EFFECTS OF PRODIGIOSIN ON BREAST CANCER CELL VIABILITY

A Thesis submitted to the Department of Material Science and Engineering,

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In partial fulfillment of requirements for the degree of

Master of Science in Material Science and Engineering

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DECLARATION

I hereby declare that this submission is my own work toward the MSE and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgment has been made in the text.

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ABSTRACT

The Luteinizing Hormone-releasing Hormone (LHRH); also known as a Gonadotropin-Releasing Hormone can be utilized for targeted therapy with cytotoxic analogs, in which prodigiosin is linked to the [D-Lys6] LHRH. Our studies demonstrate receptor-mediated actions of the cytotoxic LHRH analog in LHRH receptor positive breast cancer cells. Chemotherapy today is the only systematic therapy for patients with triple negative breast cancer cells. About 50% to 64% of human breast cancer expressed receptors for LHRH might be used as a target. This thesis focused on a new conjugate in which prodigiosin was used as a cytostatic compound and an analog or Luteinizing Hormone-releasing Hormone (LHRH) as a targeting moiety was synthesized. The molecules of the peptide were modified to allow its connection to the prodigiosin via a spacer. The objectives of our study were to synthesize a bioconjugate of LHRH analog [D-Lys]-LHRH and prodigiosin targets and inhibits breast cancer cell growth *in vitro* and *in vivo.* Prodigiosin was synthesized by bacteria (*Serratia marcescens* (subsp. *marcescen*)). High-performance liquid chromatography (HPLC) analysis on the purity was determined to be 92.8% and the ultraviolet (UV) reading was 535nm. Adhesion force measurements were also carried out to determine the adhesion force between the conjugate and the breast cancer cells. This had the highest measurement as compared to the bare, prodigiosin coated and LHRH-coated tips. The adhesion force of the conjugates measured (80±4nN) which gave the highest peak. The conjugates were prepared linking prodigiosin through its 14-0-hemiglutarate to [D-Lys6] LHRH. The results showed that the use of LHRH peptide as a targeting moiety in the anticancer drug delivery substantially enhanced the efficacy of prodigiosin which leads to amplified apoptosis induction in the tumor and minimized the side effects of the anticancer drug on healthy organs. In conclusion, prodigiosin conjugate with LHRH exhibited an antiproliferative effect for which its further testing will be implemented.

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DEDICATION

With much love, care and happiness, this work is dedicated to God almighty who has seen me through the up and downs of life and to my family. My parents, Mr. /Mrs. Albert S. Darh and my uncle Mr. Robert L. Kleekpo for all their support toward me.

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CHAPTER ONE

1.0 Introduction

Cancer is a broad term for over 200 different diseases. The increasing incidence of cancer [1] has stimulated research on the development of novel therapies that would be effective in the treatment and reduction in the rate of re-occurrence. Currently, cancer is the second leading cause of death after cardiovascular diseases [2, 3]. Among the 200 different classes of diseases, breast cancer is the second most common cause of cancer death in women [3]. By 2030, cancer will become the leading cause of death as suggested by current trends [2, 3]. The changes that occur in genes cause one cell or a few cells to grow and multiply uncontrollably leading to cancer. Where cancer starts it is a primary tumor and sometimes when it spreads to other parts of the body it is called a secondary tumor or metastasis. The conventional treatment of cancer can affect the body systems, such as blood circulation, lymphatic and immune systems, and the hormone system [1]. The conventional treatments include surgery, radiation, chemotherapy, hormone therapy, immune therapy and targeted therapy (drugs that specifically interfere with cancer cell growth) [4]. Drug delivery refers to the approaches, formulations, technologies, and systems for transporting a pharmaceutical compound in the body as needed to safely achieve its desired therapeutic effect. The use of localized drug delivery has so many advantages as compared to the conventional treatments. This treatment can avoid harm to normal cells, dramatically reduces side effects, high efficacy and controlled drug release.

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. Cancer is believed to be caused by external factors such as tobacco smoking, infectious organisms, and unhealthy diet, and internal factors, such as inherited genetic mutations, hormones, and immune conditions. Research shows that cancer has resulted in more deaths than death from AIDS, tuberculosis, and malaria combined [5]. The Global burden of cancer continues to increase largely because of the aging and growth of the world population. This is due to the increasing adaptation of cancer-causing behaviors, particularly smoking in economically developing and developed countries [5].

The cancer of the breast is one of the most prevalent cancers among women in the world. Breast cancer has two main types: (1) ductal carcinoma and (2) lobular carcinoma. Ductal carcinoma

starts in the tubes (ducts) that move milk through the breast tissue. Lobular carcinoma starts in the parts of the breast called lobules that produce milk. Breast cancer is the most frequent malignant gynecological tumor, in most cases, it is diagnosed at an early stage when surgery alone or combination with radiotherapy can achieve high cure rates. Though for some patients even after adequate treatment, they experience severe pain either due to disease progression or due to treatment-related side effects [16]. Variations in the rates of incidence as well as mortality of breast cancer are associated with factors like age, race, socioeconomic status, lifestyle, reproductive history, family history etc. [1]. International Association for the Study of Pain (IASP) estimated that the prevalence of pain in breast cancer ranges from 40-89% [5]. 20-50 % of women are affected by persistent neuropathic pain after their surgical treatment and it has been found that persistent pain after surgical treatment is quite common and is higher among young patients who are undergoing radiotherapy and axillary lymph node dissection [1]. In early breast cancer, pain does not usually occur. The first symptom may be a painless lump and in later stages, pain may occur due to the involvement of deeper structures like muscles, ribs, etc. As a result of this, it can lead to severe excruciating pain which may increase the pain of the chest. The major challenges in cancer treatment are the early detection of cancer before metastasis [7] and also the side effects associated with the available treatment methods. The current detection techniques in breast cancer include mammograms, ultrasound, magnetic resonance (MRI) and various types of scan which are not yet considered effective for early stages in cancer diagnosis. [9]. One of the best approaches that can be used to reduce the potential side effects of cancer treatments is to use localized drug delivery. This has the capacity to reduce high concentrations of cancer drugs in the tissue. Localized drug delivery can be achieved by using the conjugation of prodigiosin and LHRH.

Serratia marcescens is a gram-negative bacillus, which belongs to the family Enterobacteriaceae. It is a ubiquitous bacterium inhabiting water, soil, plants, and vertebrates and it has various characteristics including the production of the pigment prodigiosin. Prodigiosin induces apoptosis in human cancer cell lines. This is a form of cell death in which cells actively participate in their own destructive processes. Cells undergoing apoptosis shrink and lose their normal intercellular contacts and subsequently exhibit cytoplasmic and chromatin condensation and internucleosomal cleavage of DNA. Cells become fragmented into small apoptotic bodies during the final stage which are then eliminated by phagocytosis [10].

One of the various approaches in cancer therapy is the finding of receptors, peptide hormones such as somatostatin, bombesin, and LHRH. LHRH peptides are expressed on tumors in higher concentrations than on most normal cells. Analogs of these peptide hormones can be used as carrier vectors as they deliver the anticancer drugs directly to cancerous cells. This will increase the level of drug concentration in tumor tissues thus sparing noncancerous cells from unnecessary exposure [9]. In recent years, series of cytotoxic LHRH conjugates, including AN-152, which consists of LHRH agonist carriers, through a glutamic acid spacer to one molecule of doxorubicin [13], have shown a high-affinity binding. Demonstrated LHRH-R mediated internalization by MCF-7 human estrogen-dependent breast cancer cells [13] has been suggested recently. There is a cross-talk between G. Protein-coupled receptors, such as the LHRH-R and other signaling pathways [17].

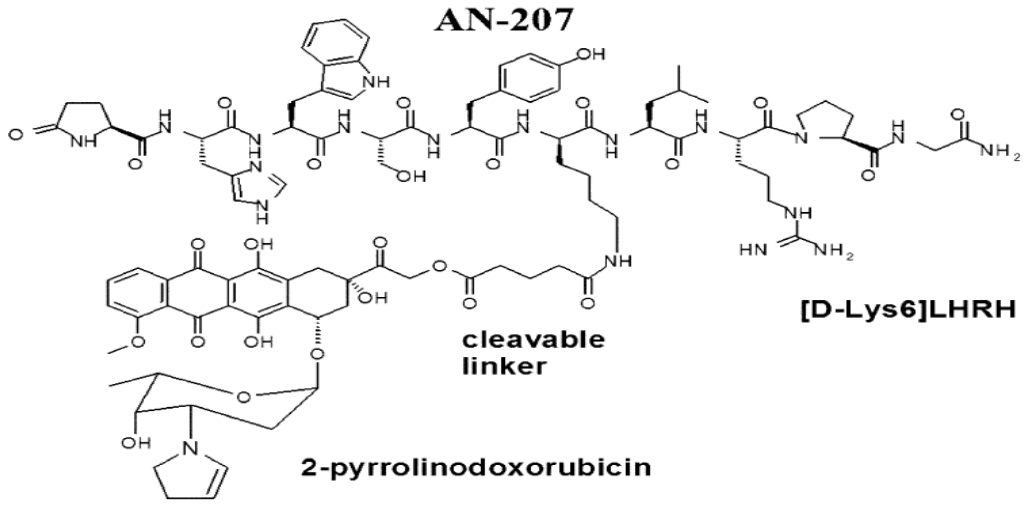


Figure 1.1 Schematic of Conjugation of Prodigiosin-LHRH

LHRH receptors are expressed in ovary and breast carcinomas while prodigiosin is an effective cytotoxic compound. The conjugation of prodigiosin-LHRH on human breast cancer cells is expected to increase the efficacy of the anticancer drug (prodigiosin). With this conjugation, we believe that the drugs will be delivered at target sites leaving normal breast cells unaffected.

1.1 Scope of Work

This thesis presents a combination of experimental and theoretical studies that provide new insights into the production of prodigiosin, a promising drug reported for its characteristics of having antifungal, immune-suppressive, anti-malaria and antiproliferation activity. We also looked at LHRH which has great potentials for the detection and treatment of cancer. LHRH in the past years has been synthesized and evaluated for therapeutic use in cancer research. Below are the objectives of the current work.

- To extract, purify, characterize, and quantify prodigiosin from *Serratia marcescen* at various incubation times in peptone glycerol agar.
- To study the effects of PH, temperature and growth dynamics in prodigiosin production.
- To conjugate prodigiosin and LHRH
- To study the interaction between the conjugate and breast cancer cell by studying the effects of adhesion.

CHAPTER TWO

2.0 Literature Review

2.1 Introduction

Prodigiosin, a family of a natural red pigment commonly characterized by a pyrrolylpyrromethene skeleton, are produced by various bacteria that first characterized from *Serratia marcescens* [1]. This pigment is a promising drug owing to its repeated characteristics of having antifungal, immunosuppressive and antiproliferative activity. Prodigiosin can also induce apoptosis in human cancer cell lines [1]. For targeted delivery of anticancer drugs, several receptors, which are over-expressed in cancer cells, are selected targets for polymer binding, such as prostate specific membrane antigen (PSMA), epidermal growth factor receptor (EGFR), and Luteinizing-hormone-releasing hormone (LHRH) receptor. LHRH is an amino acid peptide hormone secreted by the hypothalamus and regulates gametogenesis. The over-expression of LHRH receptors are detected in prostate (86%), ovarian (80%), and breast (50%) cancers and have low expression in healthy organs [2].

In recent years, LHRH and its analog have been employed in the clinical trial in the management of prostate cancer. Active targeting by LHRH is expected to be safe and efficient after systemic administration. Due to the short half-life of natural LHRH, synthetic LHRH analog with improved bioactivity has been widely used for targeting LHRH-receptors [2].

Of the various approaches to target cancer therapy, one is based on findings that receptors for certain peptide hormones such as somatostatin, bombesin, and LHRH are expressed on tumors in higher concentrations than on most normal cells. Thus, analogs of these peptide hormones can be used as carrier vectors to deliver cytotoxic agents directly to cancerous cells, thereby increasing the concentration of the drugs in tumor tissue and sparing normal, noncancerous cells from unnecessary exposure [3].

2.2 Prodigiosin Production by *Serratia marcescens*

Serratia marcescens a family of Enterobacteriaceae is a gram-negative bacterium. These bacteria can produce three special enzymes (DNAase, lipase, and gelatinase). These characteristics made them different from other genera of bacteria. However, there are a number of other traits that have been identified in this species that may contribute to pathogenesis. These traits include

swimming and swarming, motility and extracellular enzyme activities; that is nuclease, protease, and hemolysin [4]. *Serratia marcescens* can occur in water, soil, on plants, in insects, man and in animals [4]. *Serratia marcescens* is the only pathogenic species of the genera while *S. plymuthica*, *liquefaciens, rubidaea* and *odifera* rarely cause infection. *Serratia* produces prodigiosin, a secondary metabolite.

Prodigiosin is a natural product that represents one of the critical sources of chemical diversity and potential medicinal use [7]. The production of prodigiosin by the microorganism *Serratia marcescens* are a reminiscence of its secondary metabolism commonly referred to as bio-pigments. These bio-pigments have wide synthetic and commercial applications [7]. The production of pigment differs among species and is dependent on many factors such as species type and incubation time [9]. The variation depending on species type is associated with species habitats. Those species that are found in soil especially dump sites, had the incubation time ranging from 24h to 72 h. These pigments have no known or defined role in the physiology of producing strains but have been reported to have antifungal, antibacterial, algicidal, antiprotozoal, antimalarial activities, immune-suppressive, and anticancer activities [5]. The media regularly and currently used for prodigiosin biosynthesis are nutrient broth and peptone glycerol agar. A powdered l-peanuts medium showed forty fold increases in the concentration of prodigiosin [2].

There are many factors involved in the anticancer and immune-suppressive activity of prodigiosin. In therapeutic use, prodigiosin structure analogs have been designed and also the ring of pyrrole has also been reported to be very important in activity. In 2006, Murugkar *et al* had an interesting study on the red colored microbe that was isolated from mangrove soil. Purified prodigiosin was also reported by Mekheal and Yousif in 2009 showing plasmid curing activity on plasmids of E. coli HB101 and S. aureus [10].

2.2.1 Prodigiosin Structure

Prodigiosin is a family of tripyrrole red pigments that contains a common 4-methoxy, 2-2 bipyrrole ring system. The biosynthesis of the pigment is a bifurcated process in which mono and bipyrrole precursors are synthesized separately and then assembled to form prodigiosin [8]. Prodigiosin has been shown to be associated with extracellular vesicles, and cells associated or present in intracellular granules [9]. It has a chemical formula of $C_{20}H_{25}N_3O$ and a molecular

weight of 323.44 Da. It is sensitive to light and insoluble in water. It is moderately soluble in alcohol and ether, and soluble in chloroform, methanol, acetonitrile and DMSO (Figure 2.1) [1].

2.2.2 Effects of Growth Conditions on Yield of Prodigiosin

Serratia species, like other Enterobacteriaceae, grow well on ordinary media under anaerobic and aerobic conditions. They grow perfectly on synthetic media using various compounds as a single carbon source. Many types of differential and selective media have been developed for the isolation and presumptive testing for *Serratia* species. Media that are currently used for the biosynthesis of prodigiosin are nutrient broth, peptone glycerol broth [4, 5] etc. Nakamura [7], used oleic acid substitution instead of sodium oleate and has used only triolein as substrate and reported a yield of 0.69g/ml prodigiosin [7]. The idea of designing a new, nutritious and economically cheap medium was thought of for the prodigiosin biosynthesis. The maximum production of prodigiosin was seen at 28^0C and 30^0C in nutrient broth. *Serratia marcescens* did not show any pigment production at 37^0C for nutrient broth. Study with an internal adsorbent for prodigiosin in the bioreactor finally yield 13mg/ml [5]. Maltose addition to nutrient broth enhanced pigment production only by 2/4 as at 28^0C and 30^0C (**Table 2. 1**). Maltose acts as a better source of the substrate in enhancing pigment production in nutrient broth among the two sugars. The addition of glucose or maltose can cause a reduction in prodigiosin production which could be due to catabolite repression (**Table 2.1**) [5].

2.3 Luteinizing Hormone Releasing Hormone (LHRH)

LHRH also known as the Gonadotropin-releasing hormone (GnRH) is a hormonal decapeptide produced by the hypothalamus which plays a vital role in the regulation of the pituitary gonadal axis and thus reproduction. Its effects are exerted through binding to high-affinity receptors on the pituitary gonadotrophic cells and subsequently release FSH and LH. In the past few years, a large number of antagonists of luteinizing-hormone-releasing hormone (LHRH) has been synthesized and evaluated for therapeutic use [11]. LHRH analogs are widely used in oncology, and their mode of action is completely understood. The mechanism of action of these analogs is mainly based on the inhibition of pituitary and gonadal function but direct effects on various tumors may also play a role. Downregulation of LHRH receptors and uncoupling of the LHRH signal transduction mechanism can be caused by continuous treatment with LHRH. This results

in a desensitization of gonadotrophic and marked reduction in the secretion of bioactive LH and FSH. The reversible state is called “selective medical hypophysectomy”.

There are various practical applications of these LHRH analogs. These include treatment of gynecologic disorders and hormone-sensitive tumors such as prostate and breast cancers [12]. Healthy organs cytotoxic effect can be significantly diminished by employing special drug delivery systems, targeting specifically to the cancer cells [13]. Targeting cancer is usually achieved by adding to drugs delivery a ligand moiety directed to certain types of binding sites on cancer cells [14].

The receptors for LHRH are over-expressed in breast, ovarian and prostate cancer cells [10]. The LHRH receptors are not expressed detectably in most visceral organs. The advantages of this differential receptor expression are used in a modified LHRH peptide as a targeting moiety. These peptides are used to enhance drug uptake by the mentioned cancer cells and reduce the relative availability of the toxic drug to normal cells. Analogs of LHRH peptide in the mid-1980 were introduced to target LHRH receptor in prostate cancers and breast cancers. Investigations have been done extensively of the expression of LHRH receptors on human cancers cell lines and a tumor specimen has been performed worldwide during the past two (2) decades [15].

2.3.1 Chemistry of Drug-Peptide Conjugation

There have been different approaches used to conjugate carrier peptides to cytotoxic drugs. The chemistry of the conjugate has a profound impact on the stability of the conjugate. Each functional group to link the peptide and the drug has both advantages and disadvantages. This section explores common conjugation methods that have been used to make drug-peptide conjugates. Chemical bonds are normally used to link the spacer and peptide carrier or to link the drug directly to the peptide without a spacer.

1. Amide bond – drug-peptide conjugation via an amide bond is carried out by linking the carboxylic acid of the drug and primary amine of the spacer/peptide. The formation is straightforward, and the bond has relatively high chemical stability. Enzymatic cleavage of the amide bond between the drug and the peptide may be slow. There is a high probability that the conjugate will reach the target site with minor degradation. In this

conjugation, the portion of the drug may be active while it is attached to the peptide carrier [13].

2. Carboxylic Acid Ester bond – the linkage of the ester is commonly used to conjugate the drug to peptide because it can be hydrolyzed chemically or enzymatically to release the drug. Due to the instability of the ester bond, a release drug may reach before the target tissues. Several analogs of LHRH peptide have been conjugated via an ester bond to various cytotoxic agents including DOX and its derivatives [15].

3. Hydrazone Bond – this linkage can be utilized as an acid-labile bond for releasing the drug molecule from the conjugate upon a decrease in pH in tumor extracellular environments and in the lysosomes [15].

4. Enzymatic cleavage bond – enzymatic release of the drug, specifically peptide sequence may be utilized as a cleavable spacer between the drug and the carrier. One of the most widely used spacer sequences is a specific peptide substrate for the prostate specific antigen which is a serine protease enzyme that is expressed at high levels in prostate tumors [15].

5. Drug-ICAM-1 peptide conjugate – derived peptides from intercellular adhesion molecule 1 (ICAM-1) constitute a separate class of promising cell adhesion peptides that have the potential to target drugs to leukocytes. These peptides inhibited homotypic and heterotypic leukocyte adhesion mediated by leukocyte function-associated antigen-1 (LFA-1)/ICAM-1 interactions. The LFA-1/ICAM-1 mediated leukocyte adhesion can be modulated by anti-CD11a antibodies [15].

2.3.2 Drug-LHRH (GnRH) Analog Peptide Conjugates

Over-expression of LHRH receptors in breast, ovarian, endometrial and prostate cancers provides an excellent opportunity to target drugs to the cancer cell using LHRH peptides [12]. LHRH peptides that are short have been designed as receptor agonists and antagonists for releasing luteinizing hormone. Dox and 2-pyrroleno –Dox have also been antagonist peptides for selectively targeting cancer cells. The presence of D-Lys at position 6 in LHRH peptide is necessary for high receptor binding affinity and agonistic activity. The hydrophobic tripeptide, on the other hand, is a sequence at the N-terminal region of LHRH that is necessary for the

antagonist activity [16]. *In vivo* studies indicated that the drug agonist conjugates are less toxic and more potent than the corresponding individual drugs. This may be due to the selectivity of the conjugate to target cancer cells.

The structure of a drug-linker-peptide conjugate (**Figure 2.3**), X and Y represent the common functional groups used to connect either the drug or the peptide to the linker. X may be similar to or different than Y. Here, the primary focus is on the nature of the X bond, and the drug-peptide conjugation chemistry has been classified according to the nature of the X bond: (i) amide, (ii) thioether, (iii) carbamate ester, (iv) carboxylic acid ester, and (v) hydrazone bond [17].

2.4 Conjugation of Prodigiosin-LHRH

We selected [D-Lys6] LHRH as an LHRH agonist carrier, because it was well known that this peptide can be modified at the epsilon amino side chain of its D-Lys6 moiety. Cytotoxic LHRH conjugates containing prodigiosin with preserved cytotoxicity were formed by linking prodigiosin through its 14-0-hemiglutarate to [D-Lys6] LHRH, and also to LHRH antagonist sequences. These analogs will display high-affinity to receptors for LHRH on human breast cancer cells and fully preserved the cytotoxic activity of prodigiosin drugs (**Figure 2.4**) [18].

2.5 Cell Culture

The process by which cells are grown under controlled conditions generally outside of their natural environment is called cell culture. The term “cell culture” in practice, now refers to the culturing of cells derived from multicellular eukaryotes, especially animal cells, in contrast with other types of culture that also grow cells, such as plant tissue, fungal culture, and microbiological culture (of microbes). The cell line culturing world has become a profitable way for biotechnology companies to compete in the growing life science industry. The initial culture taken from an individual is referred to as the primary culture. When diluted and transferred into further containers (a process referred to as subculture or passage) it becomes a “cell line”. Cell line usage has led to many discoveries and innovations. For example, in cell culture, the growth of viruses had allowed preparation of purified viruses for the manufacture of vaccines [19]. Among the cell lines produced in laboratory environments are breast cancer cells. Cancer that starts in the tissues of the breast is the breast cancer cells. Cancer cells may be immortal that is, they can proliferate indefinitely in culture. Hela cells have been cultured in laboratories around the world. They are all descended from cells removed from cancer (of the cervix) of Henrietta

Lacks. In culture, cancer cells produce telomerase, a ribonucleic protein. It is found only in the cells of the germline, including embryonic stem cells [20].

2.6 Breast Cancer Cell Culture Methods

The condition of culturing cells varies for each type of cell. The artificial environments consist of the suitable vessel with a substrate of the medium that supplies the essentials nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (CO_2, O_2), that regulates the physiochemical environment (pH buffer, osmotic pressure, temperature). An artificial substrate or surface is required by most cells whereas others can be grown free-floating in culture medium (suspension culture). The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. The laboratory techniques used in maintaining live cell lines (a population of cells descended from a single cell and containing the same genetic makeup) separated from their original tissue source became more robust in the middle 20^{th} century [21]. Trypsin – EDTA, 2ml of fetal bovine serum (FBS), and penicillin-streptomycin are pre-warmed in a water bath to 37^0C. A growth medium ($L15^+$ medium) which contains 10% FBS plus 2%penicillin-streptomycin (i.e. antibiotics) is also prepared. The growth medium can be prepared by mixing 45ml of L15 medium with 5ml of FBS plus 1ml of penicillin-streptomycin. MDA-MB-231 cell line can be subsequently cultured in flasks containing media concentrations. The cell can be incubated in a humidified environment containing 5% CO_2-95% air at 37^0C. 70% ethanol is used for sterilization of the biosafety cabinet. Cells are to be monitored on daily basis with an inverted optical microscope. The growth medium (L15+ medium) should be changed every two days by adding 7ml of a new media. [21].

2.7 LC-50: Rate of Cell Growth and Measurement of Cell Death

Drug screening and cytotoxicity test of chemicals are measured using cell viability and cytotoxicity assays. There are various reagents used for cell viability detection which are based on various cell functions such as:

- Enzyme activities
- Cell membrane permeability
- Cell adherence

- ATP production
- Co-enzyme production
- Nucleotide uptake activities.

The widely used assay for staining is trypsin blue and the cell viability must be determined by counting the unstained cells with a microscope or other instruments. This cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions. Enzyme-based methods using MTT and WST rely on a reductive coloring reagent and dehydrogenase in a viable cell. Cell viability with a colorimetric method is easy to use, safe and has a high reproducibility. This is widely used in both cell viability and cytotoxicity test. Cellular enzymes such as lactate dehydrogenase, adenylate kinase, and glucose 6-phosphate dehydrogenase are also used as cell death markers and there are several products available on the market. However, adenylate kinase and glucose 6-phosphate are not stable and only lactate dehydrogenase does not lose its activity during cell death assays. Therefore cell death assays based on lactate dehydrogenase activities are more reliable than other enzyme-based cell death assays [21].

2.8 Trypan – Blue Assay

The most common and earliest method for measuring cell viability is the trypan blue (TB) exclusion assay. It is a Dalton molecule that is cell membrane impermeable and therefore only enters cells with compromised membranes. The trypan blue binds to the intracellular proteins upon entry into the cell and thereby render the cells a bluish color. The exclusion assay of the trypan blue allows for a direct identification and enumeration of live (unstained) and dead (blue) cells in a given population. For many years, trypan blue has been used to determine cell viability without its drawbacks. It is considered to be carcinogenic and must be handled with care and disposed of properly [21]. It is recommended that trypan blue is filtered using a 0.2micron filter prior to use because dye aggregates and crystallizes over time. Many publications have observed that trypan blue viability measurements in samples that are lower than 70% viable show higher measured cell viability when compared to fluorescent-based detection methods. Conducting viability measurements with trypan blue on samples with low viability may not be optimal. It has

been recommended that trypan blue assay is ideally used for cultured cell lines, purified/ isolated cell samples with viabilities greater than 70% [23].

2.9 Clonogenic Assay

An *in vitro* cell survival assay based on the ability of a single cell to grow into colony is called clonogenic assay or colony formation assay. A colony is defined to consist of at least 50 cells. There can be a test of every cell in the population for its ability to undergo unlimited division. The clonogenic assay is the method of choice to determine cell reproductive death after treatment with ionizing radiation. It can also be used to determine the effectiveness of other cytotoxic agents. Only a fraction of seeded cells retains the capacity to produce colonies. Before or after treatment, cells are seeded out in appropriate dilutions to form colonies in 1-3 weeks. Colonies are fixed with glutaraldehyde (6.0%v/v), stained with crystal violet (0.5%w/v) and counted using a stereo-microscope. This clonogenic assay has been used in the ensuing decades for a large variety of studies with many types of cells, using improved complex culture media. The assay can detect all cells that have retained the capacity for producing a large number of progeny after treatments that can cause cell reproductive death as a result of damage to chromosomes, apoptosis etc. Clonogenic assays have also been developed for stem cells in various tissues *in vivo*. In addition to the methods for cells in culture and cells in normal tissues, clonogenic assays have been developed for cells from tumors in animals. Cells in transplanted tumors are harvested to yield cell suspensions. Known numbers of cells are injected into recipient animals where they can develop into new tumors. If the donor tumors are irradiated before harvesting, a fraction of the cells will lose clonogenic capacity [22].

Two essentially different ways are used to perform studies using clonogenic assay. Firstly cells are plated before treatment. Cells are harvested from a stock culture and plated at appropriate dilutions into (cluster) dishes. After attachment of the cells to the dishes for 2h or more, the cells are treated. The treatment has to be performed before cells start replicating; otherwise, the numbers of the cells per dish will increase yielding more colonies. After treatment, the dishes are placed in an incubator and left there for a time equivalent to at least six potential cell divisions. This method is often used for a quick screening of the sensitivity of cells to different treatments. In the second option, cells are treated in dishes and subsequently re-plated in appropriate dilutions to assess clonogenic ability. The re-plating may be performed immediately after

treatment, or it may be delayed to allow repair processes. This method is used especially in radio-biological research to determine potential lethal and sublethal damage repairs. To assess the effects of localized cancer drugs (PG) release on MDA-MB-231 cells, a clonogenic assay was used. Breast cancer cells were cultured for 10 days in a growth medium without changing the media. The growth medium was carefully drained from the cells on the tenth day and some of these cells were stained with trypan blue (TB), while the other samples were induced with chemotherapy agent (PG). The number of colonies formed (i.e. the number of clones that evolved from single cells) prior to the number of cells plated were determined. Hemocytometer was used to determine the number of cells per unit volume (Figure 2.4 and 2.5) [22].

The surviving fractions (SF), as well as the plating efficiency (PE), can be obtained as follows:

$$PE = \frac{\text{Number of colonies observed on plate}}{\text{Number of cells plates}}$$

SF = Number of colonies observed on plate/ Number of cells seeded * (PE/100)

Where PE/100 represents the correction term for plating efficiency [22].

2.10 UV-Vis Spectrometry

The main use of ultraviolet photoelectron spectroscopy are the studies of the electronic structure of free molecules (gas phase), well-defined solid surfaces and adsorbents on solid surfaces. When the monoenergetic photons in the 10-100eV energy range strike a sample material, photoelectrons from the valence levels and low-lying core levels (i.e., having lower binding energy than the photon energy) are ejected, measurement of the kinetic energy distribution of the ejected electrons is known as ultraviolet photoelectron spectroscopy (UPS). The physics of technique is the same as XPS, the only differences being that much lower photon energies are used and the primary emphasis is on examining the valence electron levels, rather than core levels. The depth of the probe is 2-100A, and the depth profiling is over the depth probe; deeper profiling requires sputter profiling. It is vacuum compatible materials are required; also flat samples are best, and the size accepted depends on instrumentation [24].

2.11 Atomic Force Microscope (AFM)

Atomic force microscopy (AFM) is a novel which is arising out of and a form of scanning probe microscopy. It is also a major technique responsible for the emergence of modern nanotechnology and great potentials in biology particularly the study of cells. Oncogenically transformed cells are different from normal cells in terms of cell growth, morphology, cell-cell interaction, organization of cytoskeleton and interactions with the extracellular matrix. AFM is capable of detecting most of these changes and it is interesting that in the majority of these applications, AFM has not been used as a straight microscope [24]. The measurement of force between a sample surface and a very sharp probe tip mounted on a cantilever beam having a spring constant of about 0.1-1.0N/m. This is more than an order of magnitude lower than typical spring constant between two atoms. The scanning motion raster is controlled by piezoelectric tubes. The surface topography can be obtained, if the force is determined as a function of the sample's position. Detection is most often made optically by interferometry or beam deflection. In measuring AFM, the tip is held in contact with the sample. Spatial resolution is a few nanometers for scans up to 130µm, but can be at the atomic scale for smaller ranges. Conducting and insulating materials can be analyzed without sample preparation.

Figure 2.1 Structure of Prodigiosin

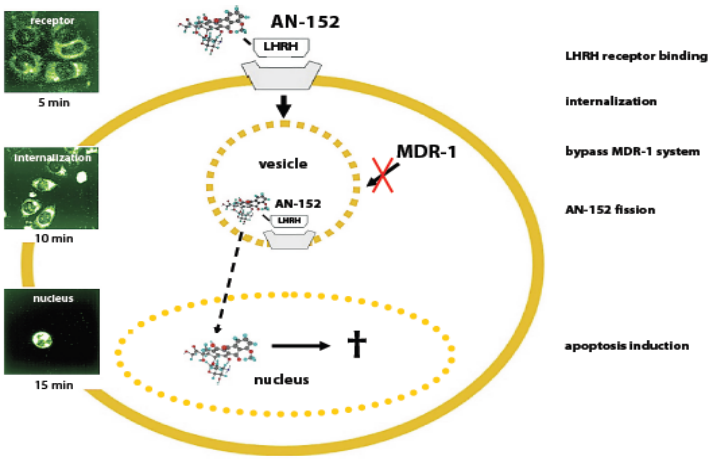


Figure 2.2 Internalization of the cytotoxic LHRH analog.

AN-152 induces multidrug resistance gene-independent apoptosis. After receptor binding, the AN-12/ LHRH receptor complexes internalized via coated vesicle bypassing the multidrug resistance-1 system. Therefore, AN-152 is split and free doxorubicin is accumulated within the nucleus, inducing apoptosis. AN-152 and doxorubicin are autofluorescent at an excitation wave length of 488nm. Detection of AN-152 and doxorubicin was performed using laser scanning microscopy [15].

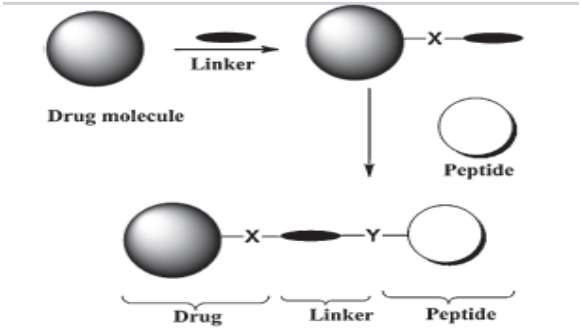


Figure 2.3 Drug-linker-Peptide Conjugate

Prodigiosin

Figure 2.4 Prodigiosin-LHRH Conjugate

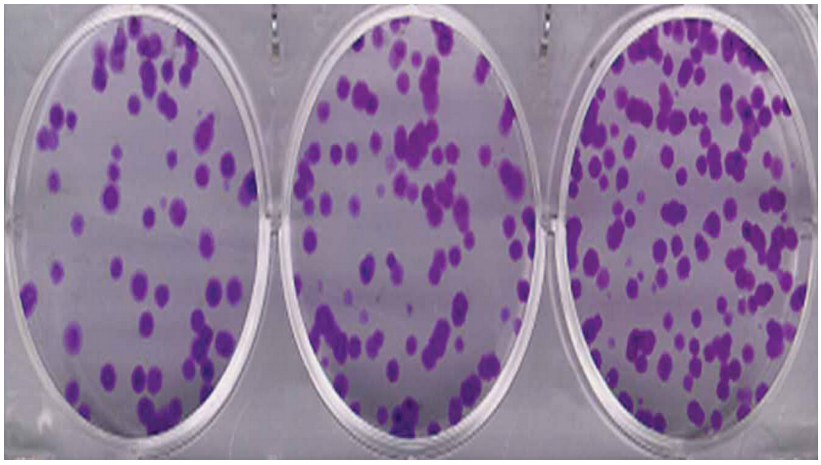


Figure 2.5 Untreated controls with 70 and Untreated controls with 70 and 115 clones, respectively,

This is formed after seeding 100 and 200 cells and the 3rd plate, too many clones are overlapping after seeding 400 cells and therefore this well is not reliable for counting [22].

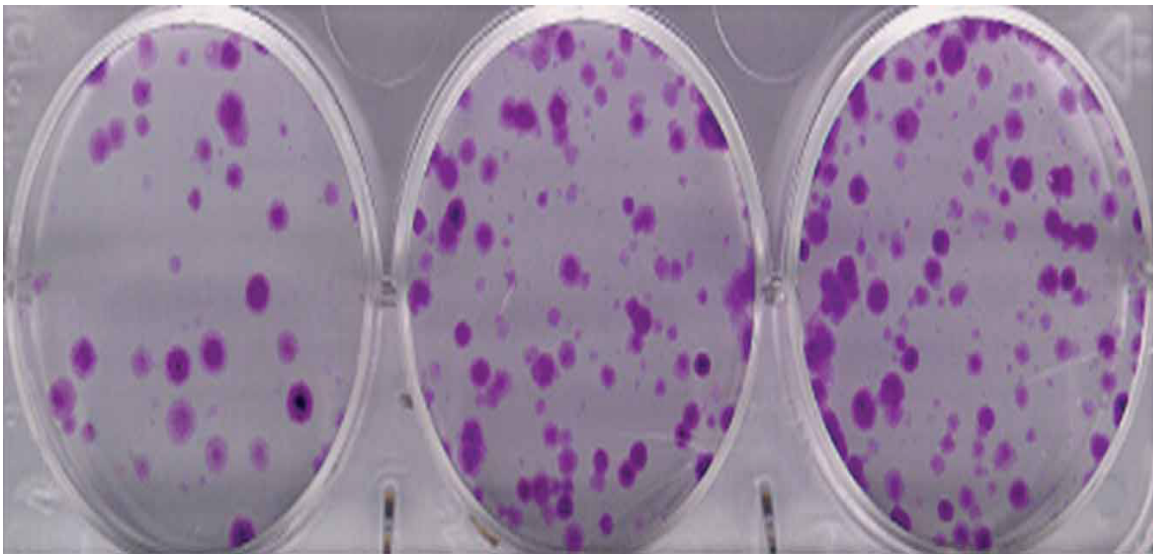


Figure 2.6 After 4 Gy radiation treatment, 39 and 66 clones are formed in plates 1and 2 after seeding 400 and 800 cells respectively.

In the 3rd plate, too many colonies are overlapping after seeding 1,600 cells and therefore this well is not countable [22].

Table 2.1: Comparative analysis of prodigiosin and extracellular protein production by Serratia marcescens in different media at 280C, 300C and 370C. [5]

S. No. Media used	28^0C $mgml^{-1}$	30^0C $mgml^{-1}$	37^0C $mgml^{-1}$
Nutrient broth	0.52	0.354	0.11
Peptone glycerol broth	0.302	0.569	0.11
Sesame seed broth	16.68	9.3	0.319
Nutrient broth with 0.5% maltose	1.836	0.79	0.104
Nutrient broth with 0.5% glucose	9.43	0.29	0.104
Sesame seed broth with 0.5% maltose	1.47	8.56	1.63
Sesame seed broth with 0.5% glucose	0.767	1.67	0.42
Sesame oil broth	38.75	1.006	0.107
Peanut seed broth	2.89	25.98	1.49
Copra seed broth	1.4	1.39	0.1736
Coconut oil broth	13	0.05	0.177
Dextrose broth			
Casein			
Ethanol and carbon source	3		

CHAPTER THREE

3.0 Prodigiosin Extraction from *Serratia marcescens*

3.1 Introduction

Prodigiosin natural red pigments from the family of *Serratia marcescens* is synthesized from various bacteria [1]. Prodigiosin, Cycloprodigiosin hydrochloride (cPrG HCl), undecylprodigiosin, metacycloprodigiosin and desmethoxyprodigiosin are the members of this family. The methoxypyrrole ring in a prodigiosin structure has several biological activities such as immunomodulatory, antibacterial, antimycotic and antimalarial activities [1]. Lots of studies recently imply that prodigiosin has a massive potential in cancer chemotherapy, which draws increasing public attention [2]. Studies on its anticancer effect mainly focus on inducing apoptosis. The induction of apoptosis by prodigiosin in various kinds of cancer cells such as hematopoietic, colorectal and gastric cancer cells because of its inhibitory effects on metastasis and invasion in the underlying mechanism have not been elucidated. One of the major causes of mortality in cancer patients is metastasis, and the treatment of this metastasis is still far from satisfaction [3]. In this present study, the diversity of prodigiosin, their production conditions in laboratory, structure, biosynthesis and the inhibitory factors for prodigiosin production, gene expression and apoptosis, toxigenic and anticancer effects of this pigment are discussed.

3.2 Materials

Peptone glycerol was purchased from Sigma chemicals Co (St. Louise, MO, USA). Distilled water obtained from the Sheda Science and Technology Complex (SHESTCO) Laboratory Abuja, Nigeria. The soil sample was collected from SHESTCO, Abuja Nigeria. The *Serratia marcescens* was supplied by SHESTCO lab, the bacterial culture was maintained on the peptone glycerol agar at 28^0C for 72h. Autoclave, HPLC, and TLC were provided by SHESTCO lab laboratory Abuja, Nigeria. All other chemicals and solvents were of analytical grade and were purchased from commercial sources in Abuja, Nigeria.

3.3 Experiments.

3.3.1 Media Preparation for *Serratia marcescens* inoculation

Different types of selected garbage soil samples were collected. One gram of soil sample from different sites was mixed in 10ml of sterile distilled water. Serial dilutions of sample were done and spread on a nutrient agar plates. A 2.4g of agar along with peptone of 0.6g, 1% glycerol and 120ml of distilled water was used to prepare the media for four plates. After dissolving the media was autoclaved for sterilization. Both the Petri dish and media were sterilized at the temperature of 121^0C and 15psi for 15 minutes. After the autoclaving, the media was taken to the biosafety cabinet and poured into four Petri dishes that were allowed to solidify. After incubation at 28°C for 24 hours, plates were observed for pigmented colonies. The colonies showing reddish pink pigment were purified on nutrient agar plates. The characteristics of the colony were studied for these isolates and those which were presumptively identified as *Serratia marcescens* using Bergey's manual of Determination Bacteriology (9^{th} edition) were identified as *Serratia marcescens* (**Figure 3.1**).

3.3.2 Extraction and purification of Prodigiosin

A media for 100plates was prepared (60g of agar, 30ml glycerol, 15g peptone and 600ml of distilled water) using the same procedure for the first four plates. *Serratia marcescens* was isolated from the four plates. The organisms were cultured which showed highly mucoid colonies and cell-associated red pigment on nutrient agar. The identification of organism was done after incubation at 28^0C for 24h. Colonies showing reddish pink pigment were extracted and placed in an ethanol solution after 72h (**Figures 3.2**).

The extraction of prodigiosin was done by scraping the *Serratia marcescens* cells into the mixture of ethanol solution. After centrifugation for about (6800g for 15 min), the solvent of the supernatant was evaporated under vacuum. At atmospheric pressure, liquid chromatography of the extract was performed on silica gel containing chloroform and methanol as solvents in a ratio 9:1. The pigment component was separated using thin layer chromatography. The pigment fractions eluted were pooled and the chloroform/methanol extracts were vacuum evaporated and later redissolved in H_2O and lyophilized. The isolated pigment was redissolved in methanol and analyzed by electrospray ionization mass spectrometry. High-performance liquid

chromatography (HPLC) was carried out using an HPLC system with a dual wavelength absorbance detector which produces spectra at 535nm. Methanol and 10nm trimethylamine (17.3v/v) were added and the PH was adjusted to 7 (using NaOH) at a low rate of 1.0ml/min through a reverse column at 40^0C. The acid was then used to improve upon the chromatographic peak shape and source of protons in the reverse phase. 500µg of each sample, standard prodigiosin and sampled prodigiosin were separately dissolved in 2ml of methanol to obtain equal concentrations of 25mg/ml [19]. The prodigiosin obtained was analyzed and the RF value of the fraction was 0.84. The prodigiosin was further purified by using silica gel chromatography [**Figure 3.3**] and HPLC. This purified fraction was monitored for the characterization by UV/Vis spectrophotometer which had the value of 535nm. Single peaks were obtained from the chromatography for sample test and also standard sample. Symmetry of the grow peaks were retained a given conformation of the prodigiosin. The area of the peak was used to determine the concentration of prodigiosin in the sample and not the heights; since the peak varied and the area remain almost constant [4]. There were other smaller samples observed in the samples due to impurities present. The greatest peak was obtained at 5.01min of retention time with a percentage of 92.42. The pure pigment extracted from the culture broth was taken for UV spectra-photometer analysis [5].

3.3.3 Estimation of Prodigiosin

Isolated prodigiosin was estimated using the following equation (Mekhael and Yousif 2008).

Prodigiosin unit/cell = $[OD_{499}-(1.381* OD]/OD_{620}$

Where OD_{499} = Pigment absorbance

OD_{620} =Bacterial cell absorbance

1.381 = Constant

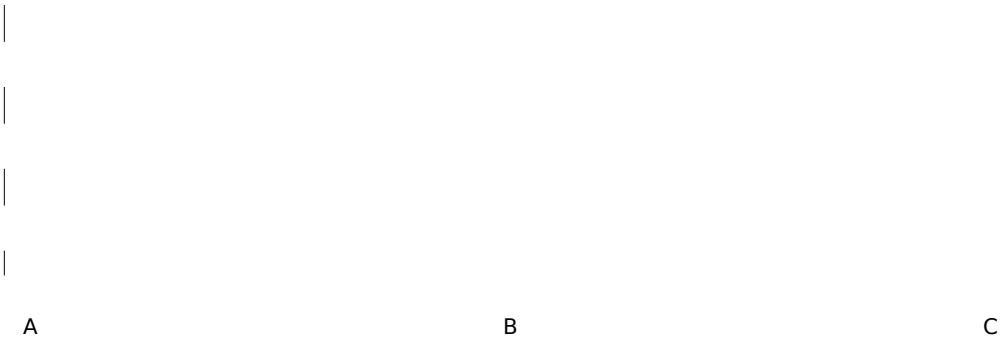
3.4 Results and Discussion

Purified pigments were characterized using UV/VIS spectrophotometer, Gas chromatography, and mass spectroscopy. Prodigiosin has tri-pyrrole ring structure which is soluble in an organic solvent which makes methanol useful for the extraction of the UV/VIS characterization of the pigment. The pigment produced by *Serratia marcescens* was identified and characterized using UV-VIS spectrophotometry and mass spectrophotometry. Pigment showed maximum absorption at 535nm and the calculated mass by GC/MS is near about 323. According to Silva *et al* (2012), the substance produced by *Serratia marcescens* which has maximum absorbance of 536nm and a molecular weight of 323m/z is characterized as prodigiosin.

3.5 Summary / Conclusion

Prodigiosin a secondary metabolite produced by *Serratia* asp which was found to be antimicrobial, antifungal and anticancer. The pigment is characterized by a common pyrrolpyrromethane skeleton, are produced by various bacteria that first characterized by *Serratia marcescens* expresses pigments under certain conditions. The optimum conditions for pigment production in nutrient broth were found to be at 30^0C and PH7 respectively. Better yield of pigment had 87% glycerol in half strain nutrient broth. A red pigment-producing bacterium was isolated from soil and identified as *Serratia marcescens*. The pigments of *Serratia marcescens* are expressed under certain conditions. Pigment expression may be easily monitored spectrophotometrically because most pigments absorb light at some defined wavelength. The promising pigment having antifungal, immunosuppressive and antiproliferative activity and considered being a protein. Prodigiosin apoptosis effect is concluded by various researchers [1]. Various members of prodigiosin in the family cycloprodigiosin have a particular compound that induces cancer cell lines including acute human T-cell leukemia, promyelocytic leukemia, human and rat hepatocellular cancer, human breast cancer and TNF-stimulated human cervix carcinoma [6].

The maximum absorbance of the pigment was 530nm and the molecular weight of 323m/z. There were other smaller samples observed in the samples due to impurities present. The greatest peak was obtained at 5.01min of retention time with a percentage of 95.42. Purified prodigiosin also showed good anticancer potential but no immunosuppressive potential.



A B C

Figure 3.1 *Serratial Marscesen* inoculated on four media plates (A), *Serratia marcescen* appearance after 24h (B); Prepared media containing Peptone, Glycerol, and Agar for the inoculation of *Serratia marcescens (C).*

A B

Figure 3.2 Prodigiosin appearance after 48h of incubation (A), Extracted prodigiosin in ethanol solution containing mixtures of media, Serratia and prodigiosin (B).

A **B**

Figure 3.3 Thin layer chromatography of prodigiosin pigment using silica gel (A), different fractions of purified prodigiosin after using HPLC (B)

Figure 3.4: Optimum conditions require for maximum production of Prodigiosin concentration were obtained at (at time 30h, 30^0C and PH 8.0) (A) Optimum conditions require for maximum production of Prodigiosin concentration were obtained at (at time 30h, 30^0C and PH 8.0) (B) Replotted from [5]

Figure 3.5: HPLC analysis of sample purity: plot of standard prodigiosin and Tested sample of Prodigiosin respectively. HPLC analysis of sample purity: plot of standard prodigiosin and Tested sample of Prodigiosin respectively [5].

CHAPTER FOUR

4.0 Conjugation of Prodigiosin and LHRH

4.1 Introduction

More than 50% of breast cancers are expressed receptors of LHRH also known as GnRH. Apart from reproductive organs (Ovaries, fallopian tubes, and uterus), other organs and hematopoietic stem cells do not express LHRH receptors. Thus these receptors can be utilized for targeted therapy to improve antitumor effects and reduce side effects compared with systemic conventional cytotoxic chemotherapy. Cytotoxic LHRH analogs have been developed, where a cytotoxic agent is covalently linked to an LHRH analog. After the findings of LHRH receptors on breast cancer cells in the mid-1980s, a group designed and synthesized cytotoxic LHRH conjugates [1]. The molecule of this hybrid was based on agonistic and antagonistic analogs of LHRH, linked to cytotoxic moieties of prodigiosin.

We selected [D-Lys6] LHRH as an LHRH agonist carrier because it is well known that this peptide can be modified at the epsilon amino side chain of its D-Lys6 moiety by large molecules without loss of its ability to bind to LHRH receptors. Bulky attachment of the hydrophobic cytotoxic compound to the D-Lys6 side chain, such as 2-hydroxymethyl anthraquinone produced a cytotoxic LHRH agonist. This study provides additional evidence that the linking of prodigiosin to an LHRH analog carrier can significantly improve the outcome of drug delivery. Findings on this conjugate will significantly inhibit the growth of estrogen-independent, prodigiosin-LHRH resistant breast cancer cells reduction.

4.2 Materials

Prodigiosin was synthesized at SHESTCO laboratory in Abuja, Nigeria. LHRH was purchased from Sigma Aldrich, USA. Stock solutions were prepared in double-distilled deionized water and stored at 4^0C in the dark. Glutamic acid was also purchased from Sigma Aldrich, USA. Human breast cancer cells were obtained from Sigma Aldrich, USA. 5% CO_2 were provided by SHESTCO laboratory in Abuja, Nigeria. Trypsin, Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Sigma Aldrich, USA. All other chemicals and solvents were of analytical grade and were purchased from commercial sources in Nigeria.

4.3 Prodigiosin – LHRH Conjugation

Cytotoxic LHRH conjugate was synthesized in our laboratory by coupling one molecule of prodigiosin to 14-O-hemiglutarate to the E-amino group of the D-Lys side chain of the carrier peptide {D-Lys] LHRH and purified by high-performance liquid Chromatography [1].prodigiosin was synthesized in SHESTCO laboratory, Abuja and D-Lys LHRH was obtained from Sigma, USA. The compound was dissolved in 5% (w/v) aqueous D-mannitol (Sigma) solution. The reactions were shared into three groups: group 1, 5% mannitol solution which was used as the control. Group 2, 5% mannitol, 2g of prodigiosin with hydrochloride and group 3 consisted of 5% mannitol, 2g of prodigiosin and 20g of LHRH.

4.4 Breast Cancer Cell Culture

Trypsin-EDTA, L15 medium, Fetal Bovine Serum (FBS) obtained from Sigma Aldrich, St. Louis, Mo, USA, and penicillin-streptomycin were pre-warmed in a water bath at 37^0C. The growth medium (L15 medium) containing 10% FBS plus 2% penicillin-streptomycin (antibiotics) were prepared by mixing 45ml of L15 medium with 5ml of FBS plus 1ml of penicillin-streptomycin. An MDA-MB-231 cell line was divided subsequently into three groups and was cultured. 7ml of L15 media was added to the one T25 flask while the other two groups in T75 flasks each contain 10ml of L15 media. After the addition of the growth media, FBS and antibiotics, cells were incubated in a humidified environment containing 5%CO_2 - 95% air at 37^0C. On a daily basis, cells were monitored with an inverted optical microscope. The growth medium was changed every two days by adding 7ml of new media. When the cells were more than 80% confluent (at least, once every two days) splitting was carried out. The remaining cells were counted and split on the sixth day. 1ml of cells solutions were resuspended into 6-well plates and diluted with 7 ml growth media to obtain $3x10^2$ cells/ml concentrations. These were incubated for 8-10days. Cells were subsequently detached with trypsin-EDTA, centrifuged at 100rsf for 8 minutes. They were subsequently stained with trypan blue and the numbers of colonies formed were counted. Cells counting were carried out using hemocytometer which was used to monitor cell viability and cell death [21].

4.5 Fluid /Drugs Concentration

Each sample of PG and LHRH was weighed to 0.1g and dissolved in 2ml methanol (100%) with a stock solution of 5mg/ml. The 5mg/ml solution was adjusted with PBS to give a final concentration of 2.5mg/ml. After the adjustment, BBT and PT were prepared similarly and were done in 2ml ethanol and 2ml DMSO respectively, and 38ml of PBS was used to adjust each solution to obtain final concentration of 2.5mg/ml. DMSO: PBS, methanol : PBS and ethanol : DW ratio were 5:95V/V% in the final drug / sample solution. The solution was later filtered twice using filter paper to remove debris. In order to estimate the exact drug content, there was a need to calibrate a standard curve. A 0.12ml of stock solution of known concentration was used to prepare a standard concentration curve, and this was done using the method of John Obayemi *et al* [4]. The solution was serially diluted and dissolved in DMSO to obtain standard prodigiosin solutions with different concentrations (0.1, 0.2, 0.5, 0.6mg/ml). The UV spectrophotometer was used to determine the absorbance at 535nm using DMSO as blank. The measured absorbances were then plotted against known concentrations of PG at any absorbance. This was used to obtain a calibration curve for determining the concentrations of PG at any absorbance respectively. A similar approach was used for LHRH to determine its absorbance.

4.6 Effect of Prodigiosin on LC 50 and Clonogenic Assay

Cell viability test on *in vitro* cells indicated that prodigiosin pigment is a potent cytotoxic agent and LC 50 assay results revealed that cytotoxicity was dependent on the concentration of prodigiosin. The concentration of prodigiosin in LC50 required to destroy 50% of cells was arrived at 2g/ml concentration of prodigiosin. When the concentration of prodigiosin increases cytotoxicity will also increases in the same proportion. The apoptotic property of the pigment was estimated by MTT assay, the lower concentration of prodigiosin (0.25g/ml) showed 10% cell death and 90% cell survival. With this concentration, 50% of cells were undergone apoptotic change. As the concentration of prodigiosin increase, the percentage of cell death also increases. These results explained the dose-dependent cytotoxicity and apoptotic property of the pigment [2].

4.7 Detection of prodigiosin –LHRH improvement – LC50/clonogenic

We prepared and described new targeted conjugates of prodigiosin along with analog of LHRH. These conjugates might improve the treatment with prodigiosin by delivering this anticancer

drug to cancer cells which express the LHRH as receptors. The peptide LHRH was chosen as a targeting moiety, because of the presence of its receptors in tumors such as prostate, ovary, breast and endometrium. The result of the receptor saturation tests are demonstrated below, the combination of the cells and LHRH was expected to have lower antiproliferative efficacy than the conjugated prodigiosin-LHRH.

UV-Visible spectrophotometer was used to estimate the concentration of PG or LHRH in the solution which was estimated from the spectroscopic measurements. The UV-VIS spectroscopy measures each solution of the samples. From Beer Lambert's law, the absorbance, (A) is directly proportional to the path length, (L), and the concentration, (C), of the absorbing species and β, is the molar absorptivity of the species. The concentrations of PG and LHRH in the solution were determined from a standard curve that was obtained from the maximum absorbance at wavelengths of 535 and 280nm respectively [3].

4.8 Cell Adhesion force measurement and characterization of Conjugated Prodigiosin-LHRH to Breast Cancer Cell and Normal Cells

The prodigiosin used in this work were synthesized and purified in SHESTCO lab Abuja, Nigeria using methods adapted from Danyuo Yiporo *et al* [2]. The conjugation of prodigiosin-LHRH methods were adapted and described from John Obayemi *et al* [4]. A simple dip-coating technique as described by Hutter and Bechheofer [6] was adapted to coat bare phosphorus n-doped silicon atomic force microscope tips with LHRH, prodigiosin, and conjugation of prodigiosin-LHRH. The peptide of LHRH was provided in a powder form and was dissolved in sterile double-distilled water to a concentration of 10ml/ml, while the antibodies were used as provided by the manufacturer at a concentration of 0.5mg/ml [4].

The tips of the probe were immersed in solutions containing the materials/particles of interest (prodigiosin, LHRH, and conjugation of prodigiosin-LHRH) and were air-dried for 15mins each. The tips were dipped three times and air-dried to ensure the tip was adequately covered with the materials of interest. After the third air dry, the tips were allowed to air dry for a maximum of 24h before being used for AFM adhesion measurements. The interactions and adhesion forces were measured using a Multimode Dimension DI Nanoscope IIIa Atomic Force Microscope.

Breast cancer cell samples were prepared for the AFM by fixation in 3.7% formaldehyde using the method as described by Hampp et al [24]. 60 x15mm Falcon Petri dishes were used for the breast cells cultured and lasted for 48h at high confluence and incubated at 37^0C at atmospheric pressure levels. Cells were grown in L-15 medium, supplemented with 100 I.U./ml penicillin/100g/ml streptomycin and 10% FBS. The samples were rinsed with sterile Dulbecco Phosphate buffered saline and fixed with a 3.7wt formaldehyde solution for 15min a temperature of 23^0C. They were rinsed again with DPBS before a final rinse with purified water. The samples were finally placed in a vacuum desiccator for 2h to dry at room temperature.

Bare (uncoated) tips of AFM and coated AFM tips were imaged using environmental scanning electron microscopy (ESEM). Phillip FEI quanta 200 FEG Environmental – SEM was used to determine the secondary/backscattered electron images of coated and bare AFM tips. An energy dispersive x-ray spectrometer, EDS, was used to acquire an elemental spectrum at the apex of the cantilever tip to confirm the presence of prodigiosin and LHRH.

Weak adhesion between the AFM tips and the coatings could result in the detachment of coatings from the AFM tips during handling or pull-off testing; the AFM tips were checked in the ESEM before and after AFM testing. The pull-off forces were related only to the coatings on the detachment of the dip coatings from the coated AFM tips occurred. The coated and uncoated AFM tips spring constants were measured experimentally using the thermal tune method [4]. This procedure was done to ensure that the actual spring constants were used to calculate the pull-off forces. Such measurements are also required to account for batch-to-batch variations in the spring constant, as well as the effects of coatings on the cantilever stiffness.

The experiments of AFM were carried out using a dimension 3100 AFM under ambient conditions (23^0C) and relative humidity of 36-45%. In any case, before the adhesion force measurements, the photodetector sensitivity was calibrated using a stiff quartz platform. The prodigiosin and LHRH were synthesized at a PH 7.0. The interactions and forces were measured for the following eight configurations using the AFM technique adopted by John Obayemi 2015[4].

- Bare AFM tip to normal breast cancer cells
- Bare AFM tip to normal breast cells

- LHRH-coated AFM tip to breast cancer cells
- LHRH-coated AFM tip to normal breast cells
- Prodigiosin coated AFM tip to breast cancer cells
- Prodigiosin coated AFM tip to normal breast cells
- Prodigiosin-LHRH-coated AFM tip to breast cancer cells
- Prodigiosin-LHRH-coated AFM tip to normal breast cells

Adhesion force measurements were carried out on each of these configurations on the prepared breast cancer cells. The pull-off forces measurement for different AFM tips and /or their configuration with breast cancer cells and normal cells are presented in figure and table with their respective tip stiffness. There was a relatively low level of adhesion between AFM tip and breast cancers; also with normal cells. The adhesion force between the coated AFM tip with prodigiosin and breast cancer cells was measured.

4.9 Results and Discussion

We treated an MCF 10 A cell line (breast cancer cells) to determine the viable and non-viable cells. The viable cells showed a complete growth after 21days (**Figure 4.1**). The viability showed that the cells were alive and capable of growth in the given area and volume. The cells used as a control were grown for 21 days and were later treated with trypan blue. When the cells were trypsinized it showed that the viable cells were alive (**Figure 4.2**). The trypsinized viable cells were later detached from the substrate; this shows the macrophage-nucleus of the breast cancer cells.

The cytotoxic effects of prodigiosin were determined in this study using different concentrations of the drug. The drug shows a morphological and apoptotic change in the breast cancer cells. The three different concentrations show apoptotic changes in the trypsinized detached macrophage-nucleus of the breast cancer cells. The lower concentration shows the lower effect of apoptotic changes as compared to the higher concentration of prodigiosin (**Figure 4.3**). The lower concentration showed 10% cell death and 95% cell survivability. As the concentration of prodigiosin increases the percentage death also increases (**Figure 4.7**). This explained the dose-

dependent cytotoxicity and apoptotic property of the pigment (prodigiosin). In this study we found that prodigiosin has a better effect on both the breast cancer cells macrophage-nucleus, detached trypsinized breast cells and drug trypsinized detached and non-detached breast cells (**Figure 4.5-4.8**). The lower concentration of drugs showed apoptotic changes, this explained that the concentrations of prodigiosin play a role in the induction of apoptosis.

In this study, we found that the apoptotic changes induced by the pigment prodigiosin were not only at the cellular level but also at nucleus level. The shrinkage of chromatin, breakage in the chromosomes and DNA fragmentation were all nucleus changes [4] (**Figure 4.4**).

Adhesion measurement for the pull-off forces for different AFM tips and their configurations with breast cancer cells are presented in (**Figure 4.9**) and (**Table 4.1**) respectively. Adhesions force of the bare AFM tip and breast cancer cells were very low which measured (10±0.5nN). The bare tip of AFM with normal breast cells also measure (5±0.25nN). This shows a very low adhesion force between bare tips, breast cancer cells and normal cells. The average adhesion force of coated AFM tip with prodigoisin interacting with breast cancer was (20±1) and for normal breast cells was (7±0.35). This shows a higher adhesion when compared to the uncoated AFM tips on both breast cancer and normal breast cells. The adhesion force of the coated (prodigoisin) AFM tips with breast cancer cells is two times the bare AFM tip measurement. The adhesion forces for the LHRH-coated AFM tip with breast cancer cells measured (75±3.75). This shows a high adhesion force when compared to a prodigiosin coated AFM tip. The normal breast cell was measured to be (15±0.75). This suggests that this high adhesion binding is because of the protein present and also the breast receptors that are present in the LHRH. The adhesion measurement between the AFM coated with prodigiosin-LHRH had the highest peak as compared to the bare, prodigiosin coated and LHRH-coated. The measurement for breast cancer cell is (80±4) and the normal breast cell is (20±1) respectively. The high binding can be attributed to the over-expression of LHRH receptors and the apoptotic property of prodigiosin (**Figure 4.9**).

The results also suggest that the adhesive forces between prodigiosin, LHRH and prodigiosin-LHRH and breast cancer cells are attributed to the van der Waals forces and hydrogen bonding. The high adhesion forces between the conjugate and the breast cancer cell are attributed to the van der Waals interactions between the peptide and its particular over-expressed receptor on the

surface of the cancer cells. The result obtained is in agreement with the results of Hampp *et al* [5].

We have prepared and described newly targeted conjugates of prodigiosin with the analog of the hormone LHRH. This conjugate might improve the treatment with prodigiosin by delivery of this anticancer drug to the cancer cells which express the LHRH receptors. The peptide LHRH was chosen as a targeting moiety, because of the presence of its receptors in tumors such as prostate, ovary, endometrium, and breast.

In this study, two conjugates were synthesized in which prodigiosin was mixed with the analog of LHRH was described. This conjugates were observed under UV-VIS and later HPLC to determine the formation of the conjugate. These conjugates were tested in human breast cancer cell line for their antiproliferation effect. One of these conjugates was more effective in the decrease of cell proliferation than prodigiosin alone. It was also demonstrated that LHRH receptors play a role in the antiproliferative effect of this conjugate. The receptors for LHRH are expressed on a high percentage of breast, ovarian, endometrial and prostate cancers but not on most healthy tissues.

4.10 Conclusion and Summary

Over the past years, a number of groups have developed media and conditions that support the growth of normal human mammary epithelial cells from a variety of lineages, and these cell systems have increased our understanding of human mammary gland biology. To isolate and culture human breast cancer cells primary tumors and metastatic specimens remain difficult. The cell lines that have been obtained exhibit an array of genetic alterations representative of the genomic diversity of uncultured human breast cancers.

The breast cancer cells are cultured under-defined conditions that allow detained cellular studies to be carried out that complement the genetic analyses of these cell lines. The work outlines the culture methods and approaches that develop the panel of cell lines. There were key features that were used for the isolation and culture of breast cancer cells. These features were all focused on developing culture conditions that allow for the slow emergence of cancer cells in the relative absence of normal cells, which proliferate rapidly under highly-growth factor enriched conditions. Growth media and methods for enriching cell populations for cancer cells selectively

while minimizing the presence of normal epithelia and stromal elements are important for the expansion of breast cancer cells *in vitro:* the improvement of the knowledge of the factors that inference breast cancer cell viability and proliferation will be important for development of novel therapeutic strategies for breast cancer.

It is well understood now that cancer therapeutic agents not only induced early apoptotic and necrotic cell death but also trigger sustained growth-arresting events through, for example, acceleration senescence and mitotic catastrophe, responses which are manifested at late times (several days) after the introduction of DNA damage. The clonogenic survival assay provides an integrated readout of all of these early and late responses and has therefore been considered as the gold standard for the assessment of cytotoxicity [7].

The prodigiosin pigment extracted from *Serratia marcescens* extracted from soil indicated the better apoptotic property. Prodigiosin also possessed mild immune stimulation property at lower doses and our findings determined a good apoptotic and antineoplastic activity and it is a better therapeutic agent for treatment of breast cancer.

The asymmetry factors reported here are considered to be good and hence peaks were considered for the analysis of the conjugation of PG-LHRH clearly. Quantitatively, peak areas or heights were used to determine the concentration of PG and LHRH in the sample. The peak areas were used to determine the content of PG because the peaks were slightly tailed. The peak heights varied, while the areas remained constant. The symmetry of the peak is also the performance characteristic for good quantitative result.

A **B**

Figure 4.1 Normal trypsinzed viable breast cancer cells when treated control 1 (A) and viable non-trypsinize breast cancer cells when treated control 2 (B).

A **B** **C**

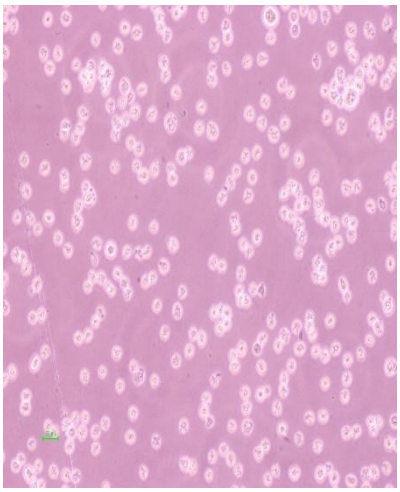
Figure 4.2 Viable breast cancer cells when treated (A), viable trypsinized breast cancer cells when (B) and Non-viable breast cancer cells when treated (C)

A **B** **C**

Figure 4.3 Detached trypsinized breast cancer cells control 1, macrophage-nucleus (A), detached trypsinized control 2 macrophage-nucleus (B) detached breast cancer cells trypsinized drug 1A macrophage-nucleus (C).

A **B** **C**

Figure 4.4 Detached breast cancer cells drug 1B trypsinized macrophage-nucleus (A), detached breast cancer cells trypsinized drug 1C macrophage-nucleus (B) and detached trypsinized drug 3A macrophage-nucleus (C).



A **B** **C**

Figure 4.5 Detached breast cancer cells trypsinized drug 3B macrophage-nucleus (A) detached breast cancer cells trypsinized drug 3C macrophage-nucleus (B) and detached trypsinized breast cancer cells macrophage-nucleus (C).

A **B** **C**

Figure 4.6 Drug 1A on viable breast cancer cells when treated after 21days (A), drug 1B on viable breast cancer cells and drug 1C on viable breast cancer cells when treated after 21days and drug 1C on viable breast cancer cells when treated after 21days (C) showing cytotocity.

A **B** **C**

Figure 4.7 Drug 3A on viable breast cancer cells after treatment for 21days (A), drug 3B on viable breast cancer cells after treatment for 21days (B) and drug 3C on viable breast cancer cells after treatment for 21days (C)showing cytotocity

A **B**

Figure 4.8 Drug 3C on breast cancer cells viability after treatment for 20days macrophage-nucleus (A) and drug 3C on breast cancer cells after 21 days treatment (B)

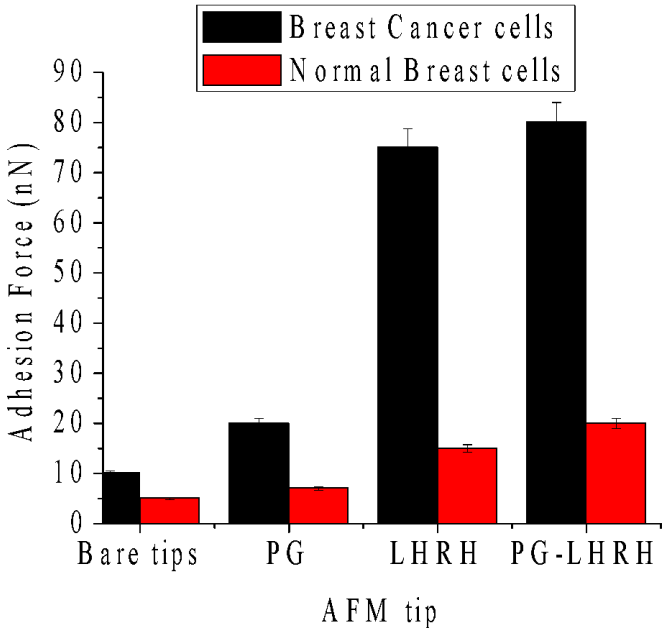


Figure 4.9: Adhesion force measurement between AFM tips with normal breast and breast cancer cells.

CHAPTER FIVE

5.0 Summary and Conclusion

This work was focused on developing strategies for regional cancer drug delivery, especially in localized breast cancer drug delivery. This work demonstrated to a large extent, the significance of LHRH and prodigiosin delivery systems that are capable of achieving controlled drug delivery. Material issues associated with localized drug release were synthesized and the research was carried out in the following steps.

We presented the results of experiments carried out on the synthesis of prodigiosin for localized breast cancer drug delivery. The maximum absorbance of the pigment was 535nm and a molecular weight of 323m/z. There were other smaller materials observed in the samples due to impurities present. The greatest peak was obtained at 5.01min of retention time with a percentage of 95.42. Purified prodigiosin also showed good anticancer potential but no verified immunosuppressive potential.

Adhesion force measurements were done between Atomic Force Microscope probe tips with breast cancer cells, normal breast cells, and bare tips. The adhesion between the coated tips with breast cancer cells had a higher measurement as compared to the bare tips. The force measurement between the prodigiosin coated tips on breast cancer cells had a higher adhesion force as compared to the same coated tip with normal breast cells. LHRH-coated tips also had higher adhesion force on breast cancer cells as compared to the normal cells. This this is due to the receptors that are present in LHRH. The conjugation of prodigiosin-LHRH shows the highest peak which indicated high adhesion force. This implies that both the drug and peptide have a binding affinity with breast cancer cells which makes it a good targeted therapy for the treatment of cancer.

We also presented the results of experiments carried out on the synthesis of LHRH-prodigiosin for localized breast cancer drug delivery. These conjugates were observed under UV-VIS and later HPLC to determine the formation of the conjugate. Thess conjugates were tested in human breast cancer cell lines for their antiproliferative effect. The UV-VIS analysis of LHRH had an absorbance of 280nm. We expected the conjugates to have more effectiveness in the decrease of

cell proliferation than prodigiosin alone. It was also demonstrated that LHRH receptors play a major role in the antiproliferative effect of this conjugate.

5.1 Recommendations for Future Work

Prodigiosin which is produced by *Serratia macenscens*, known for its anticancer, antiproliferative, antifungal, etc linked to LHRH (D-Lys) a breast receptor is recommended as a good conjugate for the treatment of breast cancer cells. Having carried out this work, we recommend that the following experiments be carried out:

- That prodigiosin-LHRH conjugates drug delivery is carried out i*n vitro* to investigate the cell viability and cell death against time. The results will be monitored using a fluorescent microscope.
- We also recommend that an animal trial be carried out. This will enable us to inject aggressive amounts of prodigiosin-LHRH to human breast carcinoma cells into the mammary fat pads of severely immunocompromised NOD SCID gamma (NSG) rats. The rats will be monitored for several weeks to study tumor shrinkage with different concentrations of drug conjugates. The results can be further analyzed with a fluorescent microscope and HPLC to determine the amount of drug present in surrounding tissues.

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