group, LMWF (100, 200 and 300 µg/ml) combined with GroA/Cro (10 µM).

4.3.2 Effects of SF and GroA on cell apoptosis in 72h treatment samples

4.3.2.1 DU-145 cell line

Results show that the percentage of viable cells in the blank treatment group was 95.84% and the total apoptotic cells were around 2%. (Fig. 17(A)).

In control groups (Fig. 17 (B)), it was found that the distribution of cell apoptosis was not changed by incubation with 10 μM Cro, but the percentage of viable cells in GroA groups dropped about 29%. Early apoptotic cells and late apoptotic cells increased 24% and 4% respectively, when compared to the blank treatment group.

In combination treatment groups, SF (500, 750 and 1000 μg/ml) combined with Cro (10 μM) for 72 hours did not make any difference in apoptotic cell values, as compared to Cro-alone treated cells. Furthermore, the combination treatments (SF (500, 750 and 1000 μg/ml) combined with GroA 10 μM) induced a slightly lower percentage of cellular apoptosis compared to that observed with single SF treatments, but did not make any changes in relation to the GroA alone treatments.

Thus, SF alone or combined with GroA did not induce DU-145 cellular apoptosis.

Fig. 17 (A) Blank treatment group

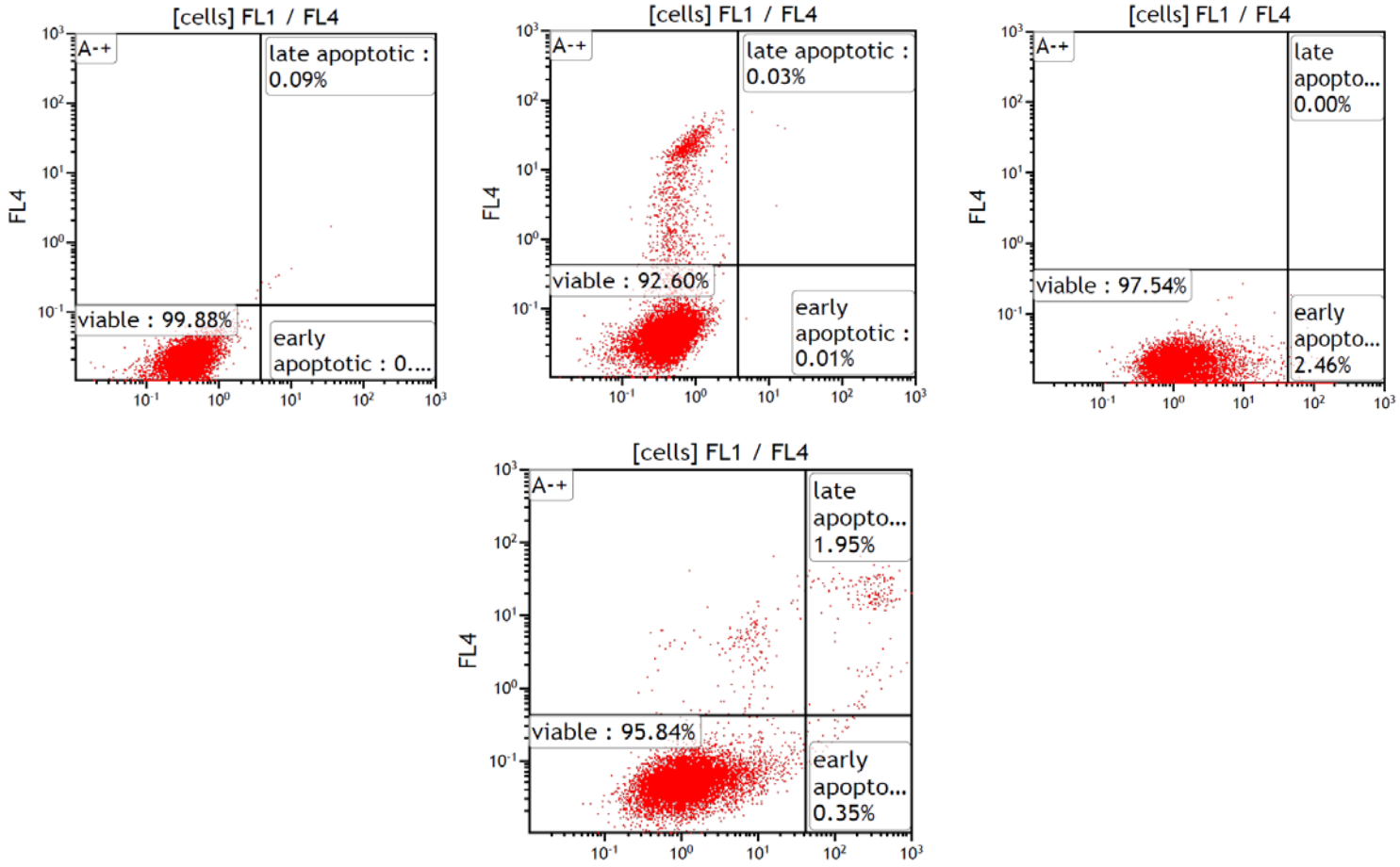


Fig. 17 (B) Control group

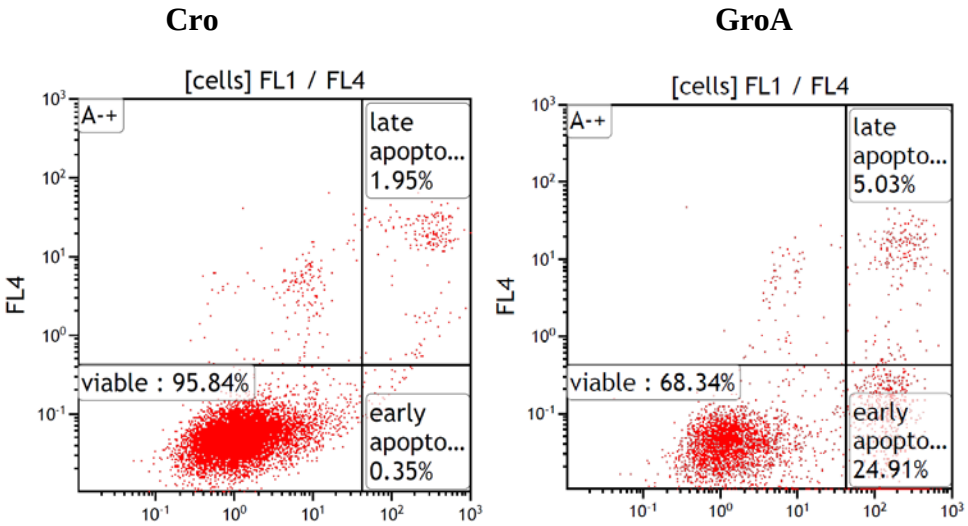


Fig. 17 (C) Joint treatment group 500F+Cro 750F+Cro 1000F+Cro

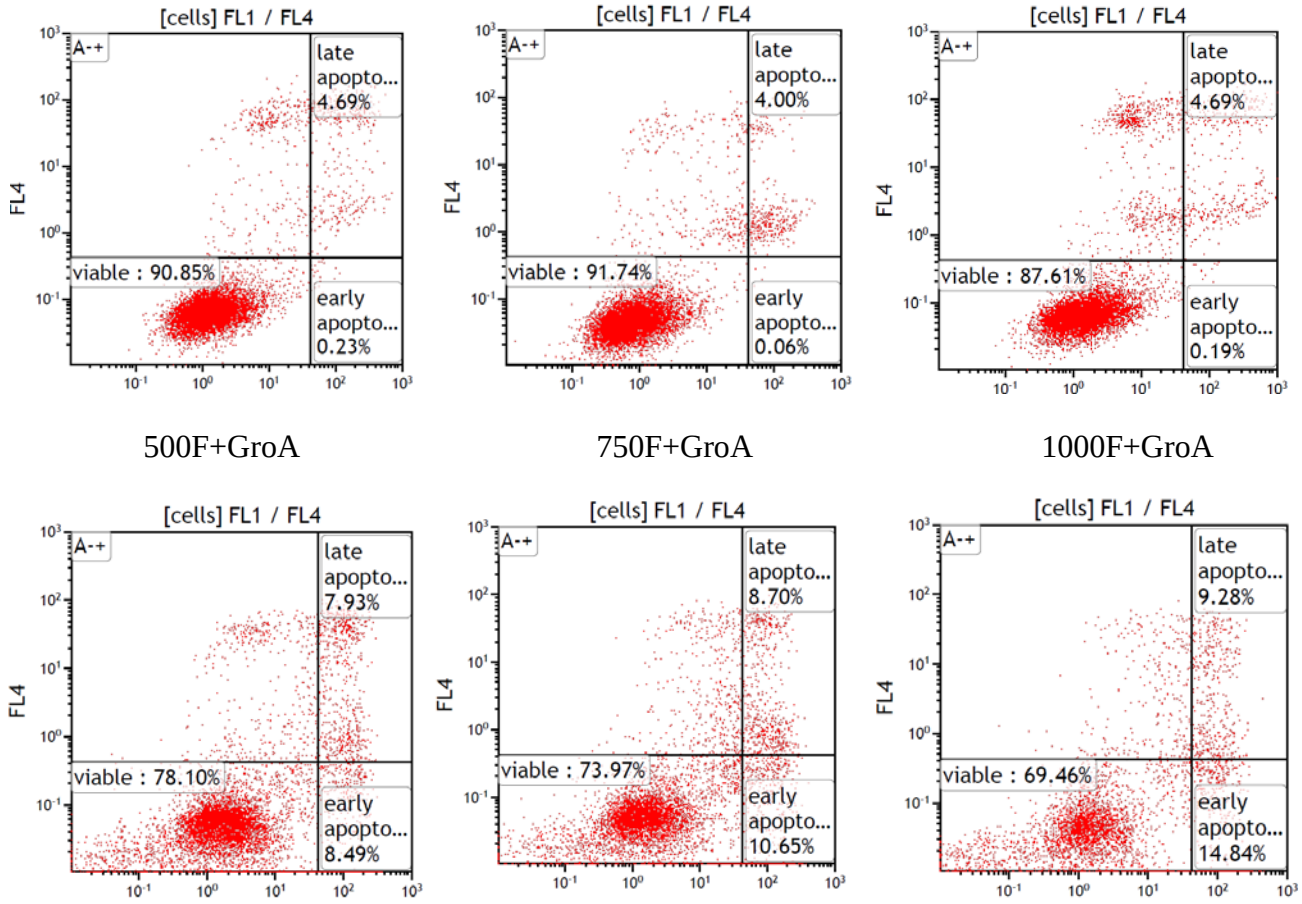


Figure 17: Apoptosis inducing effect on DU-145 cells after treatment with either GroA/Cro or SF alone, or both combined, for 72 hours. (A) Blank treatment group. (B) Control group, treated with Cro and GroA in the presence and absence of fucoidan. (C) Joint treatment group, SF (500, 750 and 1000 µg/ml) combined with GroA/Cro (10 µM).

4.3.2.2 PC-3 cell line

Results show that the percentage of viable cells in the blank treatment group was 95.61% and the total apoptotic cells were around 2%. (Fig. 18(A)).

In control groups (Fig. 18 (B)), it was found that the distribution of cell apoptosis was not changed by incubation with 10 µM Cro. The percentage of viable cells in GroA groups dropped about 20%, whereas total apoptotic cells increased about 18% compared to the blank treatment groups.

In combination treatment groups, similar to DU-145 results, SF (500, 750 and 1000 µg/ml) combined with Cro (10 µM) for 72 hours did not make any difference in PC-3 viability, when compared to Cro-alone treated cells. Furthermore, the combination treatments (SF (500, 750 and 1000 µg/ml) combined with GroA 10 µM) induced a slightly lower percentage of cell apoptosis compared to that observed with single SF treatments, but did not make any changes in viability when compared to the GroA alone treatments.

Thus, SF alone or combined with GroA did not induce PC-3 cellular apoptosis.

Fig. 18 (A) Blank treatment group

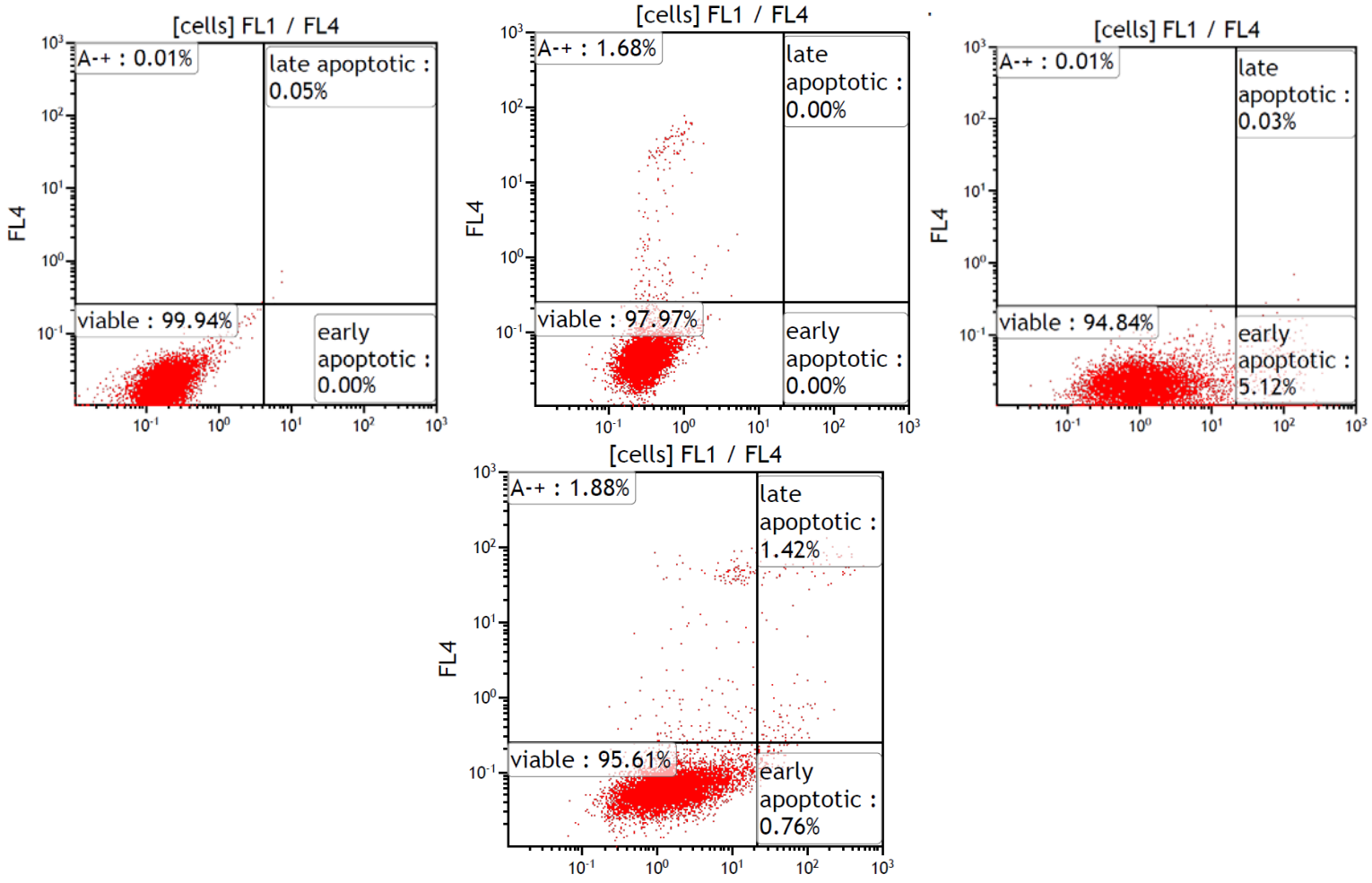


Fig. 18 (B) Control group PC-3

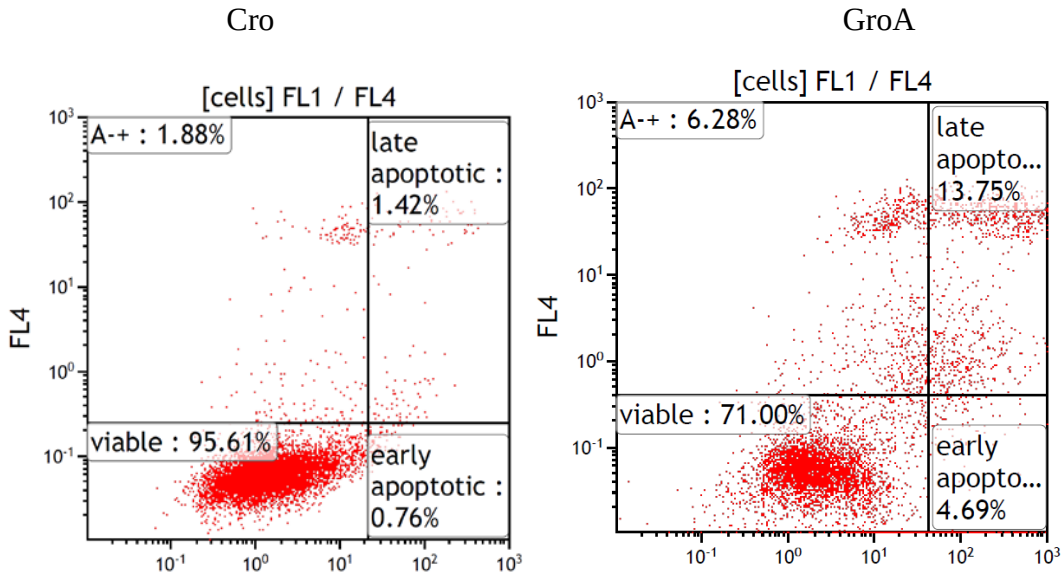


Fig. 18 (C) Joint treatment group 500F+Cro 750F+Cro 1000F+Cro

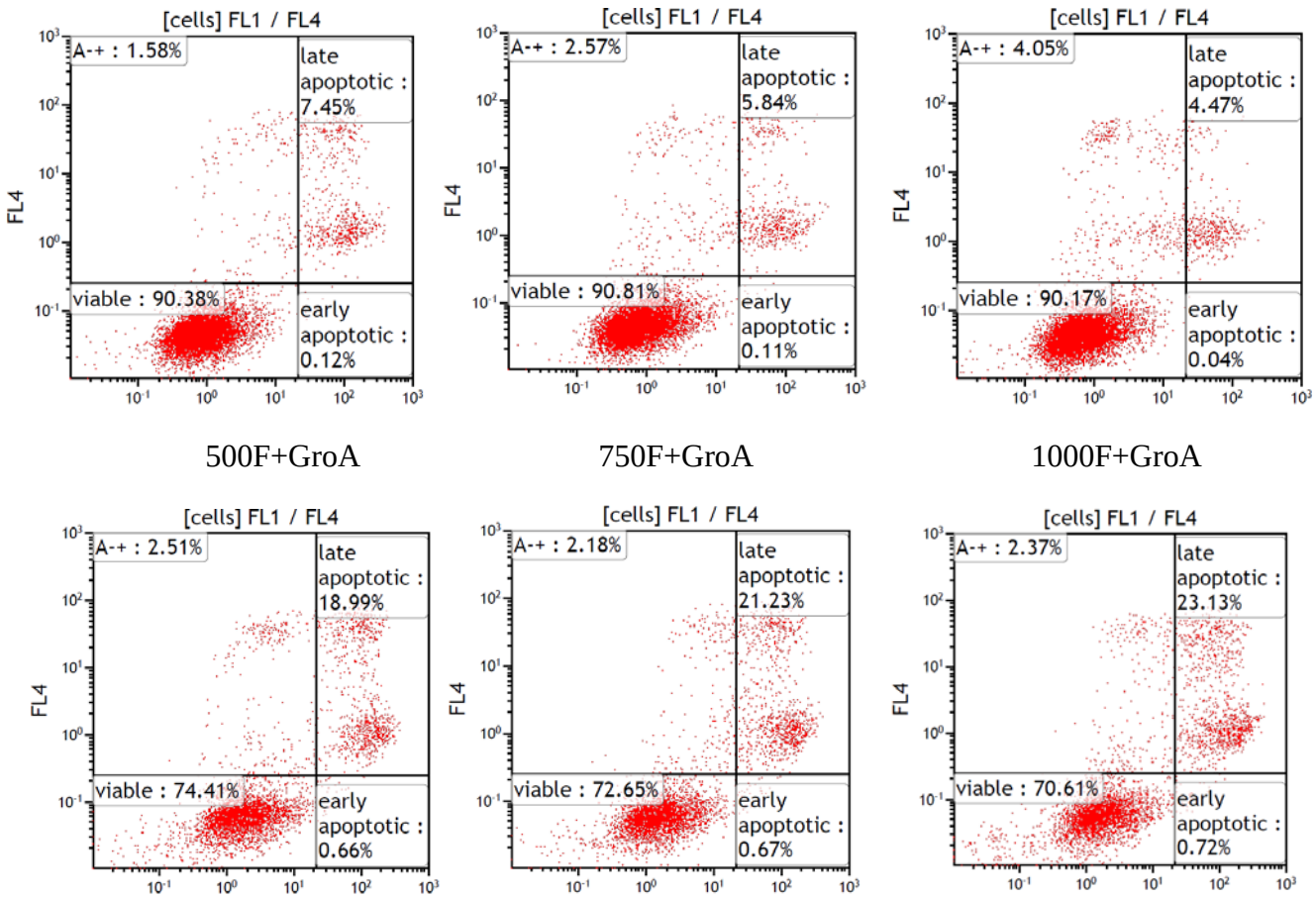


Figure 18: Apoptosis inducing effect on PC-3 cells after treatment with either GroA/Cro or SF alone, or both combined, for 72 hours. (A) Blank treatment group. (B) Control group, treated with Cro and GroA in the presence and absence of fucoidan. (C) Joint treatment group, SF (500, 750 and 1000 µg/ml) combined with GroA (10 µM).

4.4 Joint Effects of LMWF and GroA on the Alterations of Cell Cycle Progression.

Single or combination effects of GroA and LMWF on cell cycle progression of human prostate cancer cell lines PC-3 and DU-145 were studied. The cells were treated with either GroA or LMWF/SF alone, or both combined, for 72 hours. Flow cytometry was used to analyze the DNA content in each phase of the cell cycle. Each experiment was performed in triplicate.

4.4.1 Effects of LMWF and GroA on PC-3 Cell Cycle Progression after 72h treatments

4.4.1.1 PC-3 cell line

Figure 19 shows that treatment of PC-3 cells for 72 hours with Cro did have an effect on the cell line. The percentage of S phase decreased about 10%, but minimal differences were observed in other phases when compared with the blank treatment group.

The Gro A (single treatment) group results showed that the accumulation of sub-G1 phase was significantly increased by the incubation with CroA 10 μM for 72 hours as compared to the blank treatment control. The percentage of G0-G1 phase dropped about 28% and S-phase increased from 11.17% to 21.89%.

In LMWF (combined with Cro) group samples, the results illustrated that the accumulation of Sub-G1 phase was slightly increased at 72 hours with the increase of LWMF concentration as compared to the Cro alone samples. This was especially noticeable in LMWF 300μg/ml treatments, with the sub-G1 phase up to 21.41% (from 5.34% in the Cro group). In addition, LMWF did not block the cells in G0-G1 phase, slightly decreased with the increase of LMWF concentration, but did not show effect on S phase and G2-M phase compared with Cro group.

In combination treatment groups using LMWF and GroA, more cells accumulated in sub-G1 phase at 72 hours compared with GroA 10μM treated cells or LMWF with Cro groups. LMWF (100, 200 and 300 μg/ml) combined with GroA 10μM accumulated more cells in Sub-G1 phase, and the percentage of sub-G1 phase was higher with the increase of LMWF concentration, up to 37.61% at the highest

concentration of LMWF. Furthermore, the accumulations of G0-G1 phase cells decreased dramatically, up to 37.31%, in the treatments utilizing a concentration of 300 µg/ml LMWF.

Therefore, LMWF affects cell cycle progression by increasing sub-G1 phase cell populations, and the combination of LMWF and GroA further increased the sub-G1 phase cell numbers as well as demonstrating an effect on G0-G1 phase progression in the PC-3 cell line.

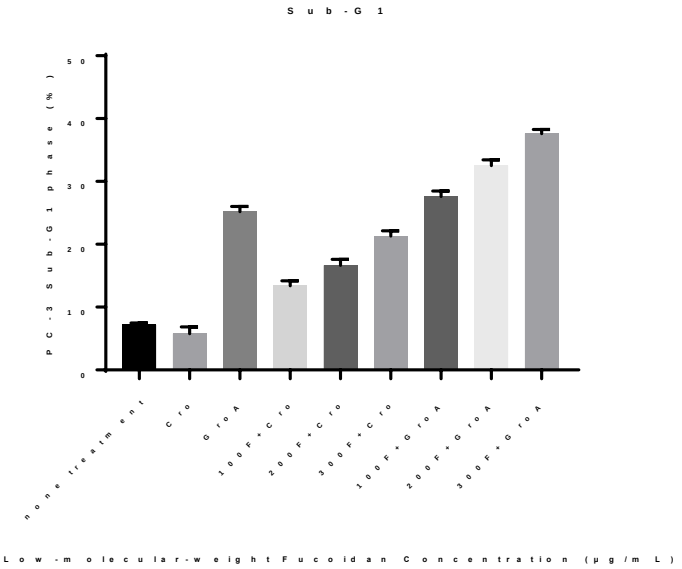


Figure 19: The PC-3 cell percentages of sub-G1 fraction in the cell cycle after treatment with either GroA or LMWF alone, or both combined on the alterations of Cell Cycle, for 72 hours.

Fig.20 (A) Control group PC-3

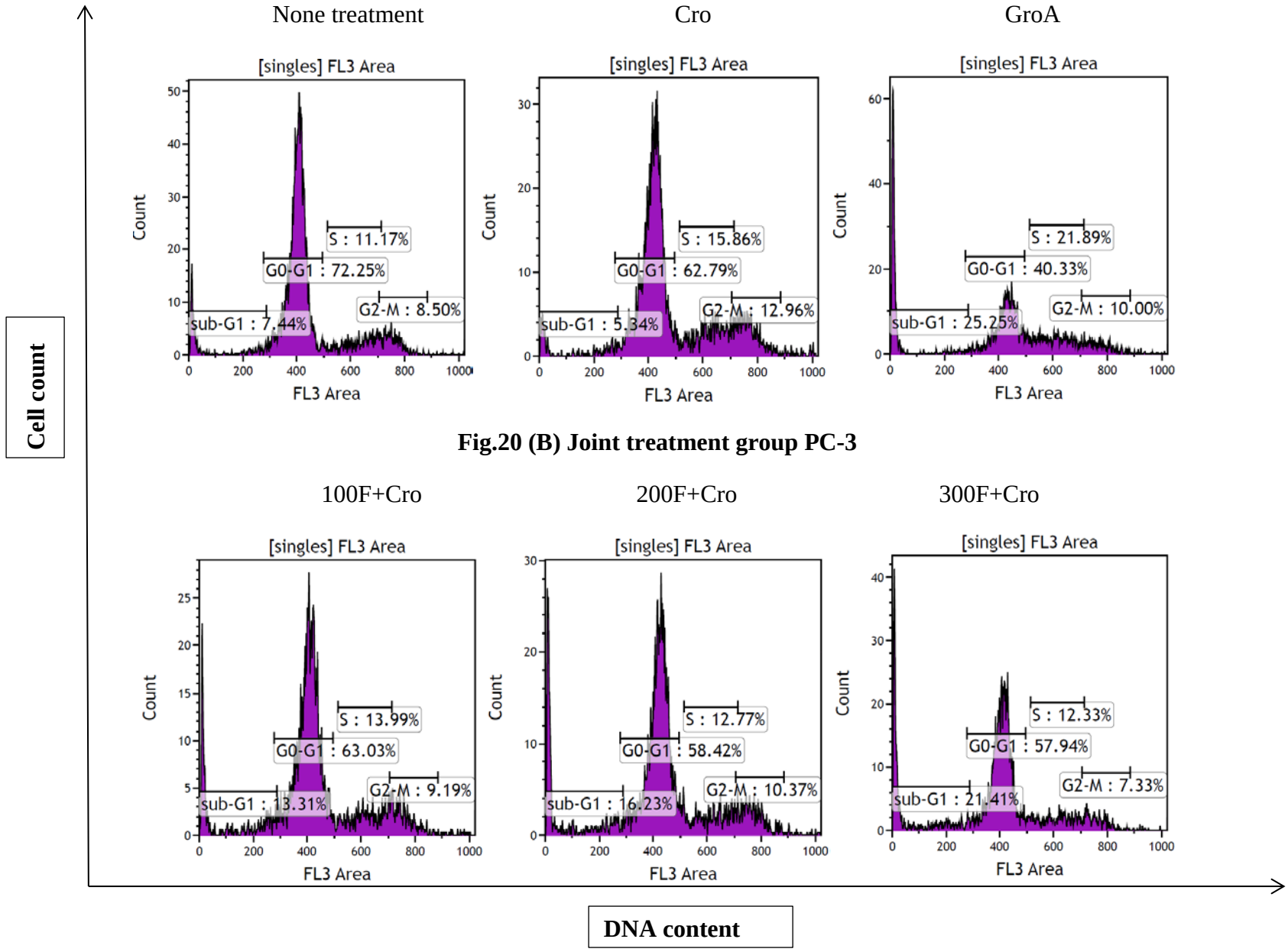


Fig.20 (B) Joint treatment group PC-3

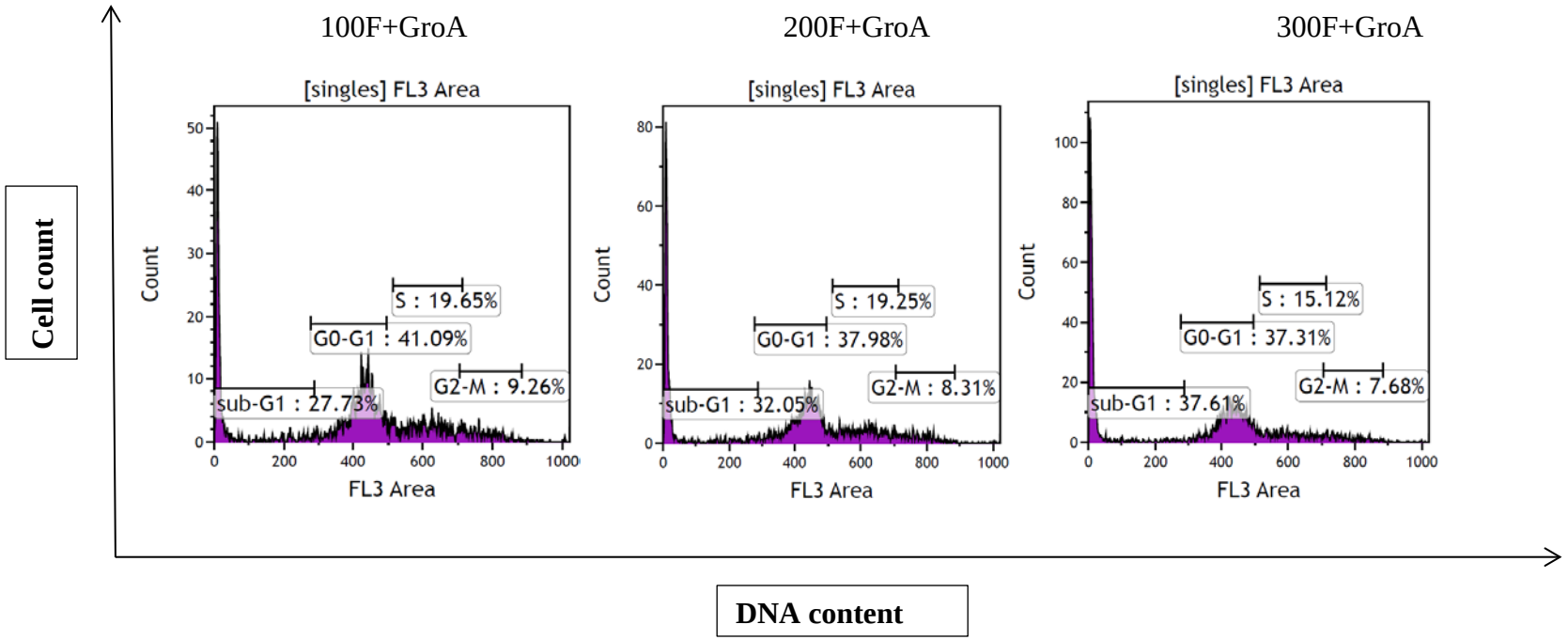


Figure 20: Cell cycle distribution of PC-3 cells after 72 hour treatments with either GroA or LMWF alone, or both combined, indicating alterations of Cell Cycle Progression. (A) Control group, treated with Cro and GroA in the presence and absence of fucoidan. (B) Joint treatment group, LMWF (100, 200 and 300 µg/ml) combined with GroA/Cro (10 µM).

4.4.1.2 DU-145 cell line

Figure 20 shows that 72 hours treatment with Cro 10 μM did not have an effect on DU-145 cells. The percentage of sub-G1 phase cells was 1.14%, and the G0-G1 phase was 64.61%.

In the Gro A group (single treatment), results indicated that the distribution of the cell cycle was changed by GroA 10 μM at 72 hours of treatment. The percentage of sub-G1 phase was increased about 7% and the percentage of G0-G1 phase decreased about 12%, compared to the blank treatment group.

In LMWF combined with Cro group samples, it was found that the distribution of the cell cycle was slightly changed by incubation with different concentrations of LMWF for 72 hours. The percentage of sub-G1 phase cells increased with the increasing concentration of LMWF, up to 12% at 300μg/ml LMWF, when compared to the Cro 10 μM alone treated cells, but no significant change in G0-G1 phase, S phase and G2-M phase was observed.

The combination treatment groups using LMWF (300 μg/ml) with Cro for 72 hours increased the percentage of cells in sub-G1 phase (27.03%), when compared with GroA 10 μM treated cells. Only slight changes in cell cycle progression were observed at LMWF concentrations of 100 and 200 μg/ml combined with Cro. In addition, G0-G1 phase cell numbers were slightly decreased with the corresponding increase in LMWF concentration. There were no significant changes in S phase and G2-M phase numbers.

Therefore, LMWF is responsible for observed cell cycle changes in DU-145 cells in sub-G1 phase progression but this is not the case in the PC-3 cell line. This result is thesame for tested combination groups, with only high concentrations of LMWF producing a rise in sub-G1 phase cell numbers compared with single treatment cell cycle progression results.

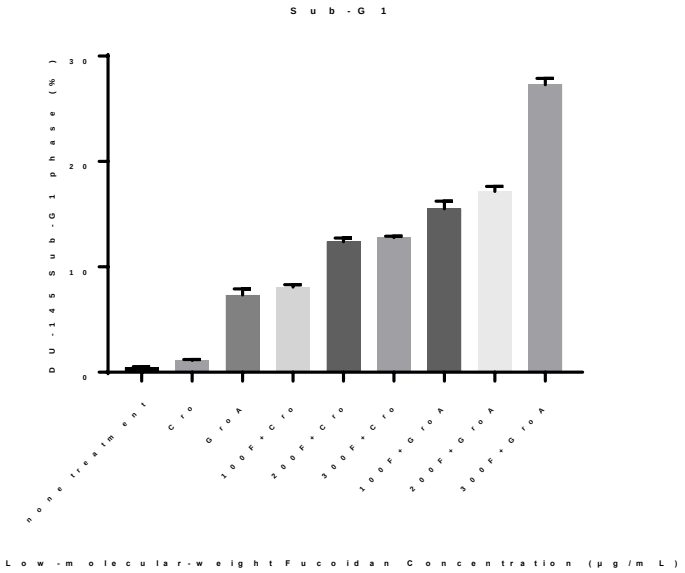


Figure 21: The DU-145 cell percentages of sub-G1 fraction in the cell cycle after treatment with either GroA or LMWF alone, or both combined on the alterations of Cell Cycle for 72 hours.

Fig.22 (A) Control group DU-145

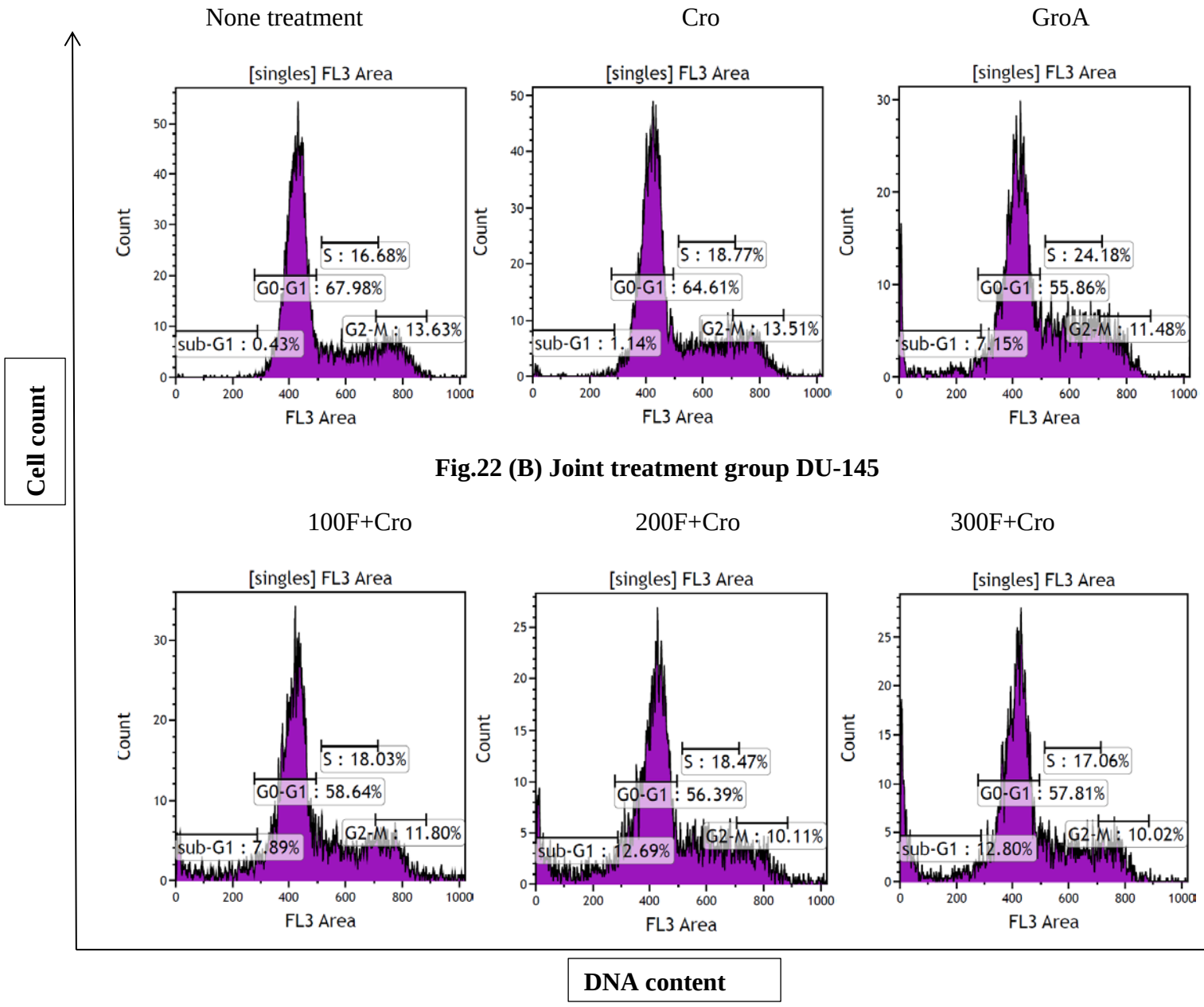


Fig.22 (B) Joint treatment group DU-145

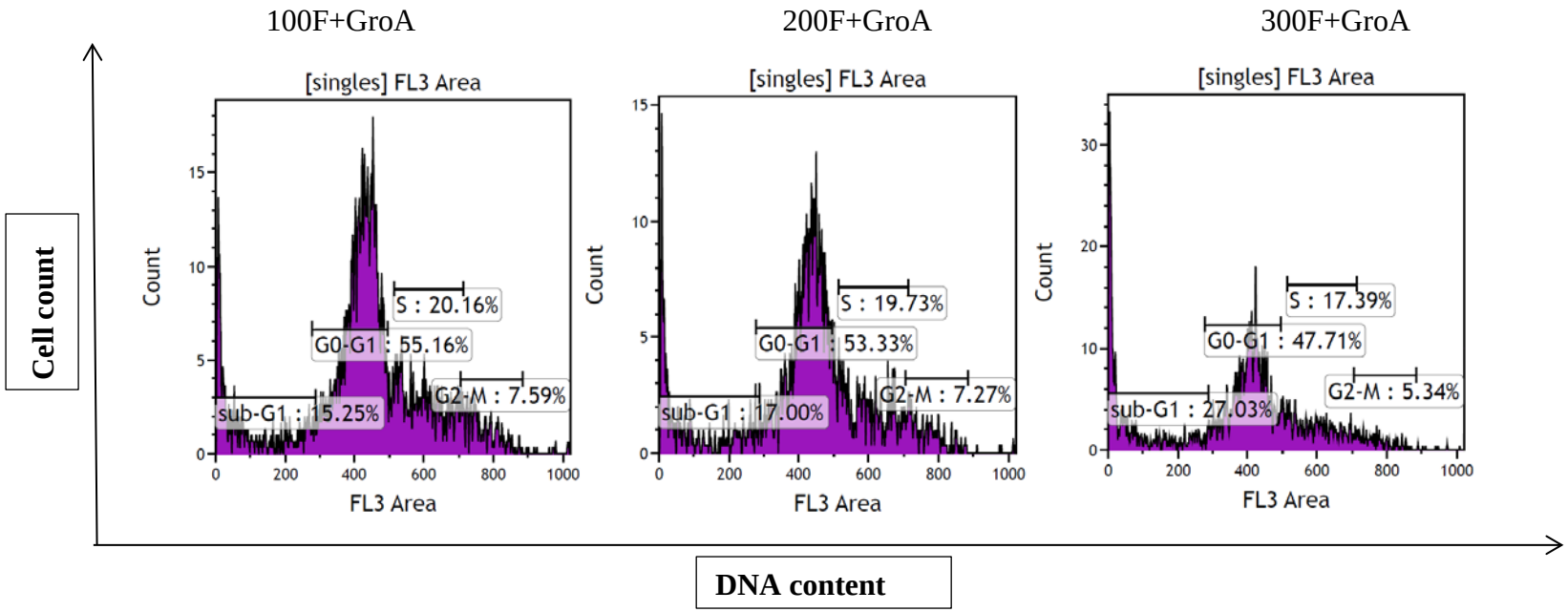


Figure 22: Cell cycle distribution of DU-145 cells after 72 hour treatments with either GroA/Cro or LMWF alone, or both combined, indicating alterations of Cell Cycle Progression. (A) Control group, treated with Cro and GroA in the presence and absence of fucoidan. (B) Joint treatment group, LMWF (100, 200 and 300 µg/ml) combined with GroA/Cro (10 µM).

Chapter 5 Discussion

Fucoidan is one of the FCSPs isolated from various brown seaweeds which are known to possess essential bioactive properties, notably growth inhibitory effects on tumor cells. FCSPs also possess sulfated galactofucans, with backbones built of (1→6)-β-D-galacto- and/or (1→2)-β-D-mannopyranosyl units. In addition to sulfate groups these backbone residues may be substituted with fucosides, single fucose substitutions, and/or glucuronic acid, xylose or glucose substitutions. The three oncogenes, ErbB receptors, Ras proteins and nucleolin, may contribute to malignant transformation of healthy cells. The epidermal growth factor receptor (EGFR; ErbB-1; HER1), one of the receptor tyrosine kinases, plays a pivotal role in regulating cell transformation, differentiation, and transformation. Previous studies have shown that fucoidan has potent anticancer-promoting activity and mainly targets the EGFR-signaling pathway, which may contribute to its chemo-preventive potential.

Nucleolin is a ubiquitously expressed acidic phosphoprotein that is involved in important aspects of cell proliferation and cell growth. It is localized primarily to the nucleoli, but it undergoes nuclear cytoplasmic shuttling and is also found on the cell surface of some types of cells. Cell surface nucleolin is found in a wide range of tumor cells, and it is used as a marker for cancer diagnosis. Ronit Pinkas-Kramarski's lab in Israel has identified non-nucleolar nucleolin as an ErbB receptor-interacting protein. This interaction leads to receptor dimerization and activation as well as to increased colony growth in soft agar. Recent studies have identified a crosstalk between nucleolin, ErbB1 and Ras proteins. Therefore, it seems that combining two drugs, Fucoidan (which inhibits ErbB1) and GroA/AS1411 (which specifically inhibits cell surface nucleolin) may have a better inhibitory effect on ErbB-1 receptor activation and thereby a stronger inhibitory effect on cancer cell growth and tumorogenicity.

In the present study, LMWF and SF, were investigated for their antiproliferative effects, apoptosis-inducing ability, and cell cycle progression activities on human prostate cancer cell lines. A joint inhibitory effect study of LMWF and SF was conducted in order to investigate whether LMWF and GroA/Cro, or SF and GroA/Cro can elicit a stronger inhibitory effect on cell viability at lower concentration ranges when combined together. There are two different fucoidan isolates analysed in this

study, LMWF and SF, both of which are from Undaria pinnatifida. Both isolates are prepared for treatments to cells by dissolving the preparations in cell culture medium (RPMI 1640 base medium with 1% Penicillin-Streptomycin, 1% L-glutamine and 10% fetal bovine serum).

LMWF was isolated from source material at Auckland University of Technology (AUT), New Zealand. The molecular weight (MW) of LMWF was less than 30 kDa. According to an unpublished study by AUT, the SF sample showed a major large MW fraction of approximately 440 kDa, and a minor small MW fraction at just over 2 kDa, as determined by high performance gel permeation chromatograph (HPGPC). Therefore, MW was considered to be the distinguishing factor between those two fucoidans. Moreover, fucoidans extracted from the same source species have been documented to differ in their chemical composition if extracted from different parts of the same seaweed. Hence, differences in structures of fucoidans from similar and different sources may be the attributes which define the observed differences in bioactivity of the molecules.

Two human prostate cancer cell lines, PC-3 and DU-145, were utilized as a study model to examine the anticancer potential of LMWF and SF. It is worth mentioning here that among prostate cancer cell lines available, PC-3 and DU-145 were established for use as androgen-independent cancer cells. The androgen-independent stage of prostate cancer is considered to be metastatically advanced cancer and therefore, to study drugs against these cell lines indicates how effective a potential agent may manifest its action against advanced prostate cancer.

To the best of my knowledge, the studies of anticancer potential for LMWF or SF or LMWF and GroA/Cro, or SF and GroA/Cro, on human prostate cancer cells were carried out for the very first time.

5.1 Comparison of Inhibitory Effects between types of Fucoidan

The Antiproliferative activities of different fucoidans on various cell lines were estimated by MTT cell proliferation assays. All cell lines were treated with various concentrations of fucoidan preparations, and the cell viability was determined as a time- (72 and 96 hours) and dose-dependent event. Cell viability was calculated as the ratio of absorbance values in treated cultures to those values in untreated control cultures. At 72

and 96 hours the results were found to be significantly different and inhibitory effects of the fucoidans on PC-3 and DU-145 cell lines were easily interpreted.

Comparison and ranking of different types of fucoidans' anticancer activity examined in this study is based on IC_{50} values, which were obtained by GraphPad Prism software, version 7. The IC_{50} value is the measure of effectiveness of a drug and it indicates the concentration of drug needed to reduce a biological process by half (i.e. 50%). The lower IC_{50} values suggest higher drug sensitivity and hence higher anticancer potential.

5.1.1 Signal treatment

To explore suitable concentrations of LMWF for use in this study, the concentration range from 1600-1 µg/ml was first investigated in cultures of PC-3 and DU-145 cells for 72 and 96 hours. These treatments resulted in very significant suppression of cell viability in a dose-dependent manner ($p < 0.001$) and the IC_{50} at 96 hours of treatment was around 400 µg/ml in PC-3 and 420µg/ml in DU-145 cells. The IC_{50} was more than 1600 µg/ml in both cell lines after 72 hours of treatment. According to one-way ANOVA testing, there was no significant difference ($p >0.05$) in cell viability between lower concentrations LMWF and control in 72 hours and 96 hours. Additionally, the concentrations of LMWF from1600 µg/ml to100 µg/ml were responsible for cell death values of around 50-60%. Moreover, LMWF was decreased by 16-fold from 1,600 to 100 µg/ml, while in both cell lines the cell viability was only about 11% lower than that observed in the highest drug concentration at 72 hours and around 5% at 96 hours. Considering these results, LMWF concentrations from 300µg/ml to 100µg/ml were chosen for the combination studies in PC-3 and DU-145 cell lines.

From the above discussion it is clear that LMWF was effective in inhibiting cell viability and a promising agent against both PC-3 and DU-145 cells. However, the higher IC_{50} value does suggest lower drug sensitivity in these cell lines. Therefore, LMWF is unlikely to be used as a sole agent for cancer treatment where there are known therapies which are more effective. Of note, LMWF is a non-toxic, edible product that is easily deliverable in its active form. Here, we will discuss research into the potential for orally-delivered fucoidan as an "adjunct" therapy to conventional treatments. Fucoidan may find a role either in reducing side effects, to enhance the

therapeutic effects of conventional therapies, or to address cancer for which there are no known therapy options.

To explore and compare the inhibitory effects of LMWF and SF, the concentrations of SF (1000-10 μg/ml) were also tested in PC-3 and DU-145 cell lines. SF inhibited the proliferation of PC-3 and DU-145 cells depending on its time of incubation and the treatment's concentration. Even though the anti-cancer ability of fucoidan has been reported in different cancer cell lines, the highest concentration of SF used in this study (1,000 μg/ml) could only induce lower than 20% cell death in PC-3 cells and DU-145 cells under 72 hour and 96 hour treatment schedules. SF was decreased by 100-fold from 1,000 to 10 μg/ml, while the PC-3 and DU-145 cell viabilities were only about 8% lower than that observed in the highest drug concentration. In the previous 24 and 48 hour treatments, there were no significant morphological changes observed under the microscope.

Based on this result, a higher SF concentration range (1000, 750, and 500 μg/ml) was applied to cell lines for 72 hour and 96 hour treatments. In the higher concentration groups numerous morphological changes were observed.

Results from antiproliferative assays revealed that SF does not exhibit a greater antiproliferative effect than LMWF against the tested prostate cancer cell lines. The highest concentration of SF (1000 μg/ml) only induced around 16% cell death in both cell lines, but LMWF at the same concentration induced about 54% cell death compared with control. The possible reasons may be associated with sulfate content in the fucoidan preparations. Previous studies show that LMWF has a sulfate content higher than 20%, and was found to exert profound anticoagulant activity as well as antiproliferative effects on a fibroblast cell line (CCL39) in a dose-dependent fashion. The antiproliferative activity decreased dramatically when sulfate groups were less than 20%, even at high concentrations of treatments: inhibition dropped to 12% and 40% for 1000 μg/mL treatments containing 12.5% and 18.4% of sulfate groups, respectively. No inhibition was observed at low concentrations (0.1–100 μg/mL) for these two fucoidan fractions (Haroun-Bouhedja, Ellouali, Sinquin, & Boisson-Vidal, 2000). Therefore, the antiproliferative activity of LMWF likely depends on the sulfate content, and the effect decreases with decreasing degree of sulfation (Haroun-Bouhedja et al., 2000).

5.1.2 Combination treatment

From previous single treatment studies, it's known that both LMWF and SF inhibited PC-3 and DU-145 proliferation, especially high concentrations of LMWF. For SF, the result was not significant. Hence, a joint inhibitory effect study of these there drugs was conducted in order to investigate whether LMWF and GroA/Cro, or SF and GroA/Cro can make a stronger inhibitory effect at lower concentration ranges when combined together. The optimum concentration of GroA/Cro in this study was 10μM, based on a previous study (Goldshmit et al., 2014). For both cell lines, GroA/Cro concentrations were consistent at 10μM, whereas LMWF concentrations were 100, 220, and 300 μg/ml, and SF concentrations were 500, 750 and 1000 μg/ml. These two drugs, LMWF and SF, with their different concentrations were concurrently combined with GroA/Cro to treat the cancer cells and control cells. Single drug treatment groups were incubated at the same time as the combination groups.

In the LMWF combination treatment study, LMWF inhibited cell viability, with 53-63% of the cells surviving the treatment times. As for cells treated with GroA alone for 72 hours incubation, the cell viability was about 60%. The cell viability in groups treated with GroA combined with 100, 220 or 300 μg/ml LMWF showed that fucoidan significantly reduced the cell viability, as compared with GroA-alone treated cells. Likewise, the joint enhanced inhibitory effect observed in 96 hour treatment groups was slightly higher than 72 hour groups, compared with GroA-alone treated cells. The proliferation rates of PC-3 cells were all under 50%. As with PC-3 trends, the DU-145 cell line showed similar inhibition rates. In all groups, increasing the concentration of LMWF combined with GroA, resulted in the gradual reduction of cell viability in PC-3 and DU-145. Hence, LMWF was able to enhance the inhibitory effect of GroA on proliferation of PC-3 and DU-145 cells in a concentration dependent manner, even at low concentrations.

The SF combination treatment study yielded different results to the LMWF combination treatments. The number of viable cells in 500, 750 and 1000 μg/ml SF with Cro did not show a remarkable inhibitory effect (10%), as compared to the control. Cells treated with GroA alone for 72 hours incubation were about 60% viable. However, when PC-3 cells were cultured in GroA and SF for the same amount of time, there was no significantly difference compare with GroA-alone, similar to the DU-145 cell line groups. In all groups, despite increasing the concentration of FS combined with GroA,

the cell viability of PC-3 and DU-145 cells showed no differences. Hence, FS was not able to enhance the inhibitory effect of GroA on proliferation of PC-3 and DU-145 cells in a concentration dependent manner, even at a very high concentration range.

The viability results demonstrate that the combination effect of LMWF and GroA on the reduction of PC-3 and DU-145 cells viability is significantly higher than either LMWF-alone (100, 220 and 300 µg/ml) or GroA-alone (10 µM) treated cells. However, the results of SF combined with GroA, indicated that only a GroA-related effect could be observed. A possible explanation for this observation might be that SF at the concentration of 500, 750, and 1000 µg/ml is ineffective and may need higher concentrations in order to be effective.

5.2 Comparison of Apoptosis Inducing Activity between types of Fucoidan combined with GroA

Apoptosis is critically important for the survival of multicellular organisms. The process of programmed cell death, or apoptosis, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. These mechanisms are characterized by cytoplasmic shrinkage and chromatin condensation, and facilitate the removal of cells without inducing inflammation (Elmore, 2007). Impairment of this native defense mechanism promotes aberrant cellular proliferation and the accumulation of genetic defects, ultimately resulting in tumorigenesis (Xiong, Kou, Yang, & Wu, 2007). Many researchers have shown that fucoidan has time- and dose- dependent cytotoxic effects on different types of cancer cells, which include hepatocellular carcinomas, prostate cancer, breast cancer, lung cancer, acute leukemia, and colon cancer in vitro (Table 1). This seems to be indicative of the fact that fucoidan induces the characteristic changes associated with apoptosis and brings about drug-induced cell death.

GroA alone is not a satisfactory option in the clinical treatment of prostate cancer. GroA in combination with other drugs has been demonstrated to target ErbB receptor activation, and can potentially reduce malignancy. Thus GroA combined with natural compounds, which have an effect on the EGFR pathway -- but with low or no cytotoxicity -- is worthy of attention. In the present investigation, GroA was used with fucoidan simultaneously, to explore their combined effects on prostate cancer cells. The cells were treated with either GroA or LMWF/SF alone, or with both drugs combined.

According to the single drug treatments, the optimal incubation time decided for combination treatments was 72 hours. At this timepoint, GroA exerted a significant inhibitory effect, and fucoidan also showed a time-dependent inhibition of proliferation. Flow cytometry allowed the measurement of several apoptotic traits in a single sample, making it a powerful tool to study the complexity of cell death. Each experiment was performed in triplicate.

In LMWF combined with GroA, the distribution of the DU-145 cell apoptosis was changed by GroA, the percentage of viable cells decreased about 30% whereas early apoptotic cells and late apoptotic cells increased 23% and 5% respectively. In combination group treatments of 72 hours, Cro with different concentrations of LMWF (100, 200 and 300 μg/ml), resulted in the enrichment of DU-145 cells in viable cells (from 97.08% to 77.76%, 68.32% and 51.43%), compared with Cro alone. In addition, the percentage of late apoptotic cells was increased with the concentration of LMWF. The same trend with the early apoptotic cells was also observed. Comparison of GroA-aolne treated cells, and LMWF (100, 200 and 300 μg/ml) combined with GroA, showed an induction of cellular apoptosis, and the percentage of total apoptotic cells was higher (from 28% to 31%, 50% and 58%). The percentage of viable cells was reduced with increased concentrations of LMWF. Therefore, LMWF combined with GroA caused a significant change in apoptosis of treated DU-145 cells.

The apoptosis observed in treated PC-3 cells was similar to that in DU-145 cells after 72 hours. There was no changed in Cro alone, but in the GroA group. The percentage of viable cells was also lower in the treatment group compared to the untreated group. In the combination treatment group, LMWF (100, 200 and 300 μg/ml) combined with Cro indicated a similar trend as seen with DU-145 cells: the total apoptotic percentage decreased with the increasing concentration of LMWF, and LMWF induced cellular apoptosis up to 20% compared with Cro alone. Moreover, compared to GroA-alone treated cells, LMWF (300 μg/ml) combined with GroA induced more cells' apoptosis, with values 35% higher than GroA alone. Other groups of combined GroA and LMWF treatments also showed remarkable changes, but not higher than the LMWF (300μg/ml) combination with GroA. Therefore, LMWF (100, 200 and 300 μg/ml) did cause about 20% loss of PC-3 and DU-145 cells to apoptosis. In addition, LMWF (100, 200 and 300 μg/ml) combined with GroA showed remarkable changes, compared with GroA alone and LMWF alone in both cell lines.

In treatment groups of SF combined with GroA, it was found that the distribution of the PC-3 and DU-145 cell apoptosis was only changed by GroA treatment. In combination treatment groups, SF (500, 750 and 1000 µg/ml) combined with Cro (10 µM) did not have a significant effect on the cell lines when compared to Cro-alone treated cells. Furthermore, the combination treatments SF (500, 750 and 1000 µg/ml) combined with GroA induced a slightly lower percentage of cell apoptosis compared to that observed with single SF treatments, however there were no changes in relation to the GroA alone treatments. Thus, SF alone or combined with GroA did not induce PC-3 or DU-145 cell apoptosis.

The results demonstrate that the combination effect of LMWF and GroA on the reduction of DU-145 and PC-3 cell viability is significantly higher than either GroA or LMWF treated cells alone. A synergistic effect was observed in the treatment groups of GroA combined with LMWF.

However, in the results of GroA combined with FS, the combination effect of the two drugs was not improved synergistically. FS did not dose-dependently increase the inhibitory effect of GroA in either of the combination groups tested. There was no significant difference between the cell viability values when GroA 10 µM was mutually combined with FS 500, 750 and 1000 µg/ml. A possible explanation for this observation might be that GroA at the concentration of 10µM is too effective and it is more cytotoxic. As a result, 1000 µg/ml FS cannot provide an additional antiproliferative effect beyond the GroA effects. Additionally, the sulfate content of the preparations may provide an explanation for some of the observed results. In a previous study of fucoidan sulfate content, LMWF sulfate levels were higher than high-molecular-weight Fucoidan, and the antiproliferative activity of LMWF was dependent on the sulfate content. This antiproliferative effect decreases with the decreasing degree of sulfation (Haroun-Bouhedja et al., 2000). Furthermore, the differences in fucoidan structures may help to explain their antiproliferative activities. There are considerable variations in the anticancer activities between fucoidan polymers which are isolated from different parts of the same species of source material. As such, the anatomical regions and growing conditions of brown seaweeds, their extraction and purification procedures, as well as the use of different cancer cell lines are all reasons for the observed effects of the treatments in this study.

5.3 Alterations of Cell Cycle on LMWF combined with GroA

Many researchers have proposed various mechanisms for explaining the antiproliferative activity agents used as treatments against cancer cells. Induction of apoptosis, cell cycle arrest, and inhibition of angiogenesis, inhibition of various growth factors and inhibition of metastasis are all mechanisms which are used as reference points to describe the effects of drug treatments. The cell cycle or cell-division cycle is a set of events that result in cell growth and division into two daughter cells. The cell cycle is a very orderly progression strictly following the sequence of G1-S-G2-M. The G1 phase stands for "GAP 1", in which the cell size increases. The S phase represents "Synthesis" that is the stage for DNA replication. The G2 phase stands for "GAP 2". Cells keep on growing in this phase. The M phase is abbreviation of "Mitosis", where chromosomes separate and cytokinesis occurs. Moreover, there is a G0 phase, which is a resting phase for the cells which are not in the cycle and stop dividing (Vermeulen, Van Bockstaele, & Berneman, 2003).

Flow cytometry is an important technique applied in cell cycle studies. Cellular DNA content is often the single parameter measured by flow cytometry for cell cycle studies. Once fluorescent molecules are specifically and stoichiometrically used to bind DNA, a linear relationship between cellular fluorescence intensity and DNA amount can be measured. The emitted fluorescence of the DNA specific dyes is proportional to DNA content present in different phases of the cell cycle. PI is the most commonly used dye to quantitatively assess DNA content. PI binds to DNA by intercalating between the bases with little or no sequence preference. Because PI also binds to RNA, nucleases such as Ribonuclease (RNAse) are necessary to distinguish between RNA and DNA (Hansen et al., 1989). To explore the possible mechanisms of single and combined effects of different fucoidan and GroA, PC-3 and DU-145 cells were analysed for cell cycle alterations by staining with PI.

The alterations of Cell Cycle of LMWF were evaluated by Flow cytometric analysis of apoptosis using PI DNA staining. Here the proportion of sub-G1 hypodiploid cells (which is proportion to cell death) was estimated after 72 hours of drugs addition for each cell lines. By comparing the % of sub-G1 fraction, obtained after cells were treated with different concentrations (100, 200 and 300 µg/ml) of LMWF or combined with Cro/GroA, the effectiveness of cell death for different combination groups was estimated. Along with determining the index of the apoptotic

DNA fragmentation (i.e. % of sub-G1) this procedure helps with simultaneous analysis of cell-cycle parameters of surviving cells (i.e. cell cycle distribution). Thus any arrest or blockage of cells at any particular cell phase was also determined. Before starting the evaluation of apoptosis inducing activity by different fucoidan preparations in this study, it was important to analyse the effect of serum starvation on each cell line.

5.3.1 Cell Synchronization by Serum Starvation

Cell synchronization induced by serum starvation has been widely used in cell cycle studies from the time when Pardee established the concept of the restriction point (Pardee, 1974). Serum elimination from culture medium is supposed to reduce basal cellular activity. Cells cultured after starvation are more homogenous, owing to their withdrawing from the cell cycle and entrance into quiescent G0-G1 phase. G1 phase plays an important part in the cell cycle and determines whether a cell commits to division or to leave the cell cycle (Corinne & Erik, 1996). Moreover, serum elimination helps reduce the analytical interference and offers more reproducible experimental conditions (Sergej & Alexander, 2011).

Appropriate serum concentration is important for starvation. High serum concentration results in imperfect starvation. If the starvation is not absolute, some leakage will occur. The cells will be able to slowly accumulate material and lead to an initiation of S phase during the process of low serum starvation (Cooper, 2003). But a previous study indicated that a certain concentration of serum which could result in a slight cell leakage under the period of serum starvation would be the most appropriate serum concentration (Song & Lu, 2003). Moreover, the starvation time is another important factor. Long starvation time leads to the deleterious effects on cell survival and results in massive DNA fragmentation (apoptosis) (Kues et al., 2000). However, if the starvation time is not long enough, it is unable to help the leakage cells complete and may then block them in G0-G1 phase.

Cell starvation carried out for the two cell lines in the present study was accomplished by withdrawal of FBS (fetal bovine serum), and using FBS-free culture medium for 24 hours. For the PC-3 and DU-145 prostate cancer cell lines, 24 hours serum-free starvation resulted in good synchronization.

For this result, it can be speculated that the starvation time and the serum concentration are suitable for DU-145 and PC-3 cells.

5.3.2 Effects of LMWF or LMWF combined with GroA on Cell Cycle Distribution

Fucoidan treatment results in sub G0/G1 cell accumulation (suggestive of dead cells/apoptotic cells) in a variety of cell types (Aisa et al., 2005; Z. Zhang, Teruya, Eto, & Shirahata, 2011). It can also induce cell cycle arrest in other phases. Riou et al. (Riou et al., 1995) and Mourea et al. (Moreau et al., 2006) reported arrest in G1 phase in a chemo-resistant non-small-cell bronchopulmonary carcinoma line by fucoidan from Ascophyllum nodosum and Bifurcaria bifurcate, respectively. In an investigation of the mechanism of the action, fucoidan demonstrated significant down regulation of cyclin D1, cyclin D2 and CDK4 in cancer cells (Banafa & Roshan, 2013; Boo et al., 2013; Haneji et al., 2005). The crude fucoidan from Fucus vesiculosus increased the level of p21/WAF1/CIP1 in PC-3 cells and down-regulated E2F, a transcription factor that controls progression of cells from G1 to S phase (Boo et al., 2013).

In the present study, LMWF was found to increase the sub-G1 phase cell population in a dose-dependent manner, but also dose-dependently decreased the G0-G1 phase in PC-3 cells. The sub-G1 phase is an index of the apoptotic DNA fragmentation (Yu et al., 2011). This result indicated that LMWF induced cell apoptosis. The same result was found in DU-145, but LMWF 100 and 200 µg/ml treatments only induced minor sub-G1 effects on DU-145 cells, while 1000 µg/ml led to more sub-G1 changes (an increase of about 27.03%). Where LMWF was found to effect PC-3 cells in G0-G1 phase after culture for 72 hours, with a slight decrease in the phase population as LMWF concentrations increased, LMWF did not show an effect on S phase and G2-M phase populations compared with the Cro treatment group. LMWF induced apoptosis of PC-3 and DU-145 cells in a time- and dose- dependent manner. Taken together, the current results in PC-3 cells are consistent with the previous reports, namely fucoidan induces a rise in sub-G1 population and the cell line undergoes apoptosis.

LMWF combined with GroA was found to help induce prostate cancer cell apoptosis in PC-3 and DU-145 cells by affecting the sub-G1 phase, up to 37.61% at the heighest concentration of LMWF. The percentages of sub-G1 cells were higher than LMWF-only or GroA-only treatments. This means, the combination of LMWF and GroA resulted in a synergistic effect. Both GroA and LMWF block cells in the sub-G1 phase of the cell cycle distributions measured, and yet the effects of each drug on cellular metabolism are different. Furthermore, the accumulations of G0-G1 phase cells

decreased dramatically, up to 37.31%, in the treatment concentration of 300µg/ml LMWF.

Therefore, LMWF affects cell cycle progression by increasing the sub-G1 phase population, and LMWF combined with GroA improved this observed result on the sub-G1 phase while also affecting the G0-G1 phase in PC-3 and DU-145 cell lines. The combination of these two drugs is supposed to result in synergistic effects. The results of this study may suggest that LMWF regulates MAPK activity; it seems that combining two drugs, LMWF and GroA, may have a better inhibitory effect on EGFR receptor activation and by that a stronger inhibitory effect on cancer cell growth and tumorogenicity.

5.4 Anti-Cancer Potential of LMWF

Fucoidans have been extensively studied due to their diverse anticoagulant, antiviral and anticancer properties. The biological activities of fucoidans are closely related to their molecular structures, which include fucose linkages, various sugar types, sulfate content, and molecular weight.

Among the characteristics of studied fucoidans that are of interest, molecular weight is one of the most important factors determining the biological activities of polysaccharides. In a study of fucoidan hydrolysates, partially hydrolyzed fucoidans (MW = 490 kDa) showed significantly improved anticancer activity compared to the native polymers (MW = 5,100 kDa), and the effect was mostly seen when fucoidans were hydrolyzed under mild conditions (Yang et al., 2008). The mechanisms whereby lower molecular weight fucoidans have greater anticancer activities appear to be very complex and are not clearly understood. Sulfate content was first identified as a possible factor by Soeda et al. (Soeda, Sakaguchi, Shimeno, & Nagamatsu, 1992), who reported that fucoidan derivatives with various sulfate contents stimulate tissue plasminogen activator (t-PA)-induced plasma clot lysis and prevent the formation of fibrin polymers. Such activities increase proportionally with the degree of sulfation. It was also suggested that oversulfated fucoidans possess higher anti-angiogenic activity than native fucoidans, and thus more effectively inhibit the growth of tumor cells by suppressing angiogenesis (Koyanagi et al., 2003). Moreover, the anticancer activity of oversulfated fucoidan derivatives was likely due to differences in their sulfate content (Cho et al., 2010).

In the current results, we demonstrated that the anticancer potential of LMWF in two human prostate cancer cell lines affects cell cycle progression according to a reduction in cell wialibity, an increase in sub-G1 phase, and induction of cell apoptosis.

Previous studies suggested that LMWF has more biological actions than native fucoidan. LMWF mediated the broad-spectrum growth inhibition of human carcinoma cells, including HeLa cervix adenocarcinoma, HT1080 fibrosarcoma, K562 leukemia, U937 lymphoma, A549 lung adenocarcinoma and HL-60 5.5 Anti-Cancer Potential of Combination Treatment (Wang, Zhang, & Zha, 2004). LMWF inhibits the invasion and angiogenesis of HT 1080 fibrosarcoma cells and induces apoptosis in MCF-7 cancer cells via a mitochondria-mediated pathway (Wang et al., 2004; Ye et al., 2005). LMWF induces apoptosis through mitochondrial mediated pathways in MDA-MB-231 breast cancer cells and also indicated interrelated roles of Ca2+ homeostasis, mitochondrial dysfunction and caspase activation (Zhang, Teruya, Eto, et al., 2013). LMWF has a sulfate content higher than 20%, and was found to exert profound anticoagulant activity as well as antiproliferative effects on a fibroblast cell line (CCL39) in a dose-dependent fashion (Haroun-Bouhedja et al., 2000).

Fucoidans are available for use in cosmetics, functional foods, dietary supplements and for inclusion in pet, livestock and aquaculture feed supplements. To date there are no approved uses for fucoidan fractions in biomedical applications, either within biomaterials, or via direct administration (intravenous, intraperitoneal, intramuscular or subcutaneous). However, research into the use of fucoidan in drug delivery, biomaterials, as a topical agent and as an orally delivered agent for a variety of pathologies, appears promising. Therefore, LMWF is unlikely to be used as a sole agent for cancer treatment where there are known effective therapies, but as a non-toxic edible product option it is an easily deliverable active supplement. The potential for orally delivered LMWF as an “adjunct” therapy to conventional treatment merits further discussion. LMWF may find a role either to reduce side effects, to enhance the therapeutic effects of conventional therapy, or to address cancer for which there are no known therapy options.

5.5 The Potential of LMWF as a Synergistic Anti-Cancer Agent

This study identifies that LMWF can act as a synergistic anti-cancer agent in the treatment of prostate cancer cell lines DU-145 and PC-3. In cell proliferation assays, a joint inhibitory effect study of these two drugs was conducted using LMWF combined with GroA. The combination elicited a stronger inhibitory effect at lower concentration ranges when combined together. In cell apoptosis assays, the combination group led to greater cell apoptosis compared with LMWF-alone or GroA-alone treatments. In cell cycle analysis, LMWF combined with GroA led to a rise in sub-G1 phase, which means a greater induction of cell apoptosis.

According to case reports, LMWF had beneficial effects on patients with colorectal, pancreatic, bladder, uterine, lung, liver, breast, and prostate cancers. Most of these patients consumed LMWF while receiving surgery, chemotherapy, or radiotherapy. These patients received a daily dosage of 1–5 g of LMWF. LMWF reduced the tumor size and the adverse effects of chemotherapy and improved the quality of life. LMWF is widely used as a food supplement for cancer patients. A double-blind randomized controlled trial documented the efficacy of low-molecular-weight fucoidan as a supplemental therapy in metastatic colorectal cancer patients, and the results demonstrate the advantages of LMWF in improving the disease control rate. The advantages of LMWF indicated by this study can provide insights into further development of cancer treatments, particularly in the combination of natural or herbal products with chemotargeting agents. LMWF may act as a synergistic anti-cancer agent in future studies.

Chapter 6 Conclusion

6.1 Overall Conclusion

The present study describes the effect of LMWF, SF or combinations of either drug with GroA, on the proliferation of human prostate cancer cell lines PC-3 and DU-145. LMWF and FS inhibited the growth of the cancer cells in a time- and dose-dependent manner in the present study, but the effect of SF was not significant at the concentrations and combinations tested. Simultaneous combination of LMWF and GroA made an additive inhibitory effect on prostate cancer cells, but in SF combined with GroA groups, SF was not able to enhance the inhibitory effect of GroA on proliferation of PC-3 and DU-145 cells in a concentration dependent manner, even at very high concentrations. Combination treatments of SF with GroA, only indicated a GroA effect at the measured timepoints. The result may suggest that LMWF has the potential to regulate MAPK activity; it seems that combining two drugs, Fucoidan and GroA, could have a better inhibitory effect on EGFR receptor activation and by that a stronger inhibitory effect on cancer cell growth and tumorogenicity. Cell apoptosis and cell cycle analysis conducted by using flow cytometry was applied to study the mechanism of action of these two drugs. This study provides further evidence that LMWF can affect the cell cycle in sub-G1 phase.

Guanosine-rich oligonucleotide (GRO，GroA) AS1411 has the sequence 5′-GGTGGTGGTGGTTGTGGTGGTGGTGG-3′ and is also known as GR026B and AGRO100, which is the first nucleic acid aptamer to be tested as a treatment for cancer. GroA (AS1411) can be a nucleolin inhibitor, which combine with fucoidan could have a better inhibitory effect on EGFR receptor activation and by that a stronger inhibitory effect on cancer cell growth and tumorogenicity.

In this study, even at low doses, LMWF significantly improved the anti-proliferative effect of GroA. LMWF, the most abundant carotenoid found in marine algae, is considered as a potential candidate for the development of anti-cancer drugs for the treatment of prostate cancer and further applications in clinical cancer chemotherapies. In summary, this study is the beginning of research intent on ascertaining the anti-proliferative activity of LMWF against prostate cancer cells.

6.2 Future research

The *in vitro* study of GroA and fucoidan on human prostate cancer cells showed synergistic effects of the drug treatments. Apoptotic and cell cycle analysis utilized kits containing RNAse A and PI, while other reagents were not chosen for this study.Only cell cycle analysis and apoptosis was applied to study the mechanism of action of drugs in this study. A number of studies for deeper investigation of the observed cell cycle inhibitions and apoptosis mechanisms, such as southern and western blotting were not carried out in this study. In order to deeply explore the mechanism of action of GroA and LMWF, further research is imperative. Additionally, experimental designs can also be improved. For example testing for protein levels of caspase 3 (one of the major apoptosis markers), using western blot, fluorescent microscopy, or flow cytometry may prove beneficial to furthering the understandings of observed mechanisms. Cell proliferation assays, testing the Ki67 protein levels (with western blot or fluorescent microscopy) may also prove useful. Testing tumorigenicity and subsequent inhibition in nude mice models is also of interest. Specific testing for protein levels of ErbB1 is also important to identifying the effects of fucoidans in the cancer cells tested thus far. Characterization or ErbB1 in PC-3 and DU-145 cell treatments, followed by identification of downstream proteins and their roles in antiproliferative and cell cycle progression activities will prove useful. Additionally, some adjustments can be made to the drug concentrations, treatment sequences and exposure times used in the initial studies as a means to improve and clarify current conclusions.

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Appendix

A1: Cell viability of PC-3 and DU-145 cells incubated in the presence of single treatment in 72 and 96 hours.

Table 16: Cell viability of PC-3 and DU-145 cells incubated in the presence of single treatment in 72 and 96 hours. Data are presented as means ± S.D, n=6.

Sigma Fucoidan(FS) (µg/ml)	PC-3 72h	PC-3 96h	DU-145 72h	DU-145 96h
0	100	100	100	100
500	90.5±0.96	84.3±0.19	93.9±0.83	88.6±0.31

700	88.8±0.42	82.7±0.31	90.2±0.09	90.5±0.51
1000	84.8±0.96	83.2±0.64	89.2±0.35	90.5±12
LMWF (µg/ml)				
100	64.7±0.02	65.3±0.47	70.3±0.68	63.8±0.88
200	62.3±0.74	58.5±0.16	69.2±0.06	62.1±0.07
300	57.6±0.15	58.1±0.45	64.2±0.81	60.7±0.24

A2: Cell viability of PC-3 cells incubated in the presence of combination treatment in 72 and 96 hours.

Table 17: Cell viability of PC-3 cells incubated in the presence of combination treatment in 72 hours. Data are presented as means ± S.D, n=6.

Sigma Fucoidan(FS) (µg/ml)	**Cro 10µM**	**GroA 10µM**
0	87.6±0.12	60.4±0.52
500	87.7±0.77	95.0±0.23
700	84.2±0.24	60.0±0.74
1000	81.6±0.10	59.4±0.39
LMWF (µg/ml)		
100	63.8±0.73	52.5±0.91
200	57.6±0.43	48.7±0.52

300	53.1±0.47	45.1±0.85

Table 18: Cell viability of PC-3 cells incubated in the presence and combination treatment in 96 hours. Data are presented as means ± S.D, n=6.

Sigma Fucoidan(FS) (µg/ml)	**Cro 10µM**	**GroA 10µM**
0	93.4±0.82	61.6±0.64
500	85.0±0.19	57.4±0.84
700	82.5±0.58	58.1±0.91
1000	81.0±0.07	57.4±0.99
LMWF (µg/ml)		
100	56.9±0.16	47.7±0.92
200	48.8±0.83	41.6±0.59
300	43.3±0.92	39.4±0.03

A3: Cell viability of DU-145 cells incubated in the presence and combination treatment in 72 and 96 hours.

Table 19: Cell viability of DU-145 cells incubated in the presence and combination treatment in 72 hours. Data are presented as means ± S.D, n=6.

Sigma Fucoidan(FS) (µg/ml)	**Cro 10µM**	**GroA 10µM**
0	93.4±0.82	66.0±0.08
500	92.7±0.54	60.8±0.31
700	91.2±0.86	66.1±0.79
1000	91.7±0.40	54.8±0.78
LMWF (µg/ml)		
100	66.0±0.85	54.5±0.04

200	60.8±0.31	52.7±0.44
300	54.8±0.78	47.4±0.08

Table 20: Cell viability of DU-145 cells incubated in the presence and combination treatment in 96 hours. Data are presented as means ± S.D, n=6.

Sigma Fucoidan(FS) (µg/ml)	**Cro 10µM**	**GroA 10µM**
0	96.4±0.82	62.6±0.37
500	91.7±0.33	62.7±0.16
700	89.1±0.89	63.2±0.62
1000	89.3±0.09	62.6±0.46
LMWF (µg/ml)		
100	51.8±0.04	40.4±0.65
200	41.2±0.39	37.2±0.03
300	38.2±0.52	37.0±0.49

A4: Apoptosis inducing effect on PC-3 and DU-145 cells after treatment with LMWF and GroA compared with SF and GroA.

Table 21: Apoptosis inducing effect on PC-3 cells after treatment with either GroA/Cro or LMWF alone, or both combined, for 72 hours. Data are presented as means ± S.D, n=3.

Treatment (µg/ml)	Viable (%)	Late apoptosis (%)	Early apoptosis (%)
No treatment	96.20±0.66	1.57±0.32	0.23±0.03
Cro	95.12±0.21	0.82±0.16	0.43±0.27
GroA	69.04±0.73	21.16±0.47	2.45±1.09
100F+Cro	78.57±0.92	11.28±0.91	2.04±0.82

200F+Cro	70.27±0.79	16.15±0.88	4.05±0.91
300F+Cro	54.24±1.22	16.00±0.97	5.45±0.43
100F+GroA	59.77±1.38	29.93±1.56	3.33±0.68
200F+GroA	46.05±2.09	40.01±1.47	2.41±0.17
300F+GroA	40.45±2.57	54.64±2.83	3.24±0.20

Table 22: Apoptosis inducing effect on DU-145 cells after treatment with either GroA/Cro or LMWF alone, or both combined, for 72 hours. Data are presented as means ± S.D, n=3.

Treatment (µg/ml)	Viable (%)	Late apoptosis (%)	Early apoptosis (%)
No treatment	97.03±0.24	1.88±0.63	0.80±0.09
Cro	97.08±0.81	0.65±0.13	1.74±0.21
GroA	69.34±0.46	23.71±1.04	4.90±0.54
100F+Cro	77.76±1.77	9.77±1.19	9.22±0.82
200F+Cro	68.32±1.05	12.15±0.85	12.04±0.91
300F+Cro	51.43±1.21	14.05±0.37	14.73±0.33
100F+GroA	54.04±1.87	23.89±1.57	8.03±0.63

200F+GroA	42.81±1.50	32.63±1.49	17.31±0.95
300F+GroA	37.61±1.29	47.15±2.08	11.20±0.79

Table 23: Apoptosis inducing effect on PC-3 cells after treatment with either GroA/Cro or SF alone, or both combined, for 72 hours. Data are presented as means ± S.D, n=3.

Treatment (µg/ml)	Viable (%)	Late apoptosis (%)	Early apoptosis (%)
No treatment	95.61±0.82	1.42±0.14	0.76±0.23
Cro	95.61±0.17	1.42±0.10	0.76±0.15
GroA	70.31±0.45	16.69±1.17	1.75±0.33
500F+Cro	90.38±1.73	7.45±0.21	0.12±0.08
750F+Cro	90.81±1,56	5.84±0.38	0.11±0.21
1000F+Cro	90.17±1.21	4.47±0.91	0.04±0.13
500F+GroA	74.41±2.65	18.99±1.72	0.66±0.11
750F+GroA	72.65±1.39	21.23±2.26	0.67±0.57
1000F+GroA	70.61±0.61	23.13±3.17	0.72±0.29

Table 24: Apoptosis inducing effect on DU-145 cells after treatment with either GroA/Cro or SF alone, or both combined, for 72 hours. Data are presented as means ± S.D, n=3.

Treatment (µg/ml)	Viable (%)	Late apoptosis (%)	Early apoptosis (%)
No treatment	95.84±1.98	1.95±0.13	0.35±0.04
Cro	95.84±0.22	1.95±0.01	0.35±0.17
GroA	71.74±1.03	19.71±1.14	6.72±0.23
500F+Cro	90.85±2.78	4.69±0.18	00.23±0.04
750F+Cro	91.74±1.25	4.00±0.09	0.06±0.19
1000F+Cro	87.61±1.73	4.49±0.17	0.91±0.45
500F+GroA	77.70±1.09	13.31±0.82	3.11±0.19

750F+GroA	73.06±2.14	16.98±1.92	1.88±0.71
1000F+GroA	68.05±2.99	19.46±1.64	4.62±0.52

A5: Cell cycle distribution of DC-3 and DU-145 cells after combination treatment (LMWF and GroA).

Table 25: Cell cycle distribution of PC-3 cells after combination treatment, for 72 hours. Data are presented as means ± S.D, n=3.

Treatment (µg/ml)	sub-G_1 (%)	G_0-G_1 (%)	G_2-M (%)	S (%)
No treatment	7.44±0.02	72.25±0.97	11.17±0.13	8.50±0.10
Cro	5.34±0.03	62.79±0.54	15.86±0.02	12.96±0.91
GroA	25.25±0.15	40.33±0.22	21.89±0.25	10.00±0.82
100F+Cro	13.31±1.17	63.03±1.03	13.99±0.94	9.19±0.14
200F+Cro	16.23±1.52	58.42±1.15	12.77±0.58	10.37±1.33
300F+Cro	21.41±0.08	57.94±0.97	12.33±0.12	7.33±0.90
100F+GroA	27.73±1.62	41.09±0.48	19.65±1.23	9.26±1.04
200F+GroA	32.05±1.56	37.98±1.12	19.25±1.77	8.31±0.71
300F+GroA	37.61±1.09	37.31±1.38	15.12±1.59	7.68±0.20

Table 26: Cell cycle distribution of DU-145 cells after combination treatment, for 72 hours. Data are presented as means ± S.D, n=3.

Treatment (µg/ml)	sub-G_1 (%)	G_0-G_1 (%)	G_2-M (%)	S (%)
No treatment	0.43±0.01	67.98±0.27	16.68±0.16	13.63±0.58
Cro	1.14±0.03	64.61±1.65	18.77±0.70	13.51±0.04
GroA	7.15±0.82	55.86±1.21	24.18±0.83	11.48±0.17
100F+Cro	7.89±0.06	58.64±0.79	18.03±0.21	11.80±0.69
200F+Cro	12.69±0.31	56.39±0.92	18.47±0.77	10.11±0.63
300F+Cro	12.80±0.28	57.81±0.11	17.06±0.65	10.02±0.24
100F+GroA	15.25±0.22	55.16±0.54	20.16±0.55	7.59±0.40

200F+GroA	17.00±1.17	53.33±1.08	19.73±1.01	7.27±0.66
300F+GroA	27.03±1.63	47.71±1.13	17.39±1.47	5.34±0.02

A6: The Voltage used for Sample Running

Table 27: The FL3 voltage set for each time sample running

	Voltage (Volts)		
Cell line	**Starvation**	**72 hours**	**96 hours**
PC-3	460	465	470
DU-145	475	480	480

A7: 1:2 Dilution plan for making cell linearity standard curve

Number	**Cell concentration (Cells/mL)**	**Cell concentration (Cells/mL)**
1	250,000	500,000
2	125,000	250,000
3	62,500	125,000
4	31,250	62,500
5	15,625	31,250
6	7,812	15,625
7	3,906	7,812
8	1,953	3,906
9	976	1,953

A8: SF dilution plan for PC-3 and DU-145

Tube Number	**Actual fucoidan concentration (µg/ml)**	**Prepared fucoidan concentration (µg/ml)**	**Dilution plan (Each 1.5 mL micro-tube has 1000 µL Fucoidan and complete culture medium mixture)**
1	0	0	1000 µL medium
2	1000	2000	1000 µL (2000 µg/ml SF stock solution) + 0 µL medium
3	750	1500	750 µL (2000 µg/ml SF stock solution) + 250 µL medium
4	500	1000	500 µL (2000 µg/ml SF stock solution) + 500 µL medium
5	400	800	400 µL (2000 µg/ml SF stock solution) + 600 µL medium
6	300	600	300 µL (2000 µg/ml SF stock solution) + 700 µL medium
7	200	400	200 µL (2000 µg/ml SF stock solution) + 800 µL medium
8	100	200	100 µL (2000 µg/ml SF stock solution) + 900 µL medium
9	50	100	50 µL (2000 µg/ml SF stock solution) + 9500 µL medium
10	10	20	10 µL (2000 µg/ml SF stock solution) + 990 µL medium

A9: LMWF 1:2 dilution plan

1:2 Dilution Tube Number	**Prepared Fucoidan Concentration (ug/ml)**	**1:2 Dilution Plan (Each 1.5 mL micro-tube has 1000 uL Fucoidan and complete culture medium mixture)**
1	3200	1.5 ml (**3200 µg/ml** LMWF stock solution)
2	1600	500 µl (take from tube 1)+ 500 µL medium
3	800	500 µl (take from tube 2)+ 500 µL medium
4	400	500 µl (take from tube 3)+ 500 µL medium
5	200	500 µl (take from tube 4)+ 500 µL medium
6	100	500 µl (take from tube 5)+ 500 µL medium
7	50	500 µl (take from tube 6)+ 500 µL medium
8	25	500 µl (take from tube 7)+ 500 µL medium
9	12.5	500 µl (take from tube 8)+ 500 µL medium
10	6.25	500 µl (take from tube 9)+ 500 µL medium

A10: LMWF dilution plan for PC-3 and DU-145

Tube Number	**Actual fucoidan concentration (ug/ml)**	**Prepared fucoidan concentration (ug/ml)**	**Dilution plan (Each 1.5 mL micro-tube has 1000 µL Fucoidan and complete culture medium mixture)**
1	0	0	1000 µL medium
2	1600	3200	1:2 Dilution Tube 1
3	800	1600	1:2 Dilution Tube 2
4	400	800	1:2 Dilution Tube 3
5	100	200	1:2 Dilution Tube 5
6	25	50	1:2 Dilution Tube 7
7	12.5	25	1:2 Dilution Tube 8
8	6.25	12.5	1:2 Dilution Tube 9
9	1	2	320 µL (1:2 Dilution Tube 10) + 680 µL medium