UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
SCHOOL OF PHARMACY
DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

CHEMOPROTECTIVE ACTIVITY OF D-RIBOSE-L-CYSTEINE (RIBOCEINE) AGAINST THE CYTOTOXIC EFFECTS OF METHOTREXATE AND DOCETAXEL ON NORMAL AND CANCER CELL LINES

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL PHARMACOLOGY DEGREE

MARCH, 2020

DECLARATION

I, Trudy Janice Philips, certify that this thesis is the result of research undertaken towards the award of the Master of Philosophy (MPhil) in Pharmacology in the Department of Pharmacology and Toxicology, School of Pharmacy, College of Health Sciences, University of Ghana. References to the works of other investigators have been duly acknowledged.

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DECLARATION BY SUPERVISORS

We hereby declare that this thesis was supervised by us, in accordance with the guidelines laid down by the University of Ghana.

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(Co-Supervisor)

ABSTRACT

Background: Methotrexate (MET) and docetaxel (DOC) are chemotherapeutic agents used for the treatment of breast and prostate cancers, respectively. Their action results in the generation of reactive oxygen species (ROS). Excessive ROS consume cellular antioxidants such as glutathione (GSH) that leads to oxidative stress in both normal and cancer cells. Riboceine (RIB), a GSH-enhancer diet supplement, has been shown to protect against oxidative stress. However, there is a dearth of information on the protective effects of RIB on normal and cancer cells during chemotherapy.

Aim: This study sought to determine the chemoprotective effects of RIB against the cytotoxic effects of DOC and MET on normal and cancer cells

Methodology: The effects of increasing concentrations of MET and DOC and their combination with RIB or N-acetylcysteine (NAC, positive control) on the cell viability of normal human prostate cell (PNT-2), human prostate cancer cell (PC3) and human breast cancer cell (MCF-7) lines were determined using the resazurin assay. Also, the effects of these agents and their combinations on the GSH content and ROS level of the normal and cancer cell lines were measured using O-phthalaldehyde (OPA) and dichlorofluorescein diacetate (DCF-DA) assays, respectively.

RESULTS

MET and DOC dose-dependently decreased ($p<0.05$) the viability of PNT-2 cell (IC_{50} = 0.552 and 0.524 μM, respectively) and PC3 cell (IC_{50} = 0.338 and 0.320 μM, respectively) lines. RIB and NAC significantly reduced ($p<0.05$) the cytotoxic effect of MET and DOC on PNT-2 cell line (IC_{50} > 10 μM). On the other hand, RIB and NAC had no significant effect on the cytotoxicity of MET and DOC on PC3 cell line. MET and DOC significantly decreased ($p<0.05$) GSH content and significantly increased ($p<0.05$) ROS level of both normal and cancer cell lines when compared to untreated cells. Similarly, RIB and NAC significantly

increased ($p<0.05$) GSH content and decreased ($p<0.05$) ROS level in both normal and cancer cell line, in the presence of MET and DOC.

CONCLUSION: The current study suggests that RIB protected the normal cells PNT-2 against the cytotoxic effects of MET and DOC. RIB and NAC also protected MCF-7 but not PC3 cell lines against the cytotoxic effects of MET and DOC. The protective effect of RIB was associated with an increase in GSH content and a decrease in ROS level in both normal and cancer cell lines. The effect of RIB was similar but less pronounced than the effect of NAC, the standard drug. Further studies should be conducted to confirm these findings at the molecular and *in vivo* levels. Also, other antioxidant defences such as catalase, superoxide dismutase and peroxidase should be investigated.

DEDICATION

"Now thank we all our God" (MHB 10). I dedicate this work to the almighty God, for He is faithful. Also, to my lovely husband, Francis Kwame Morgan Tetteh for his enormous support and to our daughters Kayleigh and Kaitlynn Tetteh.

ACKNOWLEDGMENTS

First of all, I thank God Almighty for His continuous grace and guidance.

I would like to express my most profound appreciation to my supervisors, Dr. Kwame Benoit N'guessan Banga and Prof. Regina Appiah-Opong, for their constant support, commitment, guidance and inspiration during this project. I am grateful for their resources and knowledge shared with me. I also appreciate them for their insightful criticisms and assessments that have shaped and enhanced the quality of this work. May God richly bless them.

I am eternally grateful to my father, Mr. Abeku Philips, for his prayers and encouragement. A special thank you to my mum, Miss Anna Maria Assefuah and my sister, Miss Venneta Phillips, for their enormous support. Special appreciation goes to Miss Eunice Dotse for her immense support throughout this project; words alone cannot express my heartfelt gratitude to her. I wish you success in all the days of your life. I also wish to thank all staff of the Clinical Pathology Department-NMIMR for their support.

Special acknowledgements to all my friends especially, Miss Benedicta Obenewaa Dankyi, Mrs. Diilo Cecilia Asemanyi, Miss Abigail Aning, Mr. Isaac Tuffour, Mr. Ebenezer Ofori-Attah, Mrs. Eunice Ampem-Danso, Mrs. Rhoda Ashiabi, Mrs. Mina Forson, Mrs. Sheena Neizer-Appiah and Mrs. Ernestina Tetteh for their prayers and diverse support.

Finally, a big thank you to all my wonderful classmates.

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LIST OF ABBREVIATIONS

6-MP	6-mecaptopurine
BRCA	Breast Cancer Gene
BSS	Breast Self-Exam
DCF	Dichlorofluorescein
DCF-DA	Dichlorofluorescein-diacetate
DHFR	Dihydrofolate reductase
DMEM	Dulbecoco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOC	Docetaxel
DRLC	D-ribose-L-cysteine
EDTA	Ethylene diaminetetracetic acid
FBS	Fetal Bovine Serum
GLOBOCAN	Global Burden of Cancer Study
GSH	Reduced Glutathione
H_2DCF	Dichlorohydrofluorescein
IC_{50}	Concentration that inhibits cell growth by 50%
IDC	Infiltrating Ductal Carcinoma
ILC	Infiltrating Lobular Carcinoma

LNCaP	Androgen-sensitive Human Prostate Adenocarcinoma Cells
MCF-7 line	Michigan Cancer Foundation-7. A human breast adenocarcinoma cell
MET	Methotrexate
NCI	National Cancer Institute
NMIMR	Noguchi Memorial Institute for Medical Research
OPA	O-pthalaldehyde
PC3	Human Prostate Cancer Cell Line
PNT-2	Normal Prostate Epithelium Cell Line
PSA	Prostate Specific Antigen
RibCys	Ribose Cysteine
RPMI	Rose Park Memorial Institute
−SH	Sulfhydryl group
STC	Scientific Technical Committee
UG	University of Ghana
WCRFI	World Cancer Research Fund International

CHAPTER ONE

1.0 BACKGROUND

1.1 INTRODUCTION

Non-communicable diseases (NCDs) such as cardiomyopathies, asthma, diabetes and cancer now account for most deaths worldwide. Cancer, for example, is estimated to be the major cause of death and the single most significant threat to improved-life expectancy in every country in the world in the 21st century (Bray *et al*., 2018). The incidence and mortality of cancer are rising rapidly worldwide. The International Agency for Research on Cancer (IARC) estimates that about 21.7 million cancer cases would be recorded worldwide by 2030, with cancer-related deaths estimated to reach 13 million. It also figured that cancer would be a significant cause of morbidity and mortality in developing countries. Breast and prostate cancers being the second leading forms of cancers globally, in women and men, respectively (WHO, 2018).

In Ghana, it is reported that 16,600 cancer cases occur yearly with 12,700 cancer deaths occurring in 2008, out of which 5,800 male deaths and 6,900 female deaths were recorded (GLOBOCCAN, 2008). Breast and cervical cancers are the most common in women, whereas, prostate and liver cancers are the most frequent in men, in Ghana (Laryea *et al*., 2014b).

Cancer can occur due to ageing, lifestyle modifications such as tobacco smoking, poor diet, excessive alcohol intake, obesity and also certain viruses. Examples of viruses are hepatitis B and C viruses, human papillomavirus (HPV) and human immunodeficiency virus (HIV). Some parasitic infections such as schistosomiasis were also reported to represent co-mortality factors explaining the rate of cancer-death (Cooper, 2000; Irigaray *et al*., 2007; Tuffour *et al*., 2018).

Treatment of cancer includes surgery, radiotherapy and chemotherapy (Wild & Stewart, 2014). Some current therapies in use include immunotherapy, hormonal therapy and photodynamic

therapy (Mohammad *et al*., 2018). Cancer treatments aim at destroying the cancer cells or preventing the cancer cells from growing.

Chemotherapy is the most preferred treatment option for cancer (Singh *et al*., 2018). It requires the use of therapeutic agents to destroy tumour cells by disrupting their cell function, causing damage to deoxyribonucleic acid (DNA) via apoptosis and other mechanisms leading to malignant cell death (Woods & Turchi, 2013). Methotrexate (MET) and docetaxel (DOC) are examples of chemotherapeutic drugs used for the treatment of breast and prostate cancers, respectively. Most chemotherapeutic agents (including MET and DOC) generate reactive oxygen species (ROS) that could lead to oxidative stress and ROS-mediated cellular injury in cancer and rapidly proliferating normal cells (Chen *et al*., 2007; Yang *et al*., 2018). Reactive oxygen species are chemically reactive molecules such as hydroxyl (OH^-), superoxide (O_2^-) and hydrogen peroxide (H_2O_2) that can damage cellular components such as proteins and DNA (Kunwar & Priyadarsini, 2011). Chemotherapy-induced side effects such as hepatotoxicity, neurotoxicity and myelosuppression (Singh *et al*., 2018) are generally attributed to the excessive production of the reactive oxygen species (ROS) which consume cellular antioxidants and lead to oxidative stress (Alfadda & Sallam, 2012; Deavall *et al*., 2012; Singh *et al*., 2018). Therefore, increasing the level of cellular antioxidants may reduce ROS content, decrease the oxidative stress and prevent cellular damages caused during chemotherapy (Conklin, 2004).

Antioxidants are molecules that donate one of their electrons to the free radicals to prevent them from taking electrons from other molecules; hence they are described as 'mopping up' or scavenging free radicals (W. K. Chowdhury *et al*., 2017; Glasauer & Chandel, 2014; Singh *et al*., 2018). Examples of antioxidants include reduced glutathione (GSH), catalase, superoxide dismutase, glutathione peroxidase, ubiquinol, vitamins A, C, and E among others (Birben *et al*., 2012; Fang *et al*.,2002; Ravi *et al*., 2018).

Glutathione (GSH) is the most abundant antioxidant in the cell and plays a significant role in protecting the cell from an excessive accumulation of ROS (Bansal & Celeste Simon, 2018; Traverso *et al*., 2013). It is synthesised by the sequential addition of cysteine to glutamate and then to glycine (Forman *et al*., 2009; Griffith, 1999). The sulfhydryl group (−SH) of the cysteine is involved in the reduction and conjugation reactions that are usually considered as the most essential functions of GSH. These reactions provide the means for removal of oxidants and various xenobiotic compounds (Forman *et al*., 2009). Many synthetic antioxidants (mostly used as an oral supplementation) have also proved to be effective in restoring the body's natural antioxidants. Examples of such antioxidants are N-acetylcysteine (NAC) and riboceine (RIB). Both NAC and RIB contain cysteine, a precursor required for the synthesis of GSH thereby increasing intracellular GSH in the liver, spleen, and other organs (Kader *et al*., 2014; Roberts *et al*., 1992; Saltman, 2015). A study by Chen *et al*., 2007 showed that Pre-treatment of mice with gamma-glutamyl-cysteine ethyl ester, a precursor of GHS, increases brain GSH levels and significantly decreased glutathione-S-transferase which facilitated protein oxidation and lipid peroxidation. A study by Roberts *et al*., (1992) showed that riboceine prevented acetaminophen-induced target organ injury in both the liver and kidney and its pre-treatment elevated both hepatic and renal GSH in mice. Another study by Roberts *et al*., (1987) showed an increased level of GSH in ribose cysteine-treated rat hepatocytes, which was blocked by buthionine sulfoximine pre-treatment. Riboceine (containing cysteine) by increasing cellular glutathione level could therefore reduce or prevent the oxidative stress in normal proliferating cells, thereby preventing ROS-induced cellular damages occurring during chemotherapy.

A study of the effects of riboceine on the cytotoxic activity of chemotherapeutic agents such as MET and DOC on normal and cancer cell lines will provide evidence of its potential chemoprotective activity.

1.2 PROBLEM STATEMENT

Cancer is the second leading cause of death worldwide. 18.5 million new cases of cancer were diagnosed worldwide, and 9.6 million deaths occurred because of cancer (WHO,2018). Also, over 70% of cancer deaths occur in low- and middle- income countries. In Ghana, about 23,000 new cancer cases and 15,000 cancer-related deaths were recorded in 2018 (IARC, 2019). In Ghana breast and cervical cancers are the most common cancer in women while liver and prostate cancers are the most common cancer in men (Laryea *et al*., 2014)

Treatment of cancer comes with several challenges such as cost, resistance and severe side effects of the chemotherapeutic drugs. It is well established that most chemotherapeutic agents (e.g., MET and DOC) induced an accumulation of ROS in the cell during the treatment, and this put both cancer and normal fast-proliferating cells under oxidative stress (Ozben, 2007 *Yang et al*., 2018).

It has been shown that MET induces ROS such as hydrogen peroxide and superoxide radicals which are known to induce apoptosis. Also, MET-induced accumulation of hydrogen peroxide has been shown to cause mitochondrial damages characterised by a release of cytochrome c into the cytosol (Herman *et al*., 2005; Wu *et al*., 2017). DOC has been shown to induce an accumulation of ROS (such as H_2O_2 and O^{2-}) through direct inhibition of some antioxidants systems including GSH, catalase and superoxide dismutase (H. Yang et al., 2018). The ROS generated have consequences on both cancer cells and normal rapidly proliferating cells. Reactive oxygen species generated in response to chemotherapy consume cellular antioxidants such as glutathione resulting in a decreased level of GSH, leading to oxidative stress (Singh *et al*., 2018). The ROS-induced side effects (due to damages on normal cells) make the treatment of cancer challenging. For example, therapeutic dosages of the anticancer drug are often reduced, non-compliance to cancer medications by patients and also the quality of life of patients is impaired during and after treatment (Kayl & Meyers, 2006)

1.3 JUSTIFICATION

Cancer accounts for nearly 13% of deaths worldwide, and about 1 in 6 deaths from cancer occur globally (WHO, 2018). Breast cancer is the most common cancer in women, whereas prostate cancer is the most common cancer in men in Ghana (Laryea *et al*., 2014).

Methotrexate is an approved drug used alone or in combination with other agents for the treatment of breast cancer. Docetaxel is a standard drug used for the treatment of prostate cancer and is mostly used in combination with other medications. These chemotherapeutic agents have been shown to generate excessive ROS which can lead to oxidative stress in both normal and cancer cells. The ROS-induced oxidative stress on normal rapidly proliferating cells results in injuries caused to these cells, which in return lead to the severe side effects observed during and after chemotherapy.

The use of antioxidants or their precursors to reduce the chemotherapy-induced oxidative stress on normal cells, and to decrease or prevent the severe side effects, remains a promising therapeutic approach that has been explored in several studies (Singh *et al*., 2018). It was reported that antioxidants could protect normal tissues, increase patient survival and therapeutic response when given concurrently with chemotherapeutic agents (Singh *et al*., 2018). A study by Xu *et al*., (2012) showed that GSH could be an effective protector against cisplatin-induced nephrotoxicity in various rodent models. A readily available supply of L-cysteine for GSH synthesis is essential for protection against oxidative stress (Kader *et al*., 2014). Riboceine has been shown to protect against oxidative stress by increasing cellular GSH (Roberts *et al*., 1987).

However, there is a dearth of information on the protective effects of riboceine on normal cells during chemotherapy. The present study, therefore, seeks to determine the protective effects of riboceine against the cytotoxic effects of MET and DOC on the normal and cancer cells.

1.4 HYPOTHESES

It was hypothesised that riboceine would increase the glutathione content, decrease the ROS level and increase the viability of normal cells (chemoprotection) in the presence of the cytotoxic agent MET and DOC. We also hypothesised that the effect of riboceine may be different on transformed cells such as cancer cells, and therefore, may result in a dissimilar chemoprotection.

1.5 AIM

The aim of this study was to determine the chemoprotective effect of riboceine against the cytotoxic activity of MET and DOC on normal and cancer cell lines.

1.6 SPECIFIC OBJECTIVES

The specific objectives were to:

1. determine the effect of riboceine (as compared to NAC) on the cytotoxic activities of MET and DOC on the viability of normal prostate (PNT-2), prostate cancer (PC3) and breast cancer (MCF-7) cell lines.
2. measure the glutathione content in PNT-2, PC3 and MCF-7 cells treated with RIB, NAC, MET, DOC and their combinations.
3. quantify the the ROS level in PNT-2, PC3 and MCF-7 cells treated with RIB, NAC, MET, DOC and their combinations.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 CANCER

The uncontrolled cell division, which leads to abnormal cell growth, is termed cancer. Cancer affects different tissues or organs in humans, and this accounts for the types of cancers; for example, cancers affecting the breast, prostate and blood are breast cancer, prostate cancer and leukaemia respectively. Cancer may spread from one tissue or organ to another which is referred to as metastasis and this accounts for most cancer- related mortality (WHO, 2014).

2.1.1 Cancer Statistics, Aetiology and Causes

Cancer is the second leading cause of death in the world, and it accounts for approximately 9.6 million deaths globally (WHO, 2018). Cancer deaths increased by 1.4 million from the 8.2 million deaths reported in 2012 to 9.6 million deaths reported in 2018. Nearly 1 in 6 deaths occur worldwide due to cancer, with 70% of deaths occurring in low and middle-income countries in Asia, Africa, South and Central America (WHO,2018). In Ghana, approximately 23,000 cancer cases and 15,000 cancer-related deaths were recorded in 2018 (IARC, 2019). Cancer imposes enormous pressures on the national economies. Globally, the economic burden was about US$ 1.16 trillion in 2010 (WHO, 2015).

Cancer occurs as a result of both internal and environmental or acquired factors. The internal factors include inherited mutations (genes affecting DNA repair and genes affecting cell growth or apoptosis), oxidative stress, hormones and immune conditions (Fig 2.1). The environmental factors also includes carcinogens (such as tobacco, alcohol, asbestos etc.), radiation and infectious organisms (such as Human papillomavirus, HIV, HBV, HCV, *Schistosoma haematobium* and *Helicobacter pylori*) *(*Anand *et al*., 2008; Cooper, 2000; Irigaray *et al*., 2007) (Fig 2.1). These factors overwhelm the DNA repair system, leading to persistent mutations in the genome of the somatic cell which also leads to activation of growth-

promoting oncogenes, inactivation of cancer suppressor genes and the alteration of genes that regulate apoptosis, finally leading to malignant neoplasm or cancer (Fig 2.2) (Alberts *et al*., 2002; Chukwudi Ezeani, 2017; John Mendelsohn, 2014). These oncogenic alterations in normal cells lead to the acquisition of abnormal phenotypic characteristics such as; increased cell proliferation, resistance to apoptosis, insensitivity to growth-inhibiting signals, altered cell cycle, enhanced angiogenic and invasive potentials (Fouad & Aanei, 2017; Hanahan *et al*., 2000).

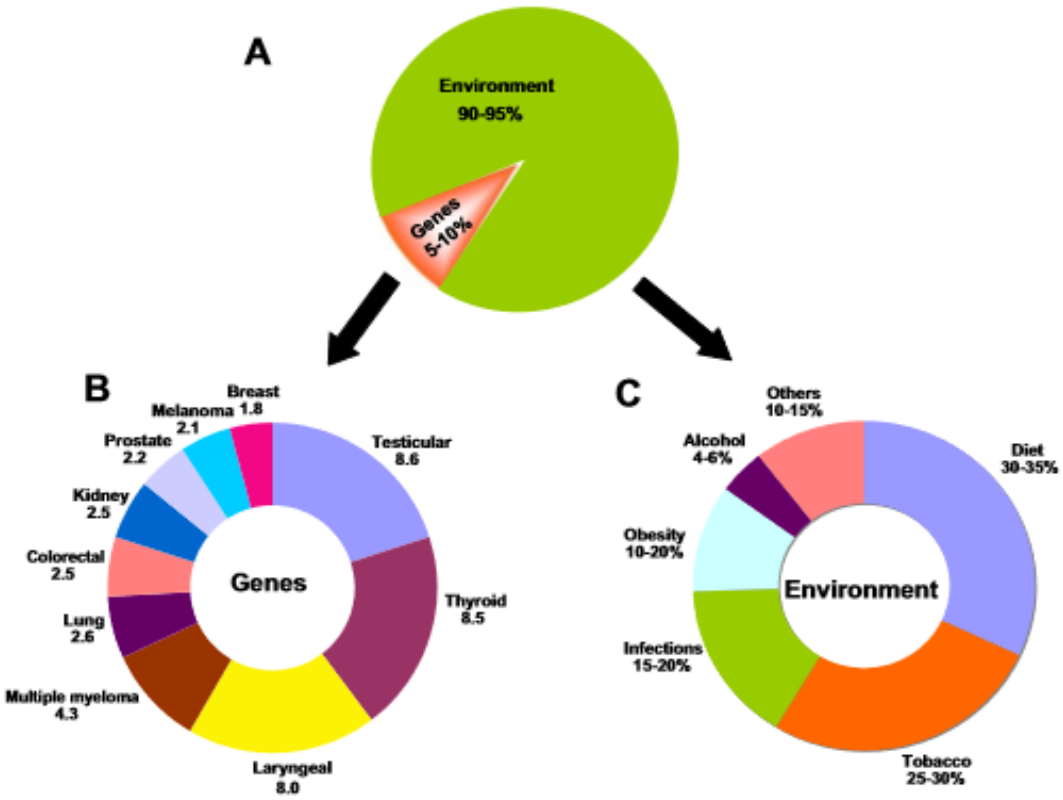

Figure 2. 1: Factors that cause cancer (Anand *et al*., 2008).

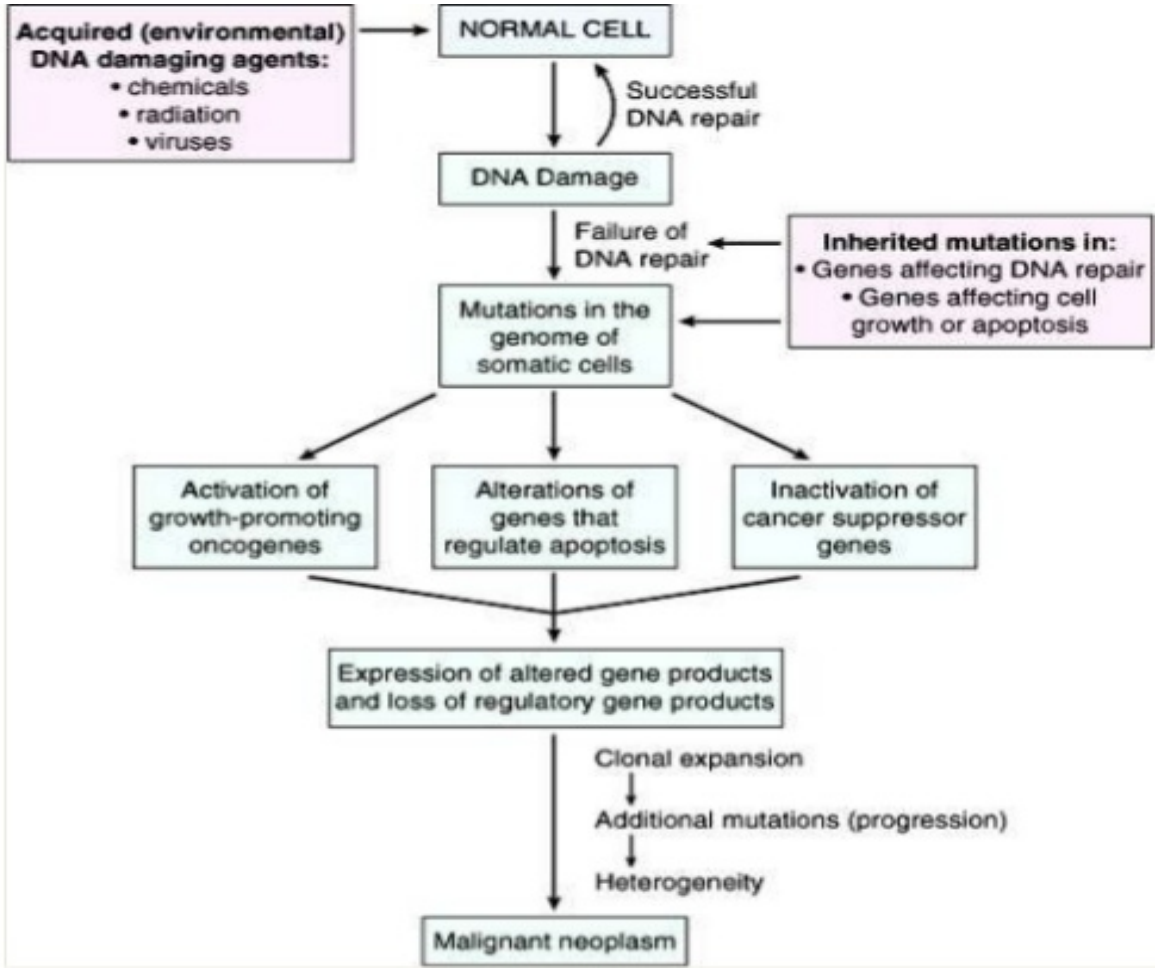

Figure 2. 2: The aetiology of cancer (Ahmed Y, 2007).

2.1.2 Types of Cancers

There are several types of cancers, and their location in the body account for the types of cancer. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, cervix and thyroid cancer are the most common among women (Bray *et al*., 2018). The description of the types of cancers in the present report is limited to prostate and breast cancers, the types of interest in our study.

2.1.2.1 Prostate Cancer

Prostate cancer, also known as prostatic carcinoma, is a type of cancer that affects the prostate. It is an adenocarcinoma that arises mainly from the glandular part of the prostate (usually the

peripheral basal cells) (Fournier *et al*., 2004). The cancer cells grow and start to multiply, spreading initially to the surrounding prostate tissue to form a tumour nodule. The tumour may either grow outside the prostate (extracapsular extension) or remain localised within the prostate for decades (McClure *et al*., 2018). It also spreads to other parts of the body mainly the bones and lymph nodes, and it is referred to as metastatic prostate cancer (Attard *et al*., 2016; Fournier *et al*., 2004). Prostate cancer is the second most predominant cancer globally and also primary cancer-causing death in men after lung cancer (WHO, 2014). The incidence of prostate cancer differs in different parts of the world (Hassanipour *et al*., 2018). About 68% of prostate cancer cases occur in the developed countries showing its predominance in these countries (WHO, 2015). Although the incidence rate of prostate cancer is low in developing regions such as Africa, some evidence have shown that prostate cancer mortalites are predominantly higher in black African populations compared to other races (Adeloye *et al*., 2016; Powell, 2007).

Age, diet, sexually transmitted diseases, obesity, family history and high serum testosterone are the risk factors associated with prostate cancer (Leitzmann & Rohrmann, 2012; Patel & Klein, 2009). It often affects older men, about 1 in 3 men older than 50 years are diagnosed with prostate cancer (Bechis *et al*., 2011; Stangelberger *et al*., 2008). Mutations of the genes BRCA 1 and BRCA2 also play a role in the development of prostate cancer (Castro & Eeles, 2012; Cavanagh & Rogers, 2015; Mitra *et al*., 2011). Prostate-specific antigen (PSA) is mostly used to detect prostate cancer. Biopsy, digital rectal exam (DRE) and prostate imaging using ultrasound and magnetic resonance imaging (MRI) are also used to diagnose prostate cancer (McClure *et al*., 2018).

2.1.2.2 Breast Cancer

Breast cancer is a form of cancer that affects the breast, primarily the lining of the milk ducts or the lobules that supply milk to the canals (Sharma *et al*., 2010). It involves the abnormal

growth and proliferation of cells originating from the breast. Breast cancer can be seen mainly in women and seldom in men.

Globally, breast cancer accounts for 10.4% of all women's cancer cases. It is also the second most common cancer and a fifth major cause of cancer-related deaths in women (WHO, 2018). Approximately 12% of females worldwide are living with breast cancer (McGuire, 2016). Also, about 580,000 deaths are associated with the disease (WHO, 2015).

The breast is made of two major tissue types; the glandular tissue and stromal tissues. The glandular tissue consists of the lobules (milk-producing glands) and the ducts (milk passages). The stromal tissues comprise the fatty and fibrous connective tissues of the breast (Sharma *et al*., 2010). Breast cancers usually start in the lining of the ducts and sometimes in the lining of the lobules (Sharma *et al*., 2010). Several types of breast cancers are classified based on the site (non-invasive vs invasive), the occurrence (lobular vs ductal carcinoma *in situ*; medullary vs mucinous vs tubular carcinoma), the origin (infiltrating lobular vs ductal carcinoma) and the inflammatory status. Also, there are several causes of breast cancer which include a previous history of breast cancer, and genetic (significant family history), hormonal, lifestyle/dietary, and environmental causes. Monthly breast self-examination (BSE) helps to detect these symptoms earlier. Mammogram and biopsy of nodules are also used to diagnose the disease.

2.2 TREATMENT OF CANCER

Prevention of cancer provides the most cost-effective long-term method of cancer control. Living a healthy lifestyle prevents cancer. An early cancer diagnosis has been the most critical stage of cancer treatment, especially when the type of cancer has a high potential for a cure (WHO, 2008). Treatment of cancer aims at curing the disease, prolonging life, and improving the quality of life for cancer survivors (WHO, 2008). Cancer treatments require the use of specific methods to kill the cancer cells or stop their growth and include surgery, radiotherapy,

chemotherapy, immunotherapy and hormonal therapy. Chemotherapy is the treatment of interest in the present study and involves the use of anticancer drugs.

2.2.1 Chemotherapy

A German scientist, Paul Ehrlich, was the first to use the term chemotherapy to describe the chemical treatment of cancer (Arruebo *et al*., 2011). The use of chemotherapy began in the early 20th century (Arruebo *et al*., 2011) and is still the most effective and widely used treatment type to date. Aside from their primary mechanisms of action, it is widely accepted that the anticancer effects of most chemotherapeutic agents lead to oxidative stress and ROS-induced cellular injury of cancer cells and rapidly proliferating normal cells (Chen *et al*., 2007; Yang *et al*., 2018) Abass and Rheman, 2018). Chemotherapeutic drugs cause tumour cells to either stop growing (cytostatic) or collapse (cytotoxic) and are primarily systemic agents (Abass and Rheman, 2018).

2.2.1.1 Classes of Chemotherapeutic Drugs

Chemotherapeutic drugs are grouped based on their chemical structure, composition, mode of action and homology to other drugs (Abass and Rheman, 2018). Some of the classes of chemotherapeutic drugs include; alkylating agents (e.g., cyclophosphamide), antimetabolites (e.g., methotrexate), anthracyclines (e.g., doxorubicin), mitotic inhibitors (e.g., taxanes and vinca alkaloids), hormone chemotherapeutic drugs (e.g., prednisone and dexamethasone), cytotoxic antibiotics (e.g., actinomycin D and bleomycin) and platinum-based antineoplastic (e.g., cisplatin and carboplatin).

2.2.1.1.1 Mitotic Inhibitors

Mitotic inhibitors are known to inhibit protein synthesis required for cell division during mitosis (Abass and Rheman, 2018). They are used to treat cancers of the lung, prostate, breast, myelomas, leukaemia and lymphomas. Some examples include taxanes (paclitaxel and docetaxel) and vinca alkaloids (Vinblastine and Vincristine).

Taxanes (Docetaxel)

Docetaxel is a semisynthetic taxane, a taxoid and a plant alkaloid, which belongs to a class of anticancer agents that binds to beta-tubulin causing tubulin assembly in microtubules and inhibiting its depolymerization, resulting in the inability of cells to replicate in the M phase (Bissery *et al*., 1999; Herbst & Khuri, 2003; Montero *et al*., 2005). Docetaxel acts as a mitotic spindle poison and induces a mitotic block in proliferating cells (Herbst & Khuri, 2003). Taxotere is the trade name of docetaxel. It is semi-synthesised from a non-cytotoxic taxoid precursor (10-diacetyl baccatin III) obtained from the needles of European yew Taxus bacta (Bissery *et al*., 1999). Docetaxel is used for the treatment of mainly androgen-receptor prostate cancer, metastatic breast cancer and non-small lung cancer (Attard *et al*., 2016; Corcoran *et al*., 2012; Engels *et al*., 2005). The recommended dose ranges from 60-100 mg x m^{-2} as a 1 h IV once every three weeks *(*Engels *et al*., 2005). Docetaxel is primarily metabolised by cytochrome P450 (CYP3A4). Some of the most common toxicities of docetaxel use are; neutropenia, neurotoxicity, hypersensitivity reactions, alopecia, cutaneous toxicity, cardiac arrhythmias and myalgia (Mikuła-Pietrasik *et al*., 2019; Omlin *et al*., 2015).

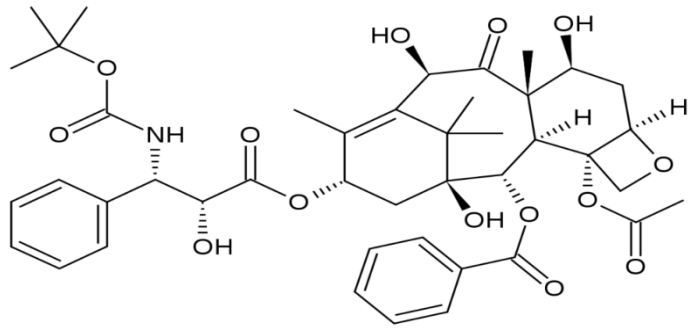

Figure 2. 3: The structure of Docetaxel (Yazdi *et al*, 2012).

2.2.3.1.2 Antimetabolites

Antimetabolites are analogues of the units of DNA and RNA. They have a structural resemblance to folic acid and the purine and pyrimidine bases involved in the synthesis of DNA, RNA, and certain coenzymes (Kwok *et al*., 2017). Antimetabolites usually affect the S-phase (DNA synthesis phase) of the cell cycle (Abass and Rheman, 2018). This, therefore, obstructs the growth of rapidly growing cells. There are three classes of antimetabolites, the folic acid analogues, purine analogues, and pyrimidine analogues. They are used to treat breast cancer, ovarian cancer, leukaemia etc. Antimetabolites are classified into three (3) groups, namely, purine and pyrimidine analogues and folic acid antagonists.

Purine and Pyrimidine Analogues

Purine analogues are derivatives of purines, and they are incorporated into the DNA instead of adenine and guanine, therefore, interfering with DNA synthesis. Examples are 6-Mercaptopurine (6-MP) and thioguanine.

Pyrimidine analogues are derivatives of pyrimidines, and they are incorporated into the DNA instead of cytosine and thymine, therefore interfering with DNA synthesis. Examples of these analogues are cytarabine, 5-fluorouracil and gemcitabine.

Folic Acid Antagonist

Folic acid is an essential vitamin that is converted into metabolically active tetrahydrofolic acid by the enzyme dihydrofolate reductase. Tetrahydrofolic acid participates in the synthesis of purines, thymidylate, and ultimately nucleic acids by transferring methyl groups to nucleotide precursors (purines and pyrimidines). Also, the conversion of deoxyuridine monophosphate to thymidine monophosphate by thymidylate synthase to aid in RNA and DNA synthesis require folic acid. Folic acid antagonist (examples; methotrexate, Pemetrexed disodium, Pralatrexate) inhibits these enzymes, therefore, interfering with DNA and RNA synthesis.

Methotrexate

Methotrexate was formerly known as amethopterin a 4-amino, 10-methyl folate analogue. It is an antimetabolite specifically an antifolate that is used for the treatment of cancers (breast cancer, lung cancer, lymphoma and leukaemia), rheumatoid arthritis, and psoriasis.

Folate binds to the enzyme dihydrofolate reductase; MET is similar in structure to the folate; therefore, binds strongly to the coenzyme DHFR in place of the folate. MET, therefore, inhibits the synthesis of DNA and RNA (De Oliveira *et al*., 2018). It also inhibits protein synthesis because reduced folates are cofactors in the conversion of glycine to serine and homocysteine to methionine (Kwok *et al*., 2017).

Methotrexate is administered intravenously and intramuscularly. Some of the adverse effects of MET use include hepatoxicity, renal impairment, myelosuppression and mucositis (van Outryve *et al*., 2002)

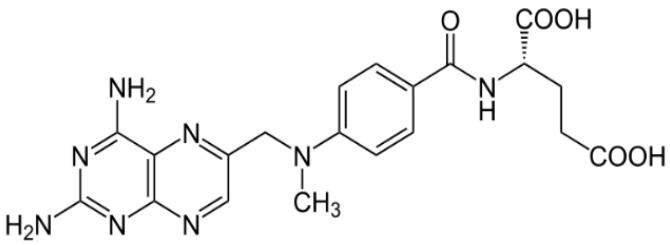

Figure 2. 4: The structure of methotrexate (Lotfi *et al*., 2016).

2.2.2 Limitations of Traditional Chemotherapeutic Agents

Many anticancer drugs are limited in their use mainly by their severe side effects and resistance. The side effects are due to non-cancer-specific damages caused by chemotherapeutic agents to normal cells that divide rapidly, like the bone marrow, cells lining the digestive tract and hair follicles (Aslam et al., 2014; Schirrmacher, 2019)Some of the side effects of chemotherapy, such as hair loss (alopecia), decreased production of blood cells (myelosuppression) and

inflammation of the lining of the digestive tract (mucositis) (Schirrmacher, 2019). Another limitation is the partial delivery of the anticancer agents due to the blood-brain barrier and drug transporters that pump out the drugs from the brain, reducing the efficacy of some chemotherapy treatment on brain tumours (Cheung-Ong *et al*., 2013).

2.2.2.1 New Pharmacological Approaches

New pharmacological approaches involve the use of cancer-cell-targeted therapies to overcome the limitations observed with traditional chemotherapeutic drugs (Abd El-Hack et al., 2019; Warsame & Grothey, 2012). Small molecules and antibodies are used to target cellular proteins and processes involved in cancer initiation and progression (Mashreghi *et al*., 2017). They include immunotherapy, hormonal therapy, use of nanoparticles and the use of antioxidants together with the anticancer agents.

2.2.2.1.1 Hormonal therapy

Hormonal therapy, which is also known as endocrine therapy, is a type of treatment that target hormones to kill the hormone-responsive cancer cell types. The treatment either blocks the body's ability to produce hormones or lowers the number of hormones in the body to stop or slow the growth of cancer (NCI, 2015). This therapy uses agents that act as a hormonal antagonist in the body. Surgical removal of endocrine organs can sometimes be a form of hormonal therapy. It is used for the treatment of cancers originating from hormonally responsive tissues such as breast and prostate cancers. Hormonal therapy may also be used to alleviate symptoms associated with other cancer treatment such as chemotherapy-associated symptoms (e.g., anorexia). Hormonal therapies are classified as inhibitors of hormone synthesis (e.g., aromatase inhibitors such as letrozole and anastrozole; gonadotropin releasing-hormone analogues such as leuprorelin and goserelin); hormone-receptor antagonists (selective estrogen receptor modulator such as tamoxifen and raloxifene; and antiandrogens such as

flutamide and bicalutamide); hormone supplementation (progestogens, estrogens and androgens).

2.2.2.1.2 Immunotherapy

Also known as biologic therapy or immuno-oncology. This therapy targets the immune system to combat cancer cells. The treatment either directs the immune system to target the antigens on the cancer cells or enhance the existing anti-tumour responses (Yang, 2015). The immune responses consist of the humoral (adaptive) and cellular (innate) immunity, which are mediated by B and T lymphocytes respectively.They both play vital roles in anticancer immune response (Zhang & Chen, 2018) $CD8^+$ cytotoxic T lymphocytes (CTLs) are known as the backbone of immune response fighting cancer (Vigneron, 2015). Tumor-infiltrating lymphocytes (TILs) contain an abundant level of CTLs capable of invading malignant cells. CTLs attacks tumor cells via perforin, granzymes and also ligands of the tumor necrosis factor (TNF) superfamily (Vigneron, 2015; Zhang & Chen, 2018)). The anti-tumor effect can also be achieved by secreting Interferon gamma (IFN-ɣ) and TNF-α from activated $CD8^+$ T cells. Naive $CD4^+$ T cells could be activated and differentiated into distinct T cell subsets such as Th1, Th2, Tregs, Th9, Th17, Th22 and also follicular helper T cells. Th1 subset of $CD4^+$ T cells play vital antitumor roles by coordinating cell mediated immunity against cancer. Th1 cells can produce IFN-ɣ and chemokines and thus enhancing $CD8^+$ T cells expansion, priming and infiltration into the tumor site by. Th1 cells can also activate inflammatory cells, such as macrophages, NK cells, granulocytes and eosinophils around the tumor. NK cells can destroy cancer cells directly via; secretion of TNF-α, perforin, cytoplasmic granules and granzymes, expression of death receptor-mediated apoptosis, and expression of CD16 which leads to antibody dependent cellular cytotoxicity (ADCC) (Zhang & Chen, 2018). Immunotherapy can be classified as passive immunotherapy (examples include; immune checkpoint inhibitors/blockade,

monoclonal antibodies, tumor infiltrating lymphocytes, T-cell therapy etc.) and active immunotherapy (examples are; vaccines, cytokines, immune adjuvants etc.). Some approved immunotherapy antibodies are rituximab, Trastuzumab ipilimumab, ofatumumab alemtuzumab etc.

2.3 REACTIVE OXYGEN SPECIES

Reactive oxygen species (ROS) are molecules with several unpaired electrons reacting to form highly reactive species. They are categorised as free radical and non-radical oxygen species. Examples of free radicals include hydroxyl radical ($OH^{\cdot}$), superoxide anions ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$). Non-radicals include hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorous acid (HOCl) and hydroperoxides (ROOH).

Reactive oxygen species are generated from both endogenous and exogenous sources. The exogenous source of ROS are pollutants, ionising radiation, oxidising chemicals and UV light. The endogenous sources of ROS are from various enzyme systems involved in cellular signalling, metabolic processes and immune response or during inflammation. They include the mitochondrial electron transport chain, NADPH oxidase complex, cytochrome P450 enzymes, cyclooxygenases, lipoxygenases, xanthine oxidase, thymidine phosphorylase and enzymes in peroxisomes (Kasiappan & Safe, 2016; Kryston *et al*., 2011; Poprac *et al*., 2017). The mitochondrial electron transport chain is the primary endogenous source of ROS. It consists of enzyme complexes I, II, III and IV with complexes I and III mainly involved in ROS production. Reactive oxygen species are byproducts of aerobic respiration in the mitochondria with approximately 2– 5% of oxygen consumed converted to ROS (Kasiappan & Safe, 2016; Kryston *et al*., 2011).

In the mitochondrion electron transport chain, electrons are transferred from NADH to an electron acceptor (oxygen molecules, O_2) forming superoxide ($O_2^{\bullet -}$), which is then converted by superoxide dismutase (SOD) to form hydrogen peroxide (H_2O_2). Hydroxyl radical ($^{\bullet}OH$) is

also produced by the catalysis of H_2O_2 via the Fenton reaction. Additionally, peroxynitrite ($ONOO^-$) is formed by the reaction of superoxide and nitric oxide ($NO^{\bullet}$) (Tong *et al*., 2015)(Fig. 2.5).

Peroxisomal oxidases catalyse the oxidation of various amino acids, purines, fatty acids, α-hydroxy acids and polyamines, followed by the formation of H_2O_2 via the transfer of hydrogen obtained from the appropriate substrate directly to O_2 (Neki, 2015; Phaniendra *et al*., 2015a; Varricchi *et al.*, 2018). Xanthine oxidase also generates $O_2^{\cdot -}$ and H_2O_2 through the catabolism of purines (Irondi *et al*., 2016; Neki, 2015; Varricchi *et al*., 2018).

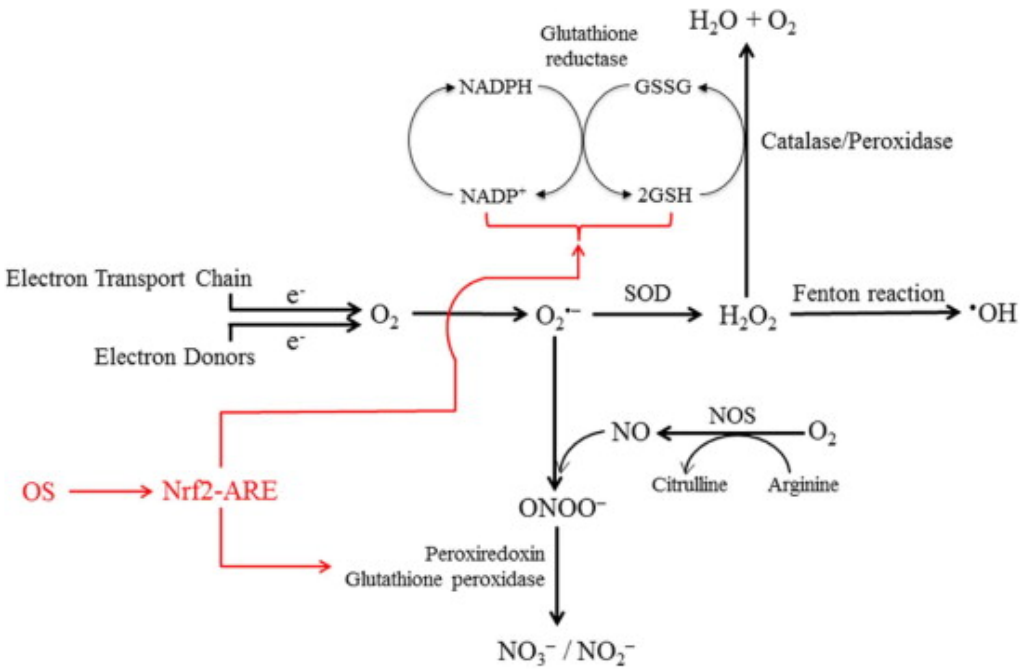

Figure 2. 5: Main reactions and signalling pathways in the generation and scavenging of ROS (Tong *et al*., 2015).

2.3.1 Reactive Oxygen Species and Cancer

Normal cells require ROS to perform several cellular activities, such as immune response mechanisms, cell signalling, enzyme activation, gene expression, the formation of disulfide

bond during the folding of new proteins in the endoplasmic reticulum, and the regulation of the caspase activity activated during apoptosis. However, an increased level of ROS modifies and disrupts several intracellular molecules (eg. DNA, RNA, proteins and lipids) which is established to be a central mechanism to several chronic diseases such as neurodegenerative diseases (e.g. Parkinson's and Alzheimer's disease), cardiovascular diseases (e.g. Muscular dystrophy), inflammatory diseases (e.g. Rheumatoid Arthritis), ageing, diabetes, cancer etc. (Nita & Grzybowski, 2016; Redza-Dutordoir & Averill-Bates, 2016; Schieber & Chandel, 2014). The effects of ROS do not only depend on their accumulation but also on their equilibrium with antioxidant defences (Redza-Dutordoir & Averill-Bates, 2016). The state of a cell, in which ROS levels (i.e. oxidants) outweighs the antioxidant defences, is known as oxidative stress (Caverzan *et al*., 2016; De Oliveira *et al*., 2018). The effects of oxidative stress on cancer cells promote cancer growth mainly through the oxidation of DNA by ROS which results in the production of 8-hydroxy-2′-deoxyguanosine, a product capable of generating DNA mutations (Guo *et al*., 2016; Qing *et al*., 2019). Reactive oxygen species have also been shown to activate growth-promoting factors or control genes responsible for apoptosis and cell proliferation (Broustas & Lieberman, 2014; Kumari *et al*., 2018; Phaniendra *et al*., 2015b). Furthermore, ROS are known to damage some protease inhibitors (such as plasminogen activator, elastin and plasmin) thereby allowing abnormal action of these proteases involved in tumour invasion and metastasis (Rakashanda *et al*., 2012). Cell survival and disruption of cell death signalling, cell cycle progression, genome instability, cell-cell adhesion and angiogenesis have also been shown to be mediated by ROS (Sabharwal & Schumacker, 2014; Kumari *et al*., 2018; Phaniendra *et al*., 2015b; Sena & Chandel, 2012). Finally, an increased generation of ROS has also been associated to increased metabolic activity, mitochondrial dysfunction, activation of signalling pathways (e.g., integrins), oncogenes activation (e.g., Cmyc, Kras and BRCA1), lipoxygenases activation, cyclooxygenases activation, increased activities of

oxidases, and loss of functional p53 of cancer cells (Liou & Storz, 2010; Rodic & Vincent, 2018; Yang *et al*., 2018; Kumari *et al*., 2018).

2.3.2 Reactive Oxygen Species and Chemotherapy

It is generally admitted that the efficiency of most traditional chemotherapeutic agents is correlated to ROS generation and accumulation (Teppo *et al*., 2017). It appears that ROS induced by chemotherapeutic agents thrust the already increased ROS level over a threshold that cause cancer cell death via apoptosis, necroptosis and autophagy (Yang *et al*., 2018; Teppo *et al*., 2017). The increased ROS level disrupts the redox homeostasis and subsequently causes cancer cell death (Kim *et al*., 2019). Most chemotherapeutic drugs either accumulate ROS through the mitochondrion ROS generation (direct ROS generation) or the inhibition of the cellular antioxidant defence system (Yang *et al*., 2018; Teppo *et al*., 2017; Kim *et al*., 2019). Anticancer drugs such as anthracyclines (eg. Doxorubicin), platinum compounds (eg. Cisplatin), vinca alkaloids (eg. Vinblastine) and antimetabolite (eg. Methotrexate) generate ROS through the mitochondrial function whereas taxanes (eg. Docetaxel) and arsenic agents (eg. pesticides) generate ROS via the inhibition of antioxidant defences (Yang *et al*., 2018) (Fig 2.7).

Anthracyclines such as doxorubicin induce the chelation of intracellular iron which accumulate ROS, specifically, hydroxyl radicals (H_2O_2) leading to apoptosis and autophagy in cancer cells. Also, cisplatin directly damages the mitochondrial DNA and impairs the electron transport chain, which leads to induced oxidative stress on the cancer cells.

Docetaxel induces endothelial dysfunction through the activation of Protein Kinase C (PKCβ) which promotes NADPH oxidase activity and ROS formation (Hung *et al*., 2015). The ROS generated disturb the electron transport chain and impair the mitochondrial membrane potential, therefore, causing cell death (Hung *et al*., 2015). Increased ROS generation in endothelial cells involves increased NADPH oxidase activity and PKCβ phosphorylation

(Chowdhury *et al*., 2005). Methotrexate induces apoptosis via the generation of ROS. The ROS generated obstruct the cell cycle and induces DNA damage leading to cancer the cell death (Albasher *et al*., 2019).

The excessive generation of ROS by these anticancer agents subsequently induces oxidative stress on the normal cells leading to cellular damages at the origin of their toxic effects. Examples of toxic effects caused by some chemotherapeutic drugs are anthracycline- mediated cardiotoxicity, MET-induced hepatoxicity, docetaxel-induced neurotoxicity and cisplatin-induced ototoxicity.

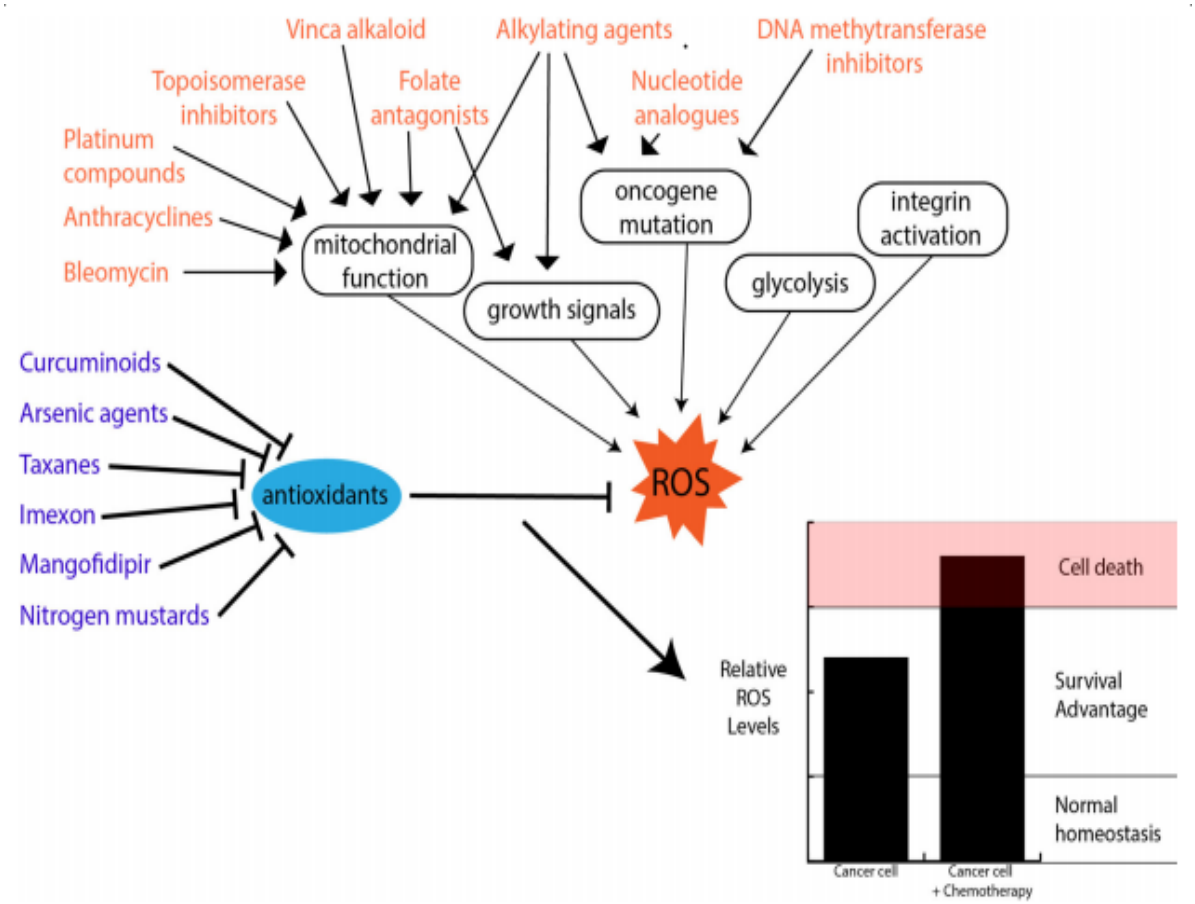

Figure 2. 6: Effects of chemotherapeutic agents on ROS level in the cell (Yang *et al*., 2018).

2.4 ANTIOXIDANTS

Antioxidants are agents that inhibit or delay oxidation (Ozougwu, 2016). They prevent cellular injury induced by free radicals (ROS), by reacting and deactivating free radicals (Ozougwu,

2016). Antioxidants neutralise free radicals by donating their electrons to the free radical and preventing the free radical from taking electrons from other molecules (Singh *et al*., 2018). They are therefore described as mopping up free radicals. They are categorised into enzymatic and non-enzymatic antioxidants.

2.4.1 Enzymatic and Non-Enzymatic Antioxidants

Enzymatic antioxidants are mainly the endogenous antioxidants. They include the catalase, superoxide dismutase and glutathione peroxidase (Singh *et al*., 2018). Superoxide dismutase breaks down superoxide into hydrogen peroxide and oxygen, catalase catalyses the breaks down of hydrogen peroxide and the glutathione peroxidase converts hydrogen peroxide to water (H_2O) and oxygen (O_2) (Ozougwu, 2016).

Non-enzymatic antioxidants are the metabolic and nutrient/dietary antioxidants (Singh *et al*., 2018). The nutrient or dietary antioxidants which are of low molecular weight are obtained from food substances, mostly from fruits and vegetables. They include carotenoids, vitamin C and E, polyphenols, flavonoids, trace elements (Zn, Se, etc.) and many others (Ozougwu, 2016; Singh *et al*., 2018). The metabolic antioxidants include glutathione, uric acid, ubiquinone, bilirubin etc.., which are also endogenous but non-enzymatic antioxidants (Ozougwu, 2016; Singh *et al*., 2018). Some antioxidants used clinically in the management of the toxic effects of anticancer agents include vitamin C, coenzyme Q10 (CoQ10) and resveratrol.

2.4.1.1 Glutathione

Glutathione (GSH) is a non-enzymatic water-soluble antioxidant that is synthesised from the amino acids glycine, cysteine and glutamate (Singh *et al*., 2018). It is an abundant low-molecular-weight thiol found in eukaryotic and other cells (Circu & Aw, 2012). It is synthesised in the cytosol and transferred to the organelles, with more than 10% found in the mitochondria (Bansal & Simon, 2018). Glutathione exists in two forms, the reduced form (GSH) and disulfide-oxidized form (GSSG). The reduced form (GSH) is the most predominant

form that is found at about 5-10 mM in the body, under normal physiological conditions (Bansal & Simon, 2018). Under oxidative stress, GSH reacts with ROS and is converted into GSSG. Glutathione is formed by a *de-novo* biosynthesis which involves a two-step ATP-dependent enzymatic reaction catalysed by two enzymes (Fig. 2.8). The first step is a rate-limiting step which consists of the ligation of glutamate to cysteine forming ɣ-glutamyl cysteine, and this reaction is catalysed by the enzyme ɣ-glutamate-cysteine ligase, followed by the addition of glycine to the dipeptide, which is catalysed by the enzyme GSH synthetase (Bansal & Simon, 2018; Bergamo *et al*., 2006; Ortega *et al*., 2011; Lu, 2013,). Glutathione is the main contributor to the cellular antioxidant defence system, and it protects cells from electrophilic compounds and free radicals generated during cellular metabolism (Circu & Aw, 2012; Pallardó *et al*., 2009; Valko *et al*., 2007). Glutathione participates in cell signalling, proliferation, regulation and redox activation of transcription factors associated with redox signalling and DNA synthesis (Karwicka, 2010; Roušar *et al*., 2012; Traverso *et al*., 2013). It modulates apoptosis, immune function and fibrogenesis (Circu & Aw, 2012; Russell *et al*., 2012; Lu, 2013). It also participates in xenobiotic metabolism, regulating carcinogenic mechanisms and ionisation of radiations (Bansal & Simon, 2018; Ortega *et al*., 2011).

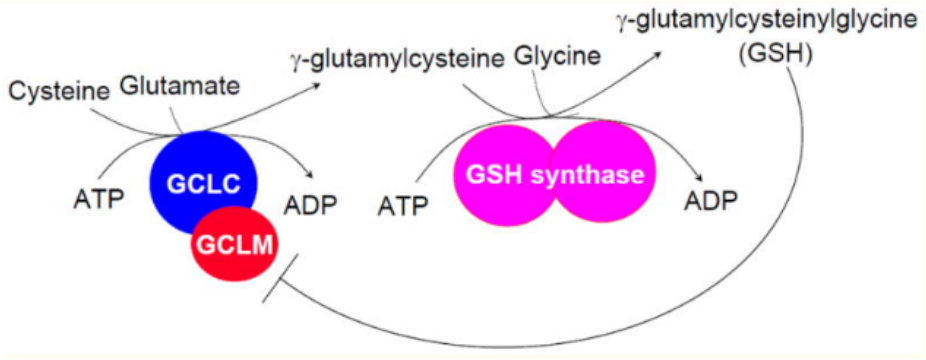

Figure 2.7: The two-step synthesis of glutathione (Lu, 2013).

2.4.1.2 N-Acetylcysteine

N-acetylcysteine (NAC) is a nutritional supplement commonly used as an antioxidant. It consist of the amino acid L-cysteine, a precursor for the synthesis of glutathione. N-acetylcysteine indirectly exerts antioxidative effects via the synthesis of glutathione which is essential for cellular defence against oxidative injury (Schmitt *et al*., 2015; Zawacka-Pankau *et al*., 2012). Glutathione, as described above, protects the cells from oxidative damage by interacting with ROS and thus acting directly as a free radical (primarily oxygen radicals) scavenger (Zawacka-Pankau *et al*., 2012). N-acetylcysteine supplementation has been shown to prevent apoptosis and oxygen-related genotoxicity in endothelial cells by increasing intracellular levels of glutathione and decreasing mitochondrial membrane potential (Mocelin *et al*., 2019).

2.4.1.3 Riboceine

Riboceine, also known as D-ribose-L-cysteine, RibCys or Ribose-Cysteine or [2(R, S)-D-ribo-(1,2,3,4-tetrahydroxybutyl)thiazolidine-4(R)-carboxylicacid], is a synthetic antioxidant that supports the production of glutathione in the cellular system (Aderemi *et al*., n.d.; Emokpae *et al*., 2020; Oz *et al*., 2007). The active ingredients of riboceine are D-ribose and L-cysteine. Cysteine is a precursor required for the synthesis of glutathione in the liver and other organs (Kader *et al*., 2014; Roberts *et al*., 1991). Ribose effectively protects and delivers cysteine to the cell enabling the production of glutathione when needed (Falana *et al*., n.d.; N'guessan *et al*., 2018). It is used as a nutritional supplement. A study by Roberts and Francetic (1991) showed that the pre-treatment of riboceine elevates both hepatic and renal GSH in mice. Another study by Roberts *et al.* (1987) showed that riboceine, by increasing cellular glutathione, protects against oxidative stress and damage by mopping up free radicals through the increase of cellular glutathione.

2.5 NORMAL AND CANCER CELL LINES CHARACTERISTICS

In this paragraph, we briefly describe essential characteristics of the cell lines (PNT-2, PC3 and MCF7) used in the present study.

2.5.1 Normal Prostate Cell Line (PNT-2)

PNT-2 are immortalised normal adult prostate epithelial cells obtained from a 33-year old male at postmortem. They were immortalised with the SV40 virus. They are known to be non-tumorigenic in nude mice. They are adherent cells which grow with an epithelial morphology. The PNT-2 cells express cytokeratin (CK) 8, 18, and 19, members of the keratin family. The keratin family is responsible for maintaining the structure of epithelial cells. The CK8 and CK18 are assembled as partners and function as plasminogen receptors (Gonias *et al*., 2001). The CK19 is expressed in the periderm and a feature of differentiated luminal cells, PNT-2 cells are therefore highly differentiated luminal epithelial cells. They grow in RPMI 1640. Examples of other normal human prostate cell lines are PNT-1A, PNT-1C, HPrEC and Rwpe1 cells.

2.5.2 Human Prostate Cancer Cell Line (PC3)

Human prostate cancer cells (PC3) were obtained from a 62-year old Caucasian male with bone metastasis of a Grade-IV prostatic adenocarcinoma. They are rapidly proliferative and highly invasive cells. They do not express luminal differentiation markers androgen receptors (AR) and prostate-specific antigen (PSA), they are, therefore, androgen-independent cells, i.e. they usually proliferate in androgen-deprived media (Tai *et al*., 2011). The PC3 cells possess a higher migratory capability and also they are more aggressive adenocarcinoma (K. Zhang & Waxman, 2010). The PC3 cells are similar to a prostatic small cell neurocarcinoma (SCNC). They are adherent cells, cultured in RPMI medium. Examples of other human prostate cancer cell lines include DU 145, LNCaP, and VCaP cells.

2.5.3 Human Breast Cancer Cell Line (MCF-7 - Michigan Cancer Foundation-7)

A human breast adenocarcinoma cell line (MCF-7) was obtained from pleural effusion of a 69-year older Caucasian woman with metastatic breast cancer (Welsh, 2013; Comsa *et al*., 2015; Lee *et al*., 2015). It is an estrogen and progesterone-receptor (ER & PR) positive cell line belonging to the luminal A molecular subtype (Levenson and Jordan, 1997; Welsh, 2013). It is a poorly aggressive and non-invasive cell line having a low metastatic potential and reduced angiogenesis. MCF-7 cells show features of differentiated mammary epithelium, positive for epithelial markers such as cytokeratin 18 (CK18), E-cadherin and β-catenin (Welsh, 2013). It is an adherent cell, cultured in DMEM medium. Examples of other human breast cancer cell lines include MDA-MB-231, MCF-10A, MCF-10F, MCF-12F, T47D, HCC1500 and HCC1599 cells.

2.6 REVIEW OF METHODS

2.6.1 Evaluation of Cell Viability Using the Resazurin Assay.

Resazurin is a redox dye that shows both colourimetric and fluorometric variations relating to cellular mitochondrial metabolic activity in live cells. This assay is based on the ability of viable, metabolically active cells to intracellularly reduce resazurin to resorufin and dihydro-resorufin (Some advantages of resazurin assay include it being readily available, relatively inexpensive and more sensitive than the tetrazolium assays. This assay has direct toxic effects on the cells as one of its limitations when compared to other methods. Some of the other cell viability assays are MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)), WTS (water-soluble tetrazolium salts), MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide).

2.6.2 Evaluation of The Glutathione (GSH) Content using Ortho-Phthalaldehyde Method

Ortho-phthalaladehyde (OPA) is a dialdehyde comprising of two formyl groups attached to adjacent carbon centres on a benzene ring (Figure 6). It is one of the vital probes that is used to measure the GSH concentration, specifically the oxidative stress-status of a cell (Bill and Tarbell, 1954). OPA is a fluorescent probe that binds explicitly to cellular GSH to form a highly fluorescent iso-indole GSH conjugate that can be measured at a wavelength of 340 nm (excitation) and 460 nm (emission) (Senft *et al*., 2000). Healthy or normal cells produce higher fluorescence as an indication of their high GSH content. Oxidative stressed cells, on the other hand, produce a lower fluorescence due to their relatively low GSH concentration. The limitation with OPA assay is that the GSH-OPA conjugate formed is unstable which affects the accuracy and precision of GSH measured. Additionally, this assay is non-specific for thiol groups. Some of the other assays used for determining GSH content in cells or tissues include DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid) and DNFB (1-Fluoro-2, 4-dinitrobenzene).

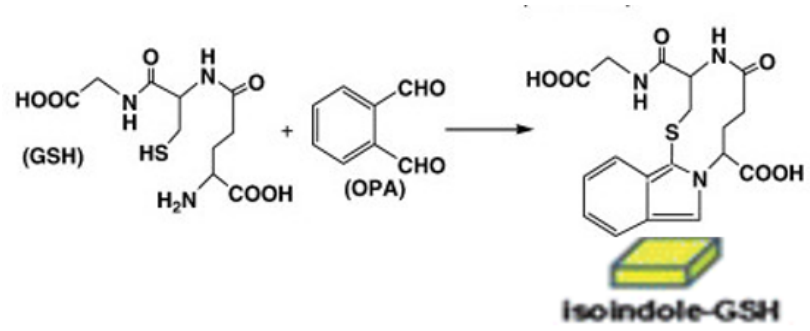

Figure 2. 7: O-phthalaldehyde (OPA) Reaction (Zhang *et al*., 2014).

2.6.3 Measurement of Reactive Oxygen Species (ROS) using Dichlorofluorescein Diacetate (DCFDA) Assay

The dichlorofluorescein diacetate (DCFDA) is a non- fluorescent probe that is taken up by the cells and acted upon by non-specific cellular esterases enzyme by hydrolysis, converting it to

2’7’ dichlorohydrofluoresence (H_2DCF), a non- fluorescent compound remaining in the cells (Kang *et al*., 2014). H_2DCF is oxidised by ROS and converted to highly fluorescent 2’7’-dichlorofluorescein. The fluorescence measured is proportional to the amount of ROS present in the cell (Kang *et al*., 2004). The limitations of this assay as compared to other assays include the indicator DCF which reacts with other oxidising intermediates leading to artificial fluorescence in cells which interferes with the ROS being measured. Also, this assay does not react directly with H_2O_2 to form a fluorescent product. Examples of other assays used to measure ROS content in cells include Amplex Red, HyPer Probe, Chemiluminescence, Electron Spin Resonance and Nitroblue Tetrazolium Salt.

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 CHEMICALS AND REAGENTS

Normal human prostate cell line; PNT-2, human prostate cancer cell line; PC3, and human breast carcinoma cell line; MCF-7 (estrogen receptor-positive) were obtained from RIKEN BioResource Center Cell Bank (Japan). Rose Park Memorial Institute (RPMI)-1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Phosphate Buffered Saline (PBS), Trypsin, O-phthalaldialdehyde (OPA), Ethylenediaminetetraacetic acid (EDTA), Dimethyl Sulfoxide (DMSO), Sodium Hydroxide (NaOH), Dichlorofluorescein-Diacetate (DCF-DA), Distilled water, Resazurin, and penicillin-streptomycin antibiotic was obtained from Clinical Pathology Department, Noguchi Memorial Institute for Medical Research (NMIMR), College of Health Sciences, University of Ghana. Riboceine, N-acetylcysteine (NAC) and the anticancer agents (methotrexate and docetaxel) were purchased from sigma. All reagents and chemicals were of molecular and analytical grade and obtained from standard suppliers.

3.2 ETHICAL CONSIDERATIONS

The study was approved by the Scientific Technical Committee (STC) of the Noguchi Memorial Institute for Medical Research (NMIMR), College of Health Sciences, University of Ghana.

3.3 CELL CULTURE

The cells, PNT-2 and PC3, were cultured in RPMI 1640 medium, and MCF-7 was cultured in DMEM medium. All media contained 10mM HEPES, 2mM L- glutamine and sodium bicarbonate ($NaHCO_3$) and the media were supplemented with 10% Fetal Bovine Serum (FBS)

and 1% Penicillin-Streptomycin. Cultured cells were maintained in a humidified incubator at 37 °C in the presence of 5% CO_2. The cells were sub-cultured every week. The cells were used at a confluent of about 80-90% when the cells were well grown (i.e. log phase) without dead cells which could interfere with the assays.

3.4 DRUG PREPARATION

An amount of 0.808 mg of DOC and 4.544 mg of MET were weighed and dissolved in 1 ml of DMSO each to obtain a stock concentration of 1 mM and 10 mM respectively. Additionally, 16.32 mg of NAC was weighed and dissolved in DMSO to obtain a stock concentration of 100 mM. An amount of 41.8 mg of RIB was also weighed and dissolved in 1 ml of DMSO to obtain a stock concentration of 100 mg/ml. The stock solutions were stored in -20^{o}C until use.

3.5 CELL VIABILITY

Cytotoxic effects of MET and DOC on the cell lines were determined using the Resazurin assay as described by Tsakalozou *et al*., (2012) with slight modifications.

3.5.1 Principle

This assay is based on the ability of viable, metabolically active cells to intracellularly reduce resazurin to resorufin and dihydro-resorufin (Tsakalozou *et al*., 2012). The oxidised, non-fluorescent blue state of the resazurin enters the cytosol. It is converted to the reduced, fluorescent pink state (resorufin) by accepting electrons from NADPH, FADH, FMNH, NADH as well as various cytochromes via mitochondrial enzyme activity (Tsakalozou *et al*., 2012). The fluorescence is measured at a wavelength of 560 nm excitation and 590 nm emission. The amount of resorufin produced is proportional to the number of viable cells. (Tsakalozou *et al*., 2012).

3.5.2 Procedure

The cells were seeded at a concentration of $1x10^5$ cells/well in a 96-well plate and incubated overnight at 37°C. The cells were treated with varying concentrations of the drugs (MET and DOC) from the highest to the lowest concentration (0.625µM - 10µM) and also treated with a combination of the anticancer agents and RIB or NAC (i.e. MET+RIB, MET+NAC, DOC+RIB and DOC+NAC). Treated cells were incubated for 24 h after which 10 µl of Resazurin solution (0.15 mg/ml in PBS) was added to each well, and incubation was continued for 4 h. The optical densities of the wells were measured at a wavelength of 560 nm excitation and 590 nm emission with a microplate reader (Tecan Infinite M200, Austria). The experiments were performed in triplicate.

3.6 GLUTATHIONE ASSAY

Glutathione level was measured using the OPA (Ortho-phthalaldehyde) assay as described by Martín *et al*., (2008).

3.6.1 Principle

OPA is a fluorescent probe that binds explicitly to cellular GSH to form a highly fluorescent iso-indole GSH conjugate that can be measured at a wavelength of 340 nm (excitation) and 460 nm (emission) (Senft *et al*., 2000).

3.6.2 Procedure

Cultured cells were seeded at a concentration of $2x10^5$ cells per well in a 24-well plate and incubated at 37°C overnight. The cells were then treated with MET, DOC, RIB, NAC (positive control) and H_2O_2 (negative control) at different concentrations and also with a combination of MET or DOC or H_2O_2 with RIB or NAC (i.e. MET+RIB, MET+NAC, DOC+RIB, DOC+NAC, H_2O_2+RIB and H_2O_2+NAC). The cells were, then, and incubated for another 24 h and were subsequently detached and homogenised by ultra-sonication with 5% trichloroacetic acid (containing 2mM EDTA) for 15 min. The cells were then again centrifuged

for 30 min at 3000 rpm (Hettich zentrifugen MIKRO 200, Germany). A volume of 50 µl of the supernatant was aliquoted into a 96 well plate, and a volume of 10µl of 1 M NaOH was added to neutralise the sample. A volume of 50 µl of sodium phosphate buffer was also added after which 10 µl of OPA (10 mg/ml) was added. The plate was incubated in the dark for 15 mins at room temperature (26^{o}C). The GSH level in the supernatant was obtained by measuring fluorescence at the wavelength of 460 nm (emission) and 340 nm (excitation).

3.7 MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS)

Intracellular ROS level was measured using dichlorofluorescein (DCF) assay as described by (Zawacka-Pankau *et al*., 2012).

3.7.1 Principle

The dichlorofluorescein diacetate (DCFDA) is taken up by the cells and acted upon by non-specific cellular esterases enzyme by hydrolysis converting it to 2'7' dichlorohydrofluoresence (H_2DCF). H_2DCF is oxidised by ROS which is converted to highly fluorescent 2'7'-dichlorofluorescein. (Kang *et al*., 2004).

3.7.2 Procedure

Cultured cells were seeded into a 24-well plate at a concentration of 2x10^5 cells per well and incubated overnight at 37 ^{o}C. The spent media was discarded and replaced with serum-free media and treated with varying concentrations of the drugs (MET, DOC, RIB, NAC, MET+NAC, MET+RIB, DOC+NAC and DOC+RIB). The plates were then incubated for 24 h after which 10µl of 5 µM of DCFDA was added and incubated for 120min. Fluorescence was read at 485nm (excitation) and 530nm (emission).

3.8 STATISTICAL ANALYSIS OF RESULTS

Results were presented as mean ± standard deviation (SD) from three independent tests. Per cent inhibition was calculated from the ratio of the activity of treated to control samples, and IC_{50} values were analysed using Graph Pad Prism. Student t-test and one-way ANOVA were used to test differences between single and multiple group data using graph pad prism. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 *IN-VITRO* CYTOTOXIC ACTIVITIES OF METHOTREXATE, DOCETAXEL, RIBOCEINE AND N-ACETYLCYSTEINE AND THEIR COMBINATIONS.

Figure 4.1 to 4.3 shows the cytoprotective effects of RIB and NAC against the cytotoxic activity of MET and DOC on PNT-2 normal cells and PC3 and MCF-7 cancer cell lines, respectively. A decrease in the percentage of cell viability reflects the cytotoxic effects of the anticancer agents. MET and DOC caused a significant decrease ($p<0.0001$) in the percentage cell viability of PNT-2 by 57% and 60%, respectively (Fig 4.1). Up to 43% and 40% of PNT-2 cells survived in the presence of MET and DOC, respectively. The cytotoxic activities of MET and DOC on PNT-2, were reduced in the presence of riboceine and N-acetylcysteine. The cell viability of PNT-2 significantly increased by 8% and 7%, when MET was combined with NAC ($p=0.0014$) and RIB ($p=0.0016$), respectively, in comparison with the treatment with MET alone. Also, the cell viability significantly increased by 16% and 13% when DOC was combined with NAC ($p<0.0001$) and RIB ($p<0.0001$), respectively, in comparison with the treatment with DOC only.

Compared to the observations done on PNT-2 cells, MET and DOC showed much more pronounced cytotoxic activities on PC3 cells (Fig. 4.2), as shown by the significant decrease ($p<0.0001$) in PC3 cell viability by 90% and 98%, respectively. Only 10% and 2% of PC3 cells survived in the presence of MET and DOC, respectively. The cytotoxic activities of MET and DOC on PC3 cells did not vary significantly in the presence of NAC ($p>0.05$) or RIB ($p>0.05$). MET and DOC decreased the cell viability of MCF-7 (Fig. 4.3) by 34% and 24%, respectively, i.e. 66% and 76% of the cells survived in the presence of MET and DOC. This decrease in MCF-7 cell viability caused by MET and DOC was not as drastic as what was observed on

PC3 cells. The combination of MET or DOC with NAC and RIB reduced their cytotoxic activities on MCF-7 cells, as shown by a significant increase in MCF-7 viability by 10% and 13%, when the cells were treated with MET combined with NAC ($p=0.0171$) or RIB ($p=0.0018$), respectively, and when compared to treatment with only MET. Also, the treatment of MCF-7 cells with DOC combined with NAC ($p=0.0003$) or RIB ($p=0.0002$) significantly increased the cell viability by 11% and 9%, respectively, when compared to the treatment with only DOC.

The IC_{50} values and selective index of MET and DOC in PNT-2, PC3 and MCF-7 cells are shown in Table 4.1. The concentration of MET and DOC needed to inhibit 50% of cell growth (IC_{50}) of PNT-2 cell were 0.552µM and 0.524 µM, respectively, and that of PC3 were 0.338µM and 0.320 µM respectively. The increasing concentration of MET and DOC could not cause a 50% decrease in cell viability of MCF-7; hence no IC_{50} could be determined. The selectivity indexes of MET and DOC were 1.633 and 1.638, respectively, on PC3 and 0.055 and 0.052, respectively on MCF-7.

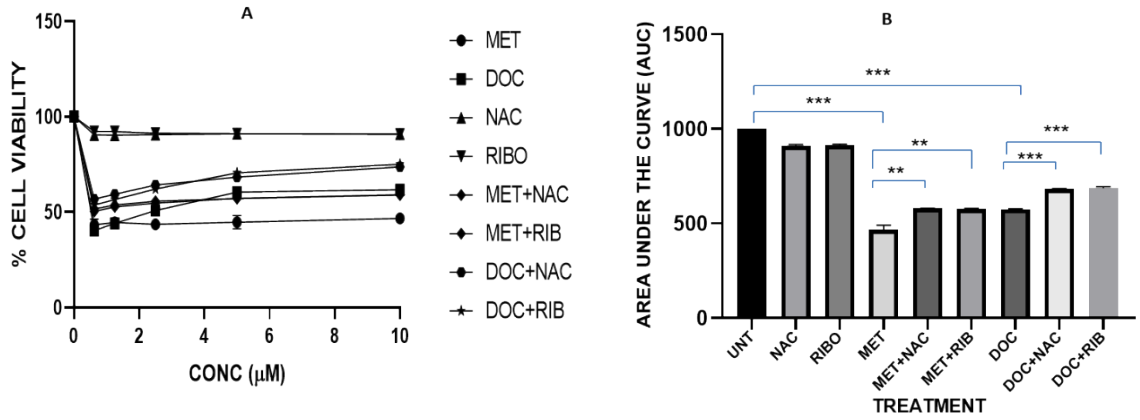

Figure 4. 1 (A) Cytoprotective effects of RIB and NAC against the cytotoxic activity of MET and DOC on PNT-2 cells; (B) Area under the curve for the line graph.

Data are means ± SEM (n=3). Statistical significance by student's t-test; P values ≤ 0.05, ≤ 0.02 and ≤ 0.001 and are denoted by the symbol *, ** and *** respectively
ROS- Reactive Oxygen Species **MET**- Methotrexate **DOC**- Docetaxel **RIB**- Riboceine, **NAC**- N-acetylcysteine

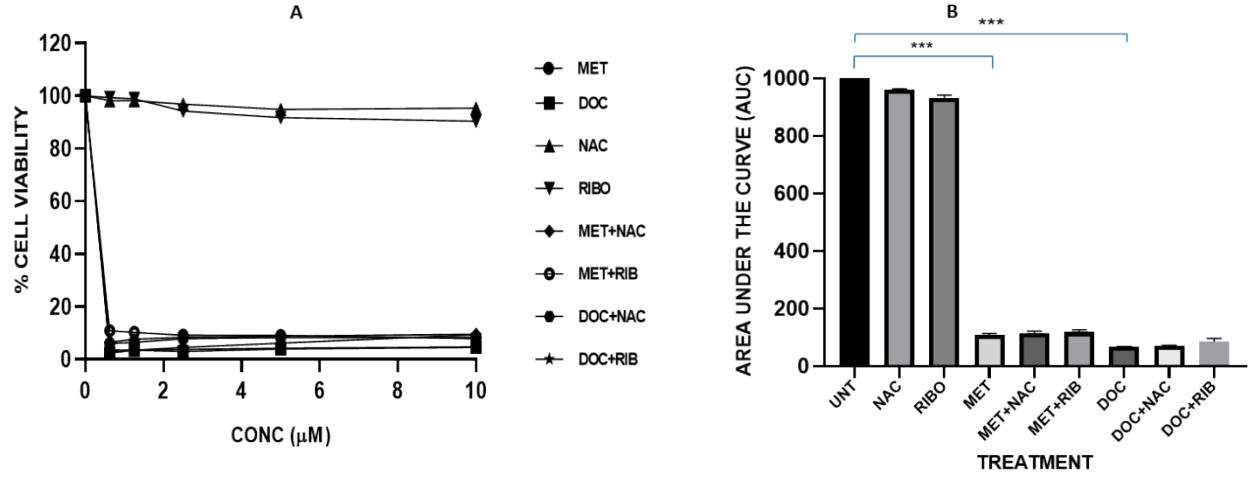

Figure 4. 2 (A) Cytoprotective effects of RIB and NAC against the cytotoxic activity of MET and DOC on PNT-2 cells; (B) Area under the curve for the line graph.

Data are means ± SEM (n=3). Statistical significance by student's t-test; P values ≤ 0.05, ≤ 0.02 and ≤ 0.001 and are denoted by the symbol *, ** and *** respectively.
ROS- Reactive Oxygen Species. MET- Methotrexate, DOC- Docetaxel, RIB- Riboceine, **NAC**- N-acetylcysteine.

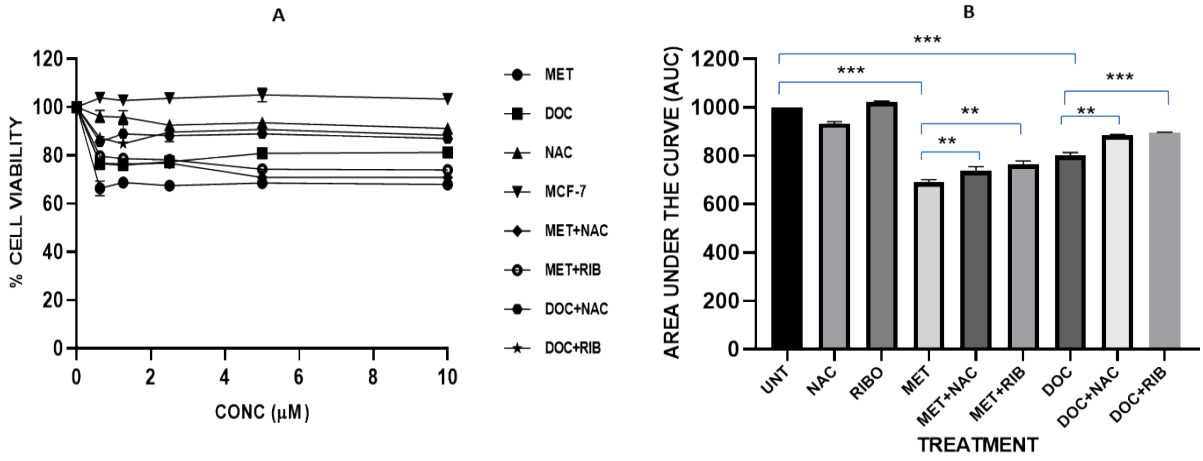

Figure 4. 3: (A) Cytoprotective effects of RIB and NAC against the cytotoxic activity of MET and DOC on MCF-7 cells (B) Area under the curve for the line graph

Data are means ± SEM (n=3). Statistical significance by student's t-test; P values ≤ 0.05, ≤ 0.02 and ≤ 0.001 and are denoted by the symbol *, ** and *** respectively.
ROS- Reactive Oxygen Species. MET- Methotrexate, DOC- Docetaxel, RIB- Riboceine. **NAC**- N-acetylcysteine

Table 4. 1: IC_{50} and Selective Index (SI) Values of MET and DOC on PNT-2, PC3 and MCF-7 Cells

Drugs / Cells	IC_{50} Values (µM)		Selective Index (SI)	
	MET	**DOC**	**MET**	**DOC**
PNT-2	0.552±0.029	0.524±0.010	-	-
PC3	0.338±0.001	0.320±0.001	1.633	1.638
MCF-7	> 10	> 10	0.055	0.052

4.2 EFFECTS OF METHOTREXATE, DOCETAXEL, RIBOCEINE AND N-ACETYLCYSTEINE AND THEIR COMBINATIONS ON GSH CONTENT

Figure 4.4 shows the effect of methotrexate, docetaxel, N-acetylcysteine, riboceine, hydrogen peroxide (H_2O_2) and their combinations on the GSH content of PNT-2 cells. At the basal level, the cancer cells (PC3 and MCF-7) recorded higher GSH content when compared to the GSH content of PNT-2. The untreated cancer cell line PC3 recorded the highest GSH content compared to the untreated MCF-7 cell lines. NAC significantly increased ($p = 0.0004$) the GSH content of PNT-2 cells by 79.9 %, when compared to the untreated cells. In contrast, the increase in GSH content of PNT-2 cells caused by RIB was not significant ($p > 0.05$), RIB increased GSH content of PNT-2 cells by only 5.02%. The control agent, H_2O_2, significantly decreased ($p = 0.0001$) the GSH content of PNT-2 cells by 82.7%, when compared to the untreated cells. The treatment of PNT-2 cells with combinations of H_2O_2+NAC and H_2O_2+RIB also caused a significant increase ($p = 0.0258$) in GSH contents, when compared to cells treated with only H_2O_2. The anticancer agent, MET, significantly decreased ($p = 0.0005$) the GSH content of PNT-2 cells by 16.1%, when compared to the untreated cell line. At the same time, DOC significantly increased ($p=0.0260$) the GSH content by 12.9%, when compared to the untreated normal cells. Treatment of PNT-2 cells with combinations of MET+NAC and MET+RIB recorded a significant increase ($p = 0.0003$ and 0.0440, respectively) in the GSH content by 80.2% and 6% respectively.

Figure 4.5 shows the effect of methotrexate, docetaxel, N-acetylcysteine, riboceine, hydrogen peroxide (H_2O_2) and their combinations on GSH content of PC3 cells. The PC3 cells recorded a significant decrease in GSH content when treated with H_2O_2, MET and DOC as well as when treated with NAC and RIB compared to the untreated cells. A combination of MET or DOC with NAC did not affect GSH content of PC3 cells when compared to cells treated with MET or DOC only. A combination of RIB and H_2O_2 or MET or DOC significantly increased GSH

content of PC3 cells, when compared to cells treated with only H_2O_2 or MET or DOC ($p = 0.0005$; 0.0223 and 0.0099, respectively).

Figure 4.6 shows the effect of methotrexate, docetaxel, N-acetylcysteine, riboceine, hydrogen peroxide (H_2O_2) and their combinations on GSH content of MCF-7 cells. The H_2O_2 and the anticancer agents, MET and DOC caused a significant decrease in GSH content of MCF-7 cells when compared to the untreated cells ($p = 0.0005$; 0.0083 and 0.0006 respectively). The treatment of MCF-7 cells with only NAC or RIB caused a significant increase in the GSH content, when compared to the untreated cells ($p = 0.0090$ and 0.0136, respectively). The combination of H_2O_2 and NAC or RIB recorded a significant increase in the GSH content of MCF-7 cells when compared to H_2O_2 ($p<0.0001$). Similarly, a combination of MET and NAC or RIB significantly increased ($p<0.0001$) GSH content when compared MET only. Also, treatment with DOC and NAC or RIB significantly increased ($p<0.0001$) the GSH content of MCF-7 cell line.

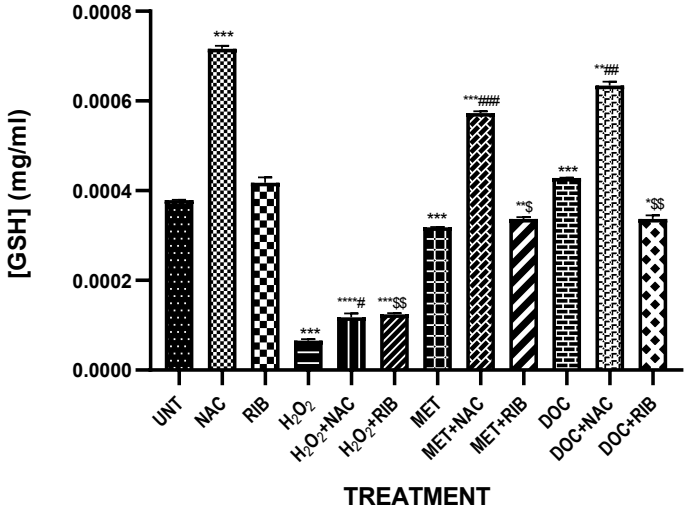

Figure 4. 4: Effects of N-acetylcysteine, Riboceine, Hydrogen peroxide, Methotrexate, Docetaxel and their combinations on GSH content of PNT-2 cells.

Data are means ± SEM (n=3). Statistical significance by student's t-test; P values ≤ 0.05, ≤ 0.02 and ≤ 0.001 and are denoted by the symbol *, ** and *** respectively when compared to the untreated (UNT) cells. The symbol # denotes comparison between MET vs MET+NAC or DOC vs DOC+NAC, $ denotes comparison between MET vs MET+RIB and DOC vs DOC+RIB.
ROS- Reactive Oxygen Species. **MET**- Methotrexate DOC- Docetaxel **RIB**- Riboceine **NAC**- N-acetylcysteine

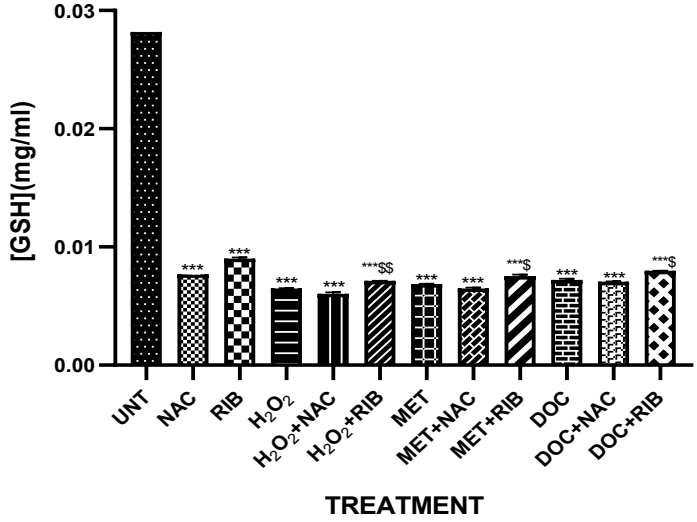

Figure 4. 5: Effects of N-acetylcysteine, Riboceine, Hydrogen peroxide, Methotrexate, Docetaxel, and their combinations on GSH) content of PC3 cells.

Data are means ± SEM (n=3). Statistical significance by student's t-test; P values ≤ 0.05, ≤ 0.02 and ≤ 0.001 and are denoted by the symbol *, ** and *** respectively when compared to the untreated (UNT) cells. The symbol # denotes comparison between MET vs MET+NAC or DOC vs DOC+NAC, $ denotes comparison between MET vs MET+RIB and DOC vs DOC+RIB.
ROS- Reactive Oxygen Species. MET- Methotrexate DOC- Docetaxel **RIB**- Riboceine **NAC**- N-acetylcysteine

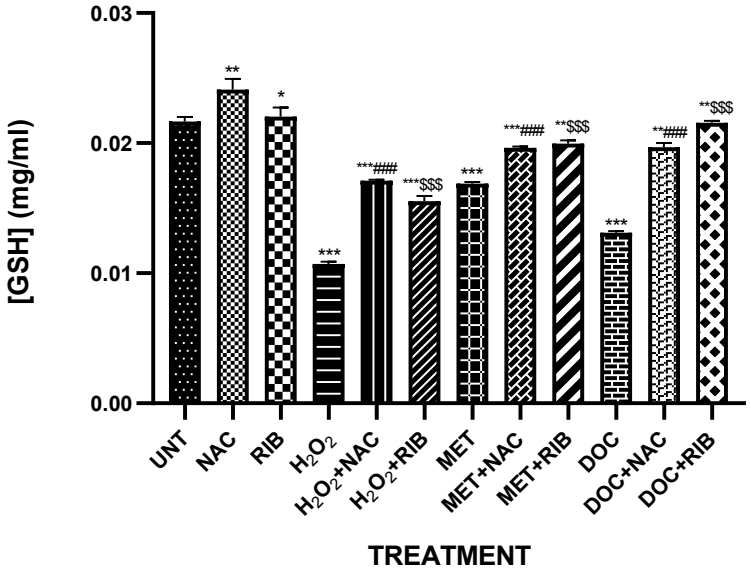

Figure 4. 6: Effects of N-acetylcysteine, Riboceine, Hydrogen peroxide, Methotrexate, Docetaxel, and their combinations on the GSH content of MCF-7 cells.

Data are means ± SEM (n=3). Statistical significance by student's t-test; P values ≤ 0.05, ≤ 0.02 and ≤ 0.001 and are denoted by the symbol *, ** and *** respectively when compared to the untreated (UNT) cells. The symbol # denotes comparison between MET vs MET+NAC or DOC vs DOC+NAC, $ denotes comparison between MET vs MET+RIB and DOC vs DOC+RIB.
ROS- Reactive Oxygen Species. MET- Methotrexate DOC- Docetaxel **RIB**- Riboceine **NAC**- N-acetylcysteine

4.3 EFFECTS OF METHOTREXATE, DOCETAXEL, RIBOCEINE AND N-ACETYLCYSTEINE AND THEIR COMBINATIONS ON ROS LEVEL

Table 4.2 shows the effects of methotrexate, docetaxel, N-acetylcysteine, riboceine and their combinations on the ROS level of PNT-2, PC3 and MCF-7 cells. The untreated cancer cell line MCF-7 recorded the highest ROS level compared to the untreated normal prostate (PNT-2) and prostate cancer (PC3) cell lines. The untreated PC3 cells, on the other hand, recorded the lowest ROS level compared to the untreated PNT-2. The ROS level of PNT-2 increased by 21.3% and 11.6% in the presence of MET and DOC, respectively; this increase was statistically significant with p-values of 0.039 and 0.0027 for MET and DOC, respectively, in comparison with the untreated cells. MET and DOC did not cause any significant increase ($p > 0.05$) in

ROS level of the PC3 cells when compared to the untreated cells; the increases were by 11.2 % and 4.2% for MET and DOC, respectively. On the other hand, in MCF-7 cells, MET and DOC caused a significant decrease in ROS level when compared to untreated cells ($p = 0.0005$ and 0.0056, respectively). The decrease was by 9.5% and 17.8% for MET and DOC, respectively.

NAC (positive control) caused significant decrease in ROS level in PNT-2 ($p = 0.0021$), PC3 ($p = 0.0234$) and MCF-7 ($p = 0.0134$) cell lines in comparison to the untreated cells. This decrease was by 34.6%, 13.1% and 34.6% for PNT-2, PC3 and MCF-7 cells, respectively. RIB also caused a significant decrease in ROS level of PNT-2 ($p = 0.0007$), PC3 ($p = 0.0202$) and MCF-7 ($p = 0.0005$) cell lines, when compared to the untreated cells. This decrease was by 28%, 15.1% and 28% for PNT-2, PC3 and MCF-7 cells, respectively.

The combination of MET+NAC and MET+riboceine, recorded a significant decrease in ROS level of PNT-2 cells ($p = 0.0203$ and 0.0436, respectively) when compared to the treatment with only MET. The ROS level PNT-2 decreased by 33.3% and 35.4% for MET+NAC and MET+riboceine, respectively. Also, a combination of DOC+NAC and DOC+ riboceine caused a significant decrease in ROS level of PNT-2 cells ($p = 0.0138$ and 0.0003, respectively) when compared to the treatment with only DOC. The ROS level of DOC+NAC and DOC+ riboceine decreased by 35.4% and 37.8% respectively. MCF-7 cells also showed a significant decrease in ROS level when treated with a combination of MET+NAC and MET+ RIB ($p = 0.0074$ and 0.0165, respectively), in comparison with the treatment with only MET. The ROS level decreased by 11.6% and 1.5% for MET+NAC and MET+ RIB, respectively. The combinations DOC+NAC and DOC+riboceine recorded a significant decrease in ROS level of MCF-7 cells ($p = 0.0306$ and 0.1003, respectively), in comparison with the treatment with only DOC. The ROS level decreased by 11.0% and 6.7% for DOC+NAC and DOC+riboceine, respectively.

The combination of MET or DOC with NAC and RIB did not cause any significant decrease in the ROS level of the PC3 cell line.

Table 4. 2: Effects of Methotrexate (MET), Docetaxel (DOC), N-Acetylcysteine (NAC), Riboceine (RIB) and their combination on ROS Level of PNT-2, PC3 and MCF-7 Cells.

	ROS Level (MIF)		
Treatment	**PNT- 2**	**PC3**	**MCF-7**
UNT	6795.5±30.41	5645 ± 137.18	16419.5±44.55
MET	8639 ± 530.33*	6360 ± 241.83	14858 ± 19.80***
DOC	7584 ± 49.50**	5914.5± 28.99	13500 ± 308.30**
NAC	4445.5±147.79**	4908 ± 106.77*	10736 ± 104.65***
RIB	4892.5±65.76***	4793.5± 104.65*	11818 ± 132.94***
MET+NAC	5760.5±263.75$^{*\#\dagger}$	5960 ± 209.30†	13138±209.30$^{**\#\#\dagger\dagger}$
MET+RIB	5582.5±768.63$^{\$}$	6007.5± 225.57‡	14634.5±36.06$^{***\$\$\ddagger\ddagger}$
DOC+NAC	5499 ± 346.48$^{*\#\#}$	5790 ± 195.16†	12013.5±215.67$^{**\#\dagger}$
DOC+RIB	4721±45.25$^{***\$\$\$}$	5966.5± 243.95‡	12595 ± 312.54**

Data are means ± SEM (n=3). Statistical significance by student's t-test; P values ≤ 0.05, ≤ 0.02 and ≤ 0.001 and are denoted by the symbol *, ** and *** respectively when compared to the untreated (UNT) cells. The symbol $^{\#}$ denotes comparison between MET vs MET+NAC or DOC vs DOC+NAC, $^{\$}$ denotes comparison between MET vs MET+RIB and DOC vs DOC+RIB, † denotes NAC vs MET+NAC and NAC vs DOC+NAC and ‡ denotes RIB vs MET+RIB and RIB vs DOC+RIB.
ROS- Reactive Oxygen Species. **MET**- Methotrexate DOC- Docetaxel, RIB- Riboceine, NAC- N-acetylcysteine

CHAPTER FIVE

5.0 DISCUSSION

Cancer chemotherapy is associated with several challenges such as drug resistance and severe side effects. Most chemotherapeutic agents are not cancer-cell-specific and target both cancer cells and rapidly dividing normal cells, resulting in severe side effects. Also, it is now well established that the chemotherapy-induced cellular damages are associated with the increased chemotherapy-induced generation of ROS (H. Yang et al., 2018). In the current study, we investigated the cytoprotective effects of D-ribose-L-cysteine (riboceine), a synthetic precursor of GSH synthesis, on the viability, ROS level and GSH content of normal and cancer cell lines, in the presence of MET and DOC. The study assessed the potential of RIB to prevent or reduce the cytotoxic effects of the anticancer agents; MET and DOC on normal cells. Also, the study determined whether the efficacy of these cytotoxic agents on the cancer cells would be impaired by their combination with RIB or NAC.

The cytotoxic effects of MET and DOC, as well as their combination with NAC and RIB, on the viability of the normal cell (PNT-2) and cancer cells (PC3 and MCF-7) were first determined using Resazurin assay. This assay is based on the ability of viable cells to reduce the non- fluorescent blue resazurin to fluorescent pink resofurin, which occurs intracellularly. The amount of resofurin produced is proportional to the number of viable cells. Our results confirmed the cytotoxic activities of MET and DOC on normal prostate cells, PNT-2, showing the non-cancer-cell specificity of these anticancer agents which could account for their severe side effects (Omlin *et al*., 2015; Pieniązek *et al*., 2013; van Outryve *et al*., 2002). The present study revealed for the first time that RIB possesses the ability to reduced the cytotoxic activities of MET and DOC on the normal prostate PNT-2 cells. This effect of RIB was similar to the effect of the standard agent, NAC, and showed their cytoprotective effects against the cytotoxic activities of MET and DOC on the normal prostate PNT-2 cells. In a related study,

Troyano *et al*., (2001) showed that NAC had a cytoprotective effect against the cytotoxic activity of cisplatin on PNT-2 cells.

Our results also showed that the cytotoxic activities of MET and DOC were much more pronounced on the prostate cancer cells, PC3, than the normal prostate PNT-2 cells, confirming their anticancer properties. The cytotoxic activity of MET on PC3 cells could be due to the specific transport mechanisms such as the reduced folate carrier (RFC), proton-coupled folate transporter (PCFT) or the folate receptors (FR), overexpressed in the PC3, but not PNT-2, cell membrane. This would subsequently result in an increased uptake of MET, hence causing the death of the cancer cells (Desmoulin *et al*., 2012). The cytotoxic effects of DOC on PC3 cells could be as a result of the phosphorylation of the antiapoptotic protein (Bcl2) that prevents its dimerisation with Bax (a pro-apoptotic protein) and induces apoptosis (Tsakalozou *et al*., 2012). In the present study, RIB showed no cytoprotective effect against the cytotoxic activities of MET or DOC on the prostate cancer cells, PC3. A similar result was obtained with NAC. As expected, the cytotoxic activities of the anticancer agents (MET and DOC) on the prostate cancer cells PC3, were not impaired by the combination with the antioxidant supplements (RIB and NAC).

Also, our study revealed that, unlike the PC3 cells, the breast cancer cells, MCF-7, displayed a resistant pattern to the cytotoxic activities of MET and DOC, which resulted in no cytotoxic activity on MCF- 7 cells. A study done by Jang *et al*., 2019 showed that MET decreased cell viability by only 30% which was similar to our finding that showed that MET reduced the cell viability of MCF-7 by 40%. The efflux of MET is done by transport proteins, breast cancer resistance protein (BCRP) and multidrug resistance proteins MRP1 and MRP5. The overexpression of these efflux proteins in cancer cells results in the insensitivity of cancer cells to MET which prevents the drug from reaching its target (Stapf *et al*., 2016). Additionally, downregulation of the reduced folate carrier, folate receptor and proton-coupled receptor by

MCF-7 leading to low uptake of MET may have accounted for the decreased cytotoxic activity of MET (Desmoulin *et al*., 2012; Ifergan *et al*., 2008). Studies have also shown that resistance to microtubule targeting agents such as DOC in breast, prostate, ovarian and lung cancers is associated to the abnormal expression of specific β- tubulin isoforms (i.e. β2, β3 and β4-tubulin) and microtubule-associated protein (MAP) (Tsakalozou *et al.,* 2012*)*. The resistance of MCF-7 cells to DOC could also be as a result of the overexpression of p-glycoprotein, a drug efflux protein called ATP binding cassette (ABC) transporter. The ABCB1 type of this transporter is the more associated with taxane resistance (Tsakalozou *et al*., 2012; Wiltshire *et al*., 2010). Our results showed that both NAC and RIB impaired the cytotoxic activities of MET and DOC on MCF-7 cells, revealing cytoprotective effects of these antioxidants on the breast cancer cells when given in combination with MET and DOC. This is similar to a study by Sahin et al., 2012 where grape oil seed (an antioxidant) decreased the cytotoxic effects of methotrexate in K-562 leukeamia cells.

The effects of MET, DOC, RIB and NAC on the GSH content of cancer cells (PC3 and MCF-7) and normal cell (PNT-2) were also determined in the present study. The results showed that MET significant decreased the GSH content of the normal prostate cells. MET is indirectly involved in liver mitochondria damage, causing inhibitory effects on G6PD and reducing NADPH availability, which leads to ROS generation (Moghadam *et al*., 2015). This could be a possible factor that contributed to reinforce the cytotoxic activity of MET on PNT-2 cells. In the opposite, it was observed from the current study that, the treatment of the normal prostate cells with DOC showed a significant increase in the GSH content of the cells. This could be due to the fact that an accumulation of DOC-induced ROS could result in a compensatory overproduction of GSH to mop up the excess ROS level.

The study also showed that normal cells treated with the NAC presented an increased content of GSH. This increase could be attributed to the efficient delivery of cysteine by NAC, for the

synthesis of GSH in PNT-2 cells, which increased the GSH content. However, RIB did not significantly increase the GSH content, and this could be attributed to a low uptake by the cell and/or a slow release of cysteine by the RIB complex. Treatment of cells with a combination of MET or DOC and RIB or NAC showed an increase in GSH content and confirmed other findings in the literature demonstrating that the first line of defence against the damaging effects of oxidative stress are the antioxidant systems that neutralise the lethal effects of ROS (Moghadam *et al*., 2015).

In this study, untreated cancers cells exhibited a high GSH content in comparison to the untreated normal cells. This result is consistent with the fact that an increased content of GSH in cancer cells, reduces the oxidative stress leading to apoptosis resistance, which results in cancer cell survival (Traverso *et al*., 2013). In the other hand, the depletion of GSH content increases cellular predisposition to apoptosis or cell programmed cell death (Traverso *et al*., 2013). Also, a decreased GSH content of cancer cells results in an increased sensitivity to chemotherapy and irradiation (Ortega *et al*., 2011). In our study, cancer cells treated with N-acetylcysteine increased the GSH content of PNT-2 and MCF-7. In the opposite, PC3 cells recorded a decrease in their GSH content. Treatment of PC3 cells with MET and DOC caused a significant reduction in GSH, and this situation could have possibly contributed to the pronounced cytotoxic activities of MET and DOC on PC3 cells. It was reported that MET has the potential to decrease various antioxidant contents such as SOD, CAT and GSH, in cancer cells (Yun *et al*., 2019). NAC and RIB, when combined with MET and DOC, had no effects on the glutathione content of PC3, and demonstrates that the antioxidants did not interfere with the cytotoxic activities of MET and DOC on PC3 cells.

On the MCF-7 cell line, the treatment with MET or DOC caused a significant decrease in the GSH content. As expected, RIB and NAC induced an increase in GSH content, and this could be due to the fact that there was more uptake of cysteine by MCF-7 cells that caused an increase

in GSH level. The MCF-7 cells treated with a combination of MET or DOC and RIB or NAC exhibited a significant increase in their GSH content, showing again that RIB, as well as NAC, impaired the cytotoxic activities of MET or DOC on MCF-7 cells.

The effects of MET, DOC, NAC and RIB on the cellular ROS level was determined using a dichlorofluorescein (DCF) assay. The results showed that in the normal prostate cells (PNT-2), MET did not significantly increase the level of ROS and could not be the leading cause of the cytotoxic effects of MET on normal cells. DOC, however, significantly increased the ROS level in the normal prostate PNT-2 cells. Our result is consistent with the findings reported by Mikuła-Pietrasik *et al*., and showing that taxane (paclitaxel and docetaxel) and platinum (cisplatin and carboplatin) based chemotherapies increase the production of ROS in normal epithelial and fibroblast cells (J Mikuła-Pietrasik *et al*., 2019). Our results showed that both NAC and RIB significantly decreased the ROS level, as expected. This could be explained by the fact that these two synthetic antioxidants are known to increase the level of GSH, which mop up more reactive oxygen species. The current study also showed that a combination of MET or DOC with NAC and RIB significantly decreased the ROS level in the normal prostate PNT-2 cells. The decrease in the ROS level could be due to the increased synthesis of GSH, therefore mopping up the extra ROS generated by the anticancer agents. Hence, RIB and NAC protected the normal prostate PNT-2 cells against the cytotoxic activities of MET and DOC. Again, from the current study, it was observed that the untreated cancer cells (PC3 and MCF-7) had a higher ROS level than the untreated normal cells (PNT-2). This finding confirmed the fact that cancer cells are under increased oxidative stress due to the increased generation of ROS caused by mitochondrion malfunction, increased metabolic activity and oncogenic transformation (Pelicano *et al*., 2004). This elevated amount of ROS has been suggested to help the cancer cells to maintain a high proliferation rate (Sosa *et al*., 2013). The ROS level measured in MCF-7 cells was higher than the ROS level determined on PC3 cells. In the current

study, it was shown that the treatment of the cancer cell lines with the anticancer drugs (MET and DOC) caused no significant increase in the ROS level. The increased ROS level of the untreated MCF-7 cells could lead to the alterations of the response of the cancer cells to chemotherapeutic agents. Wang *et al*., (2017) showed that MCF-7 cells are insensitive to taxanes especially DOC, due to an overexpression of drug efflux proteins (ATP-binding cassette transporters; ABCB1, ABCC1 and ABCG2). The efflux protein, ABCB1, is particularly implicated in taxane resistance. The MCF-7 cells are also characterized by an increased expression of β2, β3 and β4 tubulins and an altered microtubule-associated protein (MAP) which could also account for the resistance to DOC. Resistance of MCF-7 to MET could be a result of the overexpression of efflux protein, specifically, breast cancer protein (BCRP) and multidrug resistance protein (MRP1 and MRP5) (Stapf *et al*., 2016).

The results from the present study revealed that DOC and MET did not cause a significant increase in ROS level in PC3 cells. This could be explained by the fact that the excess production of ROS may not be the main factor leading to the cytotoxic activity of the anticancer agents on PC3 cells. Also, the assay used (DCF-DA assay) does not measure all the reactive oxygen species (e.g., O^{2-} and OH^{-}), hence the total amount of ROS generated by MET and DOC was probably not measured. Again, the significant decrease in the ROS level caused by NAC and RIB in the present study could be explained by the increased *de-novo* production of glutathione in the cancer cells, which mops up the ROS accumulated by the cancer cells. Our study demonstrated that neither RIB nor NAC impaired the cytotoxic activity of the MET and DOC on PC3 cells. However, RIB or NAC, in combination with MET or DOC, caused a decrease in ROS level of MCF-7 cells, which suggest a protective effect of the antioxidants on MCF-7. This result is similar to findings reported by Lyle *et al*., 2011, who showed that NAC impaired the cytotoxic effects of paclitaxel by decreasing the level of ROS in MCF-7 cells.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

The present study showed that riboceine exhibited a chemoprotective effect against the cytotoxic activities of methotrexate and docetaxel on normal prostate cells (PNT-2). This riboceine-induced chemoprotection was associated with increases in cell viability and glutathione level, and to a decrease in the ROS content of the PNT-2 cells. The effects of riboceine were similar to those obtained with the standard antioxidant (N-acetylcysteine), which, however, demonstrated a higher chemoprotection. Also, the combination of the anticancer agents (methotrexate or docetaxel) with the glutathione-enhancer dietary supplements (riboceine or N-acetylcysteine) did not impair the cytotoxic activities of these anticancer drugs on the prostate cancer cells (PC3). However, chemoprotective effects of riboceine and N-acetylcysteine were observed on breast cancer cells (MCF-7) for both methotrexate and docetaxel.

6.2 RECOMMENDATIONS

Further studies should be conducted on the effect of riboceine on other antioxidant defence systems such as catalase, peroxidase, superoxide dismutase, etc., to confirm these findings.

In vivo studies should be done to confirm the effects of riboceine on GSH and the other antioxidant defence systems. The chemoprotective effects of riboceine and N-acetylcysteine on other cancer cell lines should be performed. Finally, a comprehensive molecular study (gene and protein expressions) is highly recommended.

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APPENDICES

APPENDIX 1

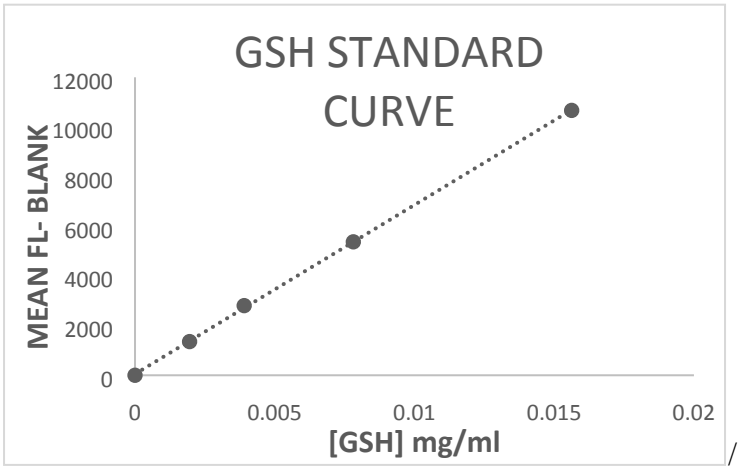

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Figure A1: Reduced Glutathione (GSH) standard curve

APPENDIX 2

PREPARATIONS OF SOLVENT

1M NaOH (sodium hydroxide):

C= n/V

n= m/M

c= m/M×V

m= c×M×V

m= 1×40×0.1

m=4g

4g of NaOH in 0.1L of distilled water

C; concentration (1M)

m; mass

M; molecular weight (NaOH-40g/mol)

V; volume (0.1L)

2mM EDTA (50ml):

m= c×M

m= 2×10-3×372.24

= 0.74448g/dm-3

1000ml= 0.74448g

50ml = (50×0.74448)/1000

= 0.037g/50ml or 37mg/50ml

c; concentration (2mM)

M; molecular weight (372.24 g/mol)

m; mass

5µM of DCF-DA:

m=C×M×V

= $5\times10^{-6}\times487.29\times2.5\times10^{-3}$

=$5\times487.29\times10^{-9}$

= 6091.125×10^{-9}g

= 0.006091125mg

0.0060911mg of DCF-DA in 2.5ml of DMSO