Assessment of cytotoxic artemisinin and its derivatives as DNA damaging inducing agents in triple-negative breast cancer cells

A thesis submitted in fulfilment of the requirements for the degree of

Master in Biochemistry

of

Rhodes University

By

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March 2022

ABSTRACT

In developing countries, including South Africa, breast cancer is the primary cause of cancer-related deaths among women. TNBC (triple-negative breast cancer) is an aggressive breast cancer subtype that is more prevalent in women of African descent. This subtype lacks the key receptors, namely the estrogen receptor (ER-), progesterone receptor (PR-), and human epidermal growth factor receptor 2 (HER2-) that are the basis of successful targeted therapies for other subtypes of the disease. To date, there are no effective, standardized targeted therapies for TNBC. Artemisinin is an anti-malarial drug and numerous derivatives of the compound have been developed to improve the potency and solubility of the parent compound. Artemisinin and its derivatives have gained attention as potential anti-cancer agents; however, such studies have not yet progressed to clinical trials and the precise mechanism of action of these compounds is yet to be fully explained. In this study, artemisinin, and its known derivative artesunate, as well as a novel derivative, WHN11, were investigated as DNA damage-inducing agents in TNBC. WHN11 was found to be the most potent of the three compounds, displaying an IC_{50} of 3.20 μM against HCC70 cells, artemisinin displayed an IC_{50} of 214.70 μM and artesunate displayed an IC_{50} of 25.48 μM. The compounds were less toxic to the MCF12A non-cancerous cells, with IC_{50} values 298.30, 87.53, and 8.35 μM for artemisinin, artesunate, and WHN11, respectively, and displayed selectivity indices of 1.39, 3.44 and 2.61 μM for artemisinin, artesunate, and WHN11, respectively. *In silico* and *in vitro* studies revealed that the artemisinin compounds bind to DNA through the minor groove. While all three compounds were able to bind to DNA, a comet assay revealed that only artemisinin and artesunate, and not WHN11, were able to cause DNA damage compared to the vehicle control, DMSO. Finally, a topoisomerase I (TOPO I) enzyme assay demonstrated that while the compounds appeared to display a degree of inhibition of TOPO I, as evidenced by a downward shift in the plasmid band on the agarose gel, they were not able to fully inhibit the enzyme to return the plasmid to the supercoiled conformation. In addition, combination studies revealed that artemisinin, artesunate, and WHN11 acted synergistically in combination with camptothecin, but displayed either an additive (artemisinin) or antagonistic (artesunate and WHN11) relationship when used in combination with etoposide. In conclusion, artemisinin, its known derivative artesunate, and novel and highly toxic derivative WHN11, all bind to DNA via the minor groove, however only artemisinin and artesunate, and not WHN11, cause DNA damage, indicating a potentially different mechanism of action of the three artemisinins. All three compounds act synergistically with camptothecin, which suggests interference with

topoisomerase activity, partially supported by slight inhibition of TOPO I activity, and could indicate either direct inhibition of the enzyme or interference with enzyme function by competitive binding to the DNA. Further studies could help explore alternate DNA damage assays, to validate these findings, and the effect of the compounds on TOPO II activity could also be assessed.

DECLARATION

I announce that this thesis is my work, and it is being submitted for the degree of Master of Science at Rhodes University. It is to the best of my knowledge that it has never been submitted for examination in any institution.

Ntando Mkhwanazi

March 2022

Grahamstown

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ACKNOWLEDGEMENTS

Firstly, I would like to thank Jehovah God for the direction, the struggles you helped me overcome, the communication we had and still have through prayer, and everything you have done for me. Prayer is of essence to the soul, and I am truly grateful for it. *"Do not be anxious over anything, but in everything by prayer and supplication along with thanksgiving, let your petitions be made known to God, and the peace of God that surpasses all understanding will guard your heart and your mental powers by means of Christ Jesus"* (Philippians 4:6-7, New World Translation Of The Holy Scriptures)

This Master's Degree was supported by a supervisor-linked bursary by Prof Adrienne L. Edkins through the National Research Foundation (NRF) for which I am very grateful, and thankful. A special thank you to my supervisor Dr. Jo de la Mare for allowing me to be under her supervision. We had numerous challenges, but she did not give up on me showing her good qualities of being a great leader. I had numerous personal challenges in accomplishing my Master's Degree which affected my academics bringing me to the verge of deregistering in numerous instances. As I thought it was all, on the 13th of January 2022, I lost my mother due to Diabetes type 2. It has not been a smooth journey but through it all, I'm grateful to have accomplished my Master's Degree.

Special thanks to Prof Richard Haynes of the Centre of Excellence for Pharmaceutical Sciences (North-West University, South Africa) for the supplementation of the compounds (artemisinin, artesunate, and WHN-11), and Dr. Laura Dingle for the generous gift of plasmid pCDNA-CL8D-His CYP2A6 from Biomedical Biotechnology Research Unit (BioBru), Department of Biochemistry and Microbiology, Rhodes University. Many thanks to my lab (BioBru) for the lab training, and sharing of ideas, skills, and techniques towards accomplishing my Master's degree.

Apart from my lab life, a platform of gratitude to Lindi, Sibongiseni, and Alulutho Nongwana for the words of courage, for allowing me in your home as I pursued my degree, and for reaching out to me whenever I was down and not feeling at my best. The love, sympathy, warmth, and making me a part of your family were and will never be taken for granted. You were a family other than my family.

I am truly, forever grateful and will always be in debt to my three ladies, my late mother, my grandmother, and my younger sister. Being raised by women as a male comes with a lot of challenges but you all did your utmost best to shape me, show me direction, love me unconditionally, always fight to achieve greatness, and make me the person I am today. Words cannot describe how honored and grateful I am and for that, I Thank you.

CHAPTER ONE

INTRODUCTION

1.1 Overview of breast cancer

Globally, breast cancer contributed to 24.5% of cancer cases and caused 15.5% of cancer-related deaths in women in 2020, such that it was ranked above all other forms of the disease in terms of both incidence and mortalities in women (Sung *et al*., 2021). In addition, it has been estimated that, by the year 2030, developing countries will experience a major increase in cancer-related deaths, while developed countries report an estimated 23.6 million new cases per year (Pacal *et al*., 2020; Chan *et al*., 2021).

African cancer mortality rates are higher than those in high-income countries (HIC), despite the higher incidence rates in HIC. A study conducted from 1995 to 2009 in terms of 5-year breast cancer survival rate based on individual data from 279 population-based registries in 67 countries, found that age-standardized net survival rates in HIC were more than 85%. Mauritius, a HIC island nation on the coast of Madagascar, reported similar survival rates of 87.4% (95% CI: 78.1–96.7). A study was also conducted by Allemani and colleagues, (2018) that compared the survival rates in Africa and it was found that Libya had a survival rate of 76.6% (95% CI:55.5–97.7), Tunisia had 68.4% (95% CI:64.5–72.2), Algeria had 59.8% (95% CI:48.6–71.1), South Africa had 53.4%, (95% CI: 0-30.1) with Gambia 11.9% (95% CI: 0–24.7), and Mali 13.6% (95% CI: 0–30.1) displaying the lowest survival rates. This indicates that almost half of all South African women diagnosed with the disease die from it (Allemani *et al.*, 2018). This highlights the disparity between incidence and mortality rates in developing countries.

Studies have shown that African women are more likely than Caucasian, Hispanic, or Asian women to be diagnosed with breast cancer at a younger age, with the disease being more common in women under 45 years of age (Monticciolo *et al*., 2021). One explanation for this is that the median age of the population is 20 years as compared to developed countries where there is a higher median age (Pilleron *et al*., 2022).

A breast cancer survival study in the United States discovered that African-American women had a worse prognosis than Asian and White-American women, which was explained by the higher prevalence of more aggressive breast cancer subtypes among women of African ancestry (Newman, 2005; Bea *et al*., 2021). Breast cancer survival was found to be lower in

African Americans in a mixed population research study compared to White-American patients (Monzavi-Karbassi *et al*., 2016). The five-year survival rate in middle- and low-income African countries is less than 60% due to the incidence of aggressive breast cancers. Clinical data from a Ugandan investigation revealed a 51.8% cumulative five-year survival rate, with the majority of cases being associated with early-stage disease (Galukande, Wabinga, and Mirembe, 2015).

The financial burden of breast cancer management in middle- and low-income countries also plays a significant role (Edge *et al*., 2014). In addition, limited access to modern diagnostic and treatment facilities, poorly maintained equipment, unmotivated oncology specialists, and a lack of healthcare policies all contribute to the higher breast cancer mortality rates in African women. Other factors include socio-cultural beliefs that lead to hesitance towards standard cancer therapy, patient non-compliance with follow-up, and a lack of reliable or updated cancer strategies that also influence the survival rates of all breast cancer. These are some of the constraints that limit research drug development or tumor-specific therapy, resulting in ineffective illness management and, as a result, higher mortality rates (Vanderpuye *et al.*, 2017).

Although there is evidence that limited resources contribute adversely to higher breast cancer mortality rates within African countries compared to developed countries, there is still a lack of knowledge regarding the genetic characteristics associated with this aggressive disease, particularly in African women (Fregene and Newman, 2005; Abdulrahman and Rahman, 2012). Recent initiatives to strengthen genomic medicine in Africa may potentially contribute to overcoming differences in health care such that the African population may benefit from specific treatment modalities such as those available in the developed regions of the world (Silverstein *et al*., 2016).

Breast cancer may be classified both according to morphology, and invasiveness and by gene expression studies for key receptors as discussed in section histological and molecular breast cancer subtype classification, respectively. There is a lack of genetic data on women of African ancestry which limits the use of targeted pharmacogenomic therapeutic techniques. As a result, the high prevalence of breast cancer mortality among African women remains a source of concern (Linnenbringer *et al*., 2017).

1.2. Histological breast cancer subtype classification

Breast cancer is not a single disease but a group of diseases showing different anatomical and molecular characteristics. Breast cancers can be classified based on the invasiveness and site of origin through histological investigations. Non-invasive breast cancers are divided into two groups, lobular carcinoma *in situ* (LCIS) and ductal carcinoma *in situ* (DCIS). DCIS is either non-invasive or pre-invasive and represents one in five new breast cancer cases being reported. Nevertheless, when DCIS is not treated, it can spread over time to become invasive cancer. The use of mammogram screening has been significant and has contributed to the increased early detection of DCIS (Thurman *et al*., 2004; Malhotra *et al*., 2010).

Several types of DCIS can be categorized due to morphological appearances, namely micropapillary, solid, cribriform, comedo, and papillary (Connolly *et al*., 1996). LCSI is where abnormal cells form and develop in the lobules and is known to be a rare condition. In contrast, invasive lobular, papillary, tubular mucinous, infiltrating ductal, ductal, lobular, and medullary carcinomas are the major invasive tumor types. The duct and globular wall are observed to be breached by the invasive form of breast cancer, which, in turn, invades the supporting tissue of the breast (Connolly *et al*., 1996).

Infiltrating ductal carcinoma (IDC) is the most dominant invasive breast cancer and is responsible for 80% of all invasive breast cancers. IDC forms in the ductal region of the breast, invade the duct wall and proliferate within the breast supporting tissue. Poorly differentiated (grade 3), moderately differentiated (grade 2) and well-differentiated (grade 1) are the three grades of IDC. The categories of grading IDC are dependent on tubule or glandular formation, mitotic index, and nuclear pleomorphism which contribute to prognosis (Lester *et al*., 2009).

1.3. Molecular breast cancer subtypes classification

There are various molecular techniques to classify breast cancer, including genomic sequencing and gene expression studies. Breast cancer classification has prognostic and clinical benefits, allowing for the prediction of clinical outcomes and the determination of suitable therapeutic strategies for patients (Correa Geyer *et al*., 2009; Taherian-Fard *et al*., 2014).

Gene expression studies by Sorlie *et al*., (2001) analyzed different types of breast cancer with the use of cDNA microarray profiling and categorized them into intrinsic molecular subtypes according to the expression of three key receptors, namely, the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) and this

allowed for classification into the four subtypes, luminal A (ER^+, PR^+/PR^-, $HER2^-$), luminal B (ER^+, PR^+/PR^-, $HER^+/HER2^-$), HER2 enriched (ER^-, PR^-, $HER2^+$) and triple-negative breast cancer (TNBC: ER^-, PR^-, $HER2^-$) (Sorlie *et al*., 2001). Estrogen and progesterone signaling greatly influence the growth of breast cancers. The expression of their cognate receptors also influences which type of therapy is used, and this impacts the prognosis of the patient (Anderson *et al*., 1989; Slamon *et al*., 1989; Boonyaratanakornkit *et al*., 2021). In particular, while hormone receptor-positive/luminal breast cancers respond to tamoxifen, which targets the estrogen receptor, and HER2 expressing tumors respond to Herceptin/trastuzumab, which targets HER2, these therapies are ineffective in TNBC due to the absence of these receptors (Cheang *et al*., 2008; Maqbool *et al*., 2021).

1.3.1 Luminal A subtype

Luminal breast tumors develop from abnormal growth of the inner luminal cells that line the mammary ducts. Luminal A breast tumors, which account for approximately 40-50% of all identified cases worldwide, are the most diagnosed subtype because they are ER^+ and either PR^+ or PR^-. Furthermore, these tumors have low levels of $HER2^-$ and a low concentration of Ki-67 proteins (<14%) (Sorlie *et al*., 2001; Goldhirsch *et al*., 2011), both of which are cell-growth markers implicated in cell proliferation. According to a study conducted in the United States, the luminal A subtype accounted for 59% of all detected breast cancer cases (Fallahpour *et al*., 2017), revealing that the subtype is the more frequently diagnosed in this developed nation.

In a Ugandan study involving 226 breast cancer patients, the luminal A subtype was shown to account for 38% of breast cancer cases (Galukande *et al*., 2014). Luminal A breast cancers are more common than other subtypes of breast cancer in African countries such as Nigeria (Desantis *et al*., 2016) and within specific racial groups (Adebamowo *et al*., 2008; Dietze *et al*., 2015), regardless of age, menopausal status, tumor stage, or other indicators. Luminal A patients account for most breast cancer survivors among African women (Galukande *et al*., 2015).

Luminal A breast tumors have the best overall prognosis, with greater survival rates and a lower chance of recurrence than other subtypes. Due to its positive hormone receptor status, tamoxifen is commonly used for the treatment of luminal A breast tumors in endocrine or hormonal therapy, especially when axillary lymph node involvement is restricted (Goldhirsch *et al*., 2011). When lymph nodes are involved, standard chemotherapy such as rituximab or cyclophosphamide has been used as an alternative treatment option (Inic *et al*., 2014).

However, clinical experts disagree on whether chemotherapy and endocrine therapy should be combined for long-term survival rates (Diessner *et al*., 2016).

Another subgroup of breast cancer, which is normal-like tumors, is a comparable breast cancer subtype to the luminal A subtype, although less defined. These tumors are ER^+ or PR^+, HER2, express low levels of Ki-67 and are identified by the presence of normal breast cells within a tumor sample (Parker *et al*., 2009). They have similar immunohistochemical characteristics to the luminal A subtype (Dai *et al*., 2015).

1.3.2 Luminal B subtype

Luminal B breast tumors make up approximately 24% of breast cancer cases (Hagen *et al*., 2010). Like luminal A breast tumors, Luminal B tumors are ER^+ and either PR^+ or PR^-, however, the HER2 expression levels in luminal B tumors differ from those in luminal A tumors. This subtype has been subdivided into luminal B $HER2^+$, which has a higher expression of HER2 and either a high or low Ki-67 index, and luminal B $HER2^-$, which has a low expression of HER2, and a high Ki-67 index (Sorlie *et al*., 2001; Goldhirsch *et al*., 2011; Inic *et al*., 2014).

Higher levels of ER-related genes, such as ER estrogen-regulated gene (*LIV-1*), and lower levels of cell proliferation genes, such as G2/mitotic-specific cyclin-B1 (*CCNB1*), a marker of proliferation Ki-67 (*MKI67*), and Myb-related protein B (*MYBL2*), have been used to distinguish luminal A from luminal B breast tumors in terms of hormone receptor expression (Sorlie *et al*., 2001; Cheang *et al*., 2009). Patients with luminal B breast cancer have a worse prognosis, higher-grade tumors, and a higher risk of local recurrence as a result of higher expression of cell proliferation genes, limiting their survival rates compared to luminal A breast cancer patients (Inic *et al*., 2014). Furthermore, patients with luminal B have a greater relapse rate in the first five years after diagnosis (Tran *et al*., 2011). After several courses of hormone therapy, $ER^+/HER2^-$ metastatic tumors (luminal A tumors) can develop into $HER2^+$ circulating tumor cells (CTCs), according to a study by Jordan and colleagues. Interestingly, the development of $HER2^+$ in CTCs was linked to enhanced cell proliferation and treatment resistance, leading to disease progression (Jordan *et al*., 2016).

For luminal B tumors, both endocrine therapies, namely tamoxifen or raloxifene (Bergamaschi *et al*., 2011) and standard chemotherapy, such as rituximab (Plosker and Figgitt, 2003), are viable treatment options. Luminal B tumors are also treated with targeted treatments for

specific signaling pathways implicated in cell proliferation, survival, migration, and metastasis (Jordan *et al*., 2016).

1.3.3 Human epidermal receptor-2 enriched

Gene microarray analysis has revealed the overexpression of the HER2 proto-oncogene in a subset of breast tumors. In particular, the *erbB2/HER2/neu* gene on chromosome 17q12 is amplified in HER2-enriched breast tumors (Sorlie *et al*., 2001). In addition, these tumors are hormone receptor-negative (ER^- and PR^-) (Sweeney *et al*., 2014). HER2-positive breast cancer makes for 15-20% of all breast cancer subtypes. Positive HER2 expression tends to be more aggressive than other subtypes. These tumors are extremely proliferative, with 75% having a high histological and nuclear grade and more than 40% having p53 mutations (Sorlie *et al*., 2001; Tsutsui *et al*., 2003). Upon activation of HER2, the cells are provided with potent anti-apoptosis and cell proliferative signals (Gutierrez and Schiff, 2011). Notably, greater HER2 protein expression in tumors causes resistance to hormone-based therapies, which accounts for the lower susceptibility to hormone-based therapies in both the luminal B and $HER2^+$ tumor subtypes (Higgins and Baselga, 2011). Amplification of the *erb-B2* gene has also been observed in other malignancies, such as ovarian and lung carcinomas (Slamon *et al*., 1989; Presmanes *et al*., 2008). Overexpression of the *erb-B2* gene has been linked to unregulated cell growth and proliferation, aggressive tumors, and enhanced metastasis in breast carcinomas (Tan *et al*., 2007).

Hormone receptor-targeted drugs like tamoxifen (which targets the ER) cannot be utilized to treat $HER2^+$ tumors because the ER and PR receptors are not present. Instead, HER2 inhibitors like Herceptin, which is a monoclonal antibody that is used to treat breast as well as stomach cancer (Harries and Smith, 2002), tyrosine kinase inhibitors, namely erlotinib or lapatinib, which inhibit HER2 and epidermal growth factor receptor (EGFR), or other classic chemotherapy drugs such as docetaxel and paclitaxel are commonly used (Kümler *et al*., 2014). The survival and relapse rates of this subtype are 46.1% and 47.9%, respectively, indicating less favorable outcomes for these patients in comparison with the luminal subtypes (Hagen *et al*., 2010; Yan *et al*., 2015).

1.3.4 Triple-Negative Breast Cancer

Triple-negative breast cancer (TNBC), which accounts for 10 – 15% of all breast cancers diagnosed globally, is immunohistochemically classified according to the absence of expression of all three clinically relevant receptors (ER^-, PR^- and $HER2^-$) (Dass *et al*., 2021). These tumors also express cytokeratin (CK5/6, CK14, and CK17) and EGFR (Sorlie *et al*., 2001; Cheang *et al*., 2008; Gusterson, 2009). This form of the disease is more common among black women (Dent *et al*., 2007; Friebel-Klingner *et al*., 2021; Luo *et al*., 2021), has a poor prognosis, and is associated with a 10-14% overall survival (OS) rate in patients with metastatic TNBC (Hagen *et al*., 2010; Hsu *et al*., 2022) and a 40% recurrence rate (Eldridge, 2021), with a high mortality rate during the first five years of diagnosis (Dent *et al*., 2007). This was also observed in a study conducted by Stead and colleagues where they analyzed data on published studies based on tumor grade, stage, ER/PR/HER2 status, patient age, BMI index, racial group, and discovered that the risk of TNBC in black women was 3-fold higher as to white women (Stead *et al*., 2009).

The TNBC subtype is classified by poor cellular differentiation, mutations in the *TP53* tumor suppressor gene, and high cellular proliferation (Sorlie *et al*., 2001; Lehmann and Pietenpol *et al*., 2014). TNBC is characterized by large tumors, high tumor histological grades, and axillary lymph node involvement (Dent *et al*., 2007). The subtype is a very aggressive cancer, and it has been reported that approximately 46% of TNBC patients will develop distant metastases. After metastasis, the median survival period is commonly a year. The brain and visceral organs are frequently involved in metastasis. The majority of distant metastases develop in the third year after diagnosis and some reports suggest that all patients with TNBC eventually die from the disease (Lin *et al*., 2008).

The gene profiling analysis commonly categorizes TNBC as a subtype of basal-like breast cancer (BLBC). TNBC and BLBC gene expression profiles coincide in about 56% of cases. The overlap ratio between TNBC and BLBC can be as high as 60–90%, compared to only 11.5 % between non-TNBC and BLBC (Bertucci *et al*., 2008). TNBC commonly occurs in premenopausal young black women under 40 years old (Boyle, 2012), and is more commonly diagnosed in women of African ancestry [including African American women (AA)] as well as Hispanic women than in other ethnic groups, according to epidemiological research (Brewster *et al*., 2007; Boyle, 2012).

Figure 1 displays the results of a study conducted by Siddharth and Sharma, (2018) of 1428 women where the frequency of American women diagnosed with TNBC (displayed in %) was compared to that of women from different regions of Africa, that is East, North, and West Africa. It was found that European American women (EA; shown in pink) had a lower frequency (16%) of women diagnosed with TNBC as compared to AA (30%; shown in blue). Even though AA women had a higher TNBC diagnosis, it was low compared to women of Africa. East African countries (displayed in green) such as Kenya (44%) had the highest percentage of women diagnosed with TNBC followed by Tanzania (38%) and Uganda (36%) having the least percentage frequency of TNBC. North African countries (displayed in purple) have Egypt (29%) as being the highest, followed by Tunisia (23%), Algeria (20%), and Morocco (17%) almost equivalent to EA. Ghana had the highest percentage of women diagnosed with TNBC in the study as this was observed with a frequency of TNBC of 61% in women followed by Mali (46%), Nigeria (29%), and Senegal (27%) (Siddharth and Sharma, 2018). It was concluded from the study that African women have a higher prevalence of TNBC as compared with AA and EA women.

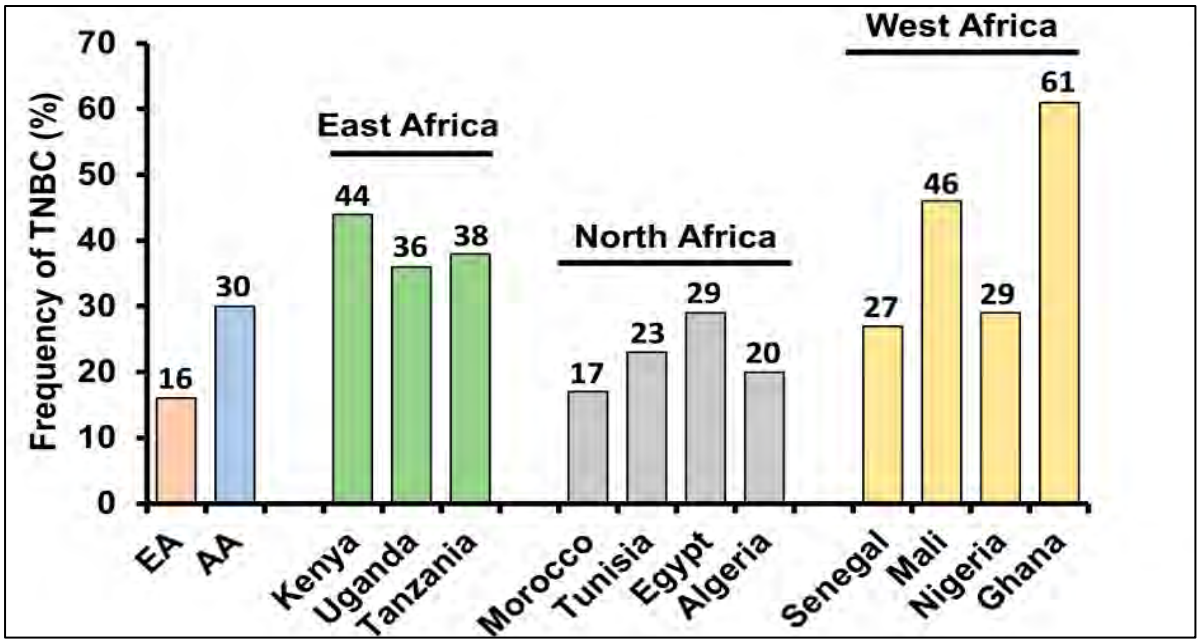


Figure 1.1: The frequency of TNBC displayed in percentage is more common in women from various countries in Africa as compared to European Americans (EA), and African Americans (AA) (Siddharth and Sharma, 2018).

A study conducted by Huo and colleagues demonstrated that basal-like TNBC was the most common malignancy in Africa (Huo *et al*., 2009), based on the distribution of molecular subtypes of invasive breast cancers in women (mean age 44.8 years) from diverse geographical

locations of Nigeria and Senegal. In Bamako University Hospital in Mali, where the average age of breast cancer patients was 46 years, 46% of tumors were determined to be triple-negative (Ly *et al*., 2012). In another case study of 1216 breast cancer patients from Soweto, South Africa, 90% of women diagnosed with TNBC were black, which was similar to the observed frequency of TNBC in AA women (McCormack *et al*., 2013). Studies that have been conducted in smaller hospitals have also shown a high prevalence of TNBC in Ghanaian women compared to white American women (Stark *et al*., 2010).

Several factors contribute to or influence the risk of being diagnosed with TNBC (Almansour, 2022). For example, physiological changes in endogenous sex hormones increase the risk of TNBC in premenopausal and postmenopausal women (Zhang *et al*., 2013). *BRCA1* gene mutations have been discovered to be highly associated with TNBC (Shiovitz *et al*., 2015). TP53, CDH1, PTEN, and STK11 mutations have also been associated with TNBC (Shiovitz *et al*., 2015; Wood *et al*., 2020). Differences in population age, lifestyle risk factor distribution, and access to mammography screening are all factors that could account for variances in the incidence and prevalence of TNBC among women of African ancestry (Stark *et al*., 2010; McComack *et al*., 2013).

Table 1.1 describes a study that was conducted by Lehmann *et al*., (2011), where six subtypes of TNBC were classified using gene expression profiling. The subtypes were basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal (M), mesenchymal stem-like (MSL), immunomodulatory (IM), and luminal androgen receptor (LAR) (Lehmann *et al*., 2011). In the BL1 subtype, gene expression profiling revealed abnormal expression of cell cycle-regulating and DNA repair-related genes (high amplification of *MYC, PIK3CA, CDK6*, *AKT2*, *KRAS*, *FGFR1*, *IGF1R*, *CCNE1*, and *CDKN2A/B*), as well as a high frequency of heterozygous or homozygous deletion of DNA repair-related genes like *BRCA2*, *PTEN*, *MDM2*, *RB1*. The BL2 subtype has abnormal activation of signaling pathways such as the EGFR, MET, NGF, Wnt/β-catenin, and IGF-1R pathways (Lehmann and Pietenpol, 2014). The M subtype is also known as metaplastic breast cancer because it has highly activated cell migration-related signaling pathways that are regulated by actin, in addition to the upregulated extracellular matrix–receptor interaction and differentiation pathways. Examples of the latter include the Wnt anaplastic lymphoma kinase and transforming growth factor (TGF)-β signaling pathways (Lehmann and Pietenpol, 2014). The M subtype is characterized by sarcoma-like or squamous epithelial cell-like tissue and is prone to chemotherapeutic treatment resistance (Gibson *et al*., 2005). The MSL subtype expresses fewer cell proliferation-related genes and more stemness-

related genes (*ABCA8*, *PROCR*, *ENG*, *ALDHA$_1$*, *PER$_1$*, *ABCB$_1$*, *TERT$_2$IP*, *BCL$_2$*, *BMP$_2$*, and *THY*), HOX genes (*HOXA$_5$*, *HOXA$_{10}$*, *MEIS$_1$*, *MEIS$_2$*, *MEOX$_1$*, *MEOX$_2$*, and *MSX$_1$*), and mesenchymal stem cell-specific markers than the M gene subtype (*BMP$_2$*, *ENG*, *ITGAV*, *KDR*, *NGFR*, *NT$_5$E*, *PDGFR*, *THY$_1$*, and *VCAM$_1$*) (Lehmann and Pietenpol, 2014). Immune cell-associated genes and signaling pathways, such as the Th1/Th2 route, NK cell pathway, B cell receptor signaling system, dendritic cell (DC) pathway, T cell receptor signaling, interleukin (IL)-12 pathway, and IL-7 pathway are considerably enriched in the IM subtype. As a result, the IM subtype resembles medullary carcinoma of the breast (Bertucci *et al*., 2006; Lehmann and Pietenpol, 2014). Lastly, the gene expression profile of the LAR subtype differs significantly from those of other TNBC subtypes. Although the LAR subtype lacks the ER receptor, it has highly activated hormonal signaling pathways (including steroid synthesis, porphyrin metabolism, and androgen/estrogen metabolism). The androgen receptor (AR) is strongly expressed in the LAR subtype of breast cancer, with an mRNA level nine times higher than in other TNBC subtypes. In the LAR subtype, immunohistochemistry also detects high levels of AR as well as a large number of downstream metabolic indicators of AR and their auxiliary activators (DHCR24, ALCAM, FASN, FKBP5, APOD, PIP, SPDEF, and CLDN8) (Hayes *et al*., 2008).

The examples of treatments used for each subtype are also displayed in Table 1.1 (Lehmann *et al*., 2011); however, they are not effective especially in African women as they are less responsive to neoadjuvant therapy (Frasci *et al*., 2009), and patients eventually die from the disease (Taskindoust *et al*., 2021). Thus, novel therapies are thus required to treat this aggressive breast cancer subtype. Additionally, the treatments (shown in the table) have adverse effects that cannot be ignored. For example, the poly (ADP-ribose) polymerase (PARP) inhibitor, Olaparib, failed to achieve any response in patients diagnosed with TNBC that have germline and somatic mutations (Gelmon *et al*., 2011). Camptothecin (genotoxic agent) has demonstrated that it has potent activity in cancer cell lines, but the drug failed in clinical trials due to low solubility (Weingart *et al*., 1995; Barnieh *et al*., 2021). mTOR targeted therapy, namely, rapamycin as well as its analogs have been observed not to be effective in that the analogs release the mTOR negative feedback to the upstream kinases which leads to the activation of AKT/PI3K as well as ERK/MAPK signaling pathways which this leads to mTOR inhibition being bypassed and TNBC not treated (Carracedo *et al*., 2008; Yu *et al*., 2011). P1$_3$K inhibitors have been unsuccessful in treating patients as the drugs display high levels of systemic toxicity as well as the inability to downregulate the pathway that is needed for the

tumor response in a clinical setting (Wright *et al*., 2021). PD1 and PDL1 therapies are not highly effective due to the genetic instability of tumor cells in TNBC making PD1, and PDL1 poor therapies for the success of immunotherapies (Saraiva *et al*., 2017). The use of gonadotropin (anti-androgen receptor therapy) to treat cancer has side effects such as causing ovarian hyperstimulation syndrome (OHSS), a syndrome observed by the enlargement of ovaries as well as the accumulation of fluid in the abdomen (Gürbüz *et al*., 2020).

Table 1.1: Examples of treatment used on genetic characteristics for each subtype of TNBC and BLBC (Lehmann *et al*., 2011)

TNBC sub-type	Genes overexpressed	Examples of treatment
Basal-like 1 (BL1)	Genes involved in cell cycle-regulating and DNA repair	PARP inhibitors and genotoxic agents (Lehmann and Pietenpol, 2014)
Basal-like 2 (BL2)	Growth factor signalling genes	mTOR inhibitors and growth factor inhibitors (lapatinib, gefitinib, and cetuximab) (Lehmann and Pietenpol, 2014)
Mesenchymal (M)	Genes involved in extracellular matrix-receptor interaction pathways, and differentiation pathways	mTOR inhibitors or drug targeting epithelial-mesenchymal transition (Gibson *et al*., 2005; Lehmann and Pietenpol, 2014)
Mesenchymal stem-like (MSL)	Cell proliferation related genes	P13K inhibitors. Src antagonists, or anti-angiogenic drugs (Lehmann and Pietenpol, 2014)
Immunomodulatory (IM)	Immune cell processes related genes	PD1, PDL1, CTLA-4, and other immune checkpoints inhibitors (Bertucci *et al*., 2006; Lehmann and Pietenpol, 2014)
Luminal androgen receptor (LAR)	Genes involved in luminal gene expression pattern	Anti-AR therapy (Hayes *et al*., 2008)

In a study conducted where platin therapy was assessed in germline BRCA (breast cancer gene) mutated patients in the phase II INFORM trial, it was found that cisplatin had an 18% pathological complete response in 117 patients enrolled in the trial (Tung *et al*., 2019). In another study where sacituzumab govitecan (antibody-drug conjugate) was assessed in an I/II trial (NCT01631552), it achieved a poor effective response rate of 34.3% in TNBC patients (Logan, 2020). Anthracycline and taxanes are known to be platinum-based chemotherapeutic

drugs. Anthracycline drugs such as epirubicin and doxorubicin are associated with high toxicity which results in alopecia (hair loss), myelotoxicity (a condition in which bone marrow is decreased resulting in less white blood cells, red blood cells, and platelets) and there has been no improvement observed in overall survival in TNBC patients (Rhea *et al*., 2015; Vera *et al*., 2015; Yun *et al*., 2015). Vinorelbine has been observed to cause leukopenia (low white blood cell count), and gastrointestinal toxicities when used to treat TNBC (Valerio *et al*., 2021). The use of erlotinib (tyrosine kinase inhibitor) has been observed to cause prolonged and severe lymphopenia (Dickler *et al*., 2009; Layman *et al*., 2013). Perrault *et al*., (1988) and Zhao *et al*., (1988) on examining the effect of flutamide, an anti-androgen, and it was found that the drug did not show meaningful activity when tested in TNBC tumors (Perrault *et al*., 1988; Zhao *et al*., 1988). Docetaxel and paclitaxel are examples of taxanes that have been observed to cause diseases such as neuropathy (Rivera and Cianfrocca, 2015). In a study conducted [Impassion131 study (NCT031259020], where atezolizumab combined with paclitaxel was compared with placebo combined with paclitaxel, both combinations failed to show a PFS and OS in TNBC patients (Nagayama *et al*., 2021). Capecitabine, an anti-metabolite, has been observed to cause hand-foot syndrome, alopecia, and myelosuppression and it is also known to be highly toxic (Yamamoto *et al*., 2015).

These therapies are either costly, cause other diseases such as OHSS or fail in clinical trials as mentioned above. Therefore, novel therapies are thus required to treat this aggressive breast cancer subtype. Despite the novel treatment strategies explored for TNBC and TNBC subtypes, as described above, these therapies have largely failed in the clinic. TNBC contains high levels of *BRCA* and *TP53* mutations (Cristall *et al*., 2021) which proposes that TNBC and its subtypes may be more sensitive to DNA damage induction, which could represent an effective therapeutic approach. Topoisomerases are enzymes that maintain DNA topology during DNA replication and transcription thereby preventing DNA damage during these cellular processes (Kadioglu *et al*., 2017). The inhibition of these enzymes serves as a potential therapeutic strategy since highly proliferative cells rely more heavily on topoisomerases to relieve torsional strain due to increased levels of transcription and more rapid cell division (McKie *et al*., 2021).

1.4 Artemisinin and its derivatives

1.4.1 Development of artemisinin derivatives and their activity in Malaria

Artemisinin is a sesquiterpene lactone that contains a peroxide bridge (Efferth *et al*., 2015). It is produced from the leaves of *Artemisia annua*, a medicinal plant that has been used for centuries against chills and fever (Klayman *et al*., 1984). The search for new anti-malarial treatments began in the 1960s in response to *Plasmodium falciparum's* developing resistance to chloroquine (CQ). In the year 1976, dihydroartemisinin (DHA) was the first derivative synthesized from the parent, artemisinin, made by reducing the carbonyl groups to form a hydroxyl group at carbon number 10 of artemisinin (Haynes *et al*., 2002; Posner *et al*., 2002). Figure 1.2 shown below displays the structures of the parent compound, artemisinin, and dihydroartemisinin with the endoperoxide bridge in red. The synthesis of other derivatives soon followed, including the more water-soluble artesunate and the more oil-soluble derivatives artemether and artemotil. These compounds were ten times more effective than artemisinin against malaria (Klayman, 1985). Artemisinin and its derivatives are selectively taken up by parasite-infected erythrocytes and eventually localized in parasite membranes such as the mitochondria and digesting vacuole (Meshnick *et al*, 1996).

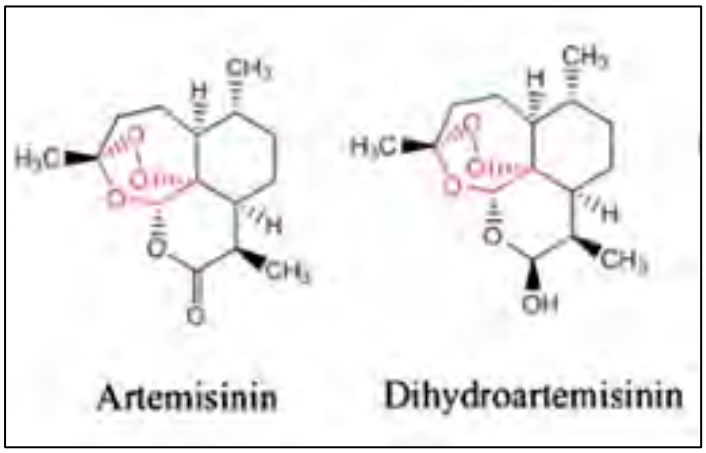


Figure 1.2: The structures of artemisinin and dihydroartemisinin with the endoperoxide bridge displayed in red (Khanal, 2021).

The action of artemisinin and its derivatives have been attributed to a unique structure, the endoperoxide bridge (C-O-O-C), which produces free radicals in the presence of heme or iron (Meshnick *et al*, 1996). Malaria membrane-associated proteins are alkylated by these free radicals, killing the parasite. Artemisinin and its derivatives have been discovered to be effective against a variety of malaria strains, particularly those resistant to the current malaria

treatment such as chloroquine. The compounds are highly effective in *in vitro* studies requiring only nanomolar concentrations and with therapeutic effects appearing as soon as 20 hours after treatment (Meshnick *et al*., 1996).

1.4.2 Artemisinin and its derivatives activity in other diseases

Numerous studies have reported that artemisinin and its derivatives have inhibitory activity in a range of other infectious diseases, other than malaria such as viruses [herpes, Hepatitis B and C, human immune virus (HIV), and human cytomegalovirus] and fungi (*Candida albicans*, *Cryptococcus neoformans*). Studies suggest that these compounds also display activity against non-communicable human diseases, (Andong Koung Edzidzi, 2016). Systemic lupus erythematosus (SLE) is a chronic autoimmune illness characterized by an excessive build-up of autoreactive T cells and the formation of autoantibodies against self-antigen, which leads to immune complex-mediated glomerulonephritis and renal failure. Female MRL/lpr mice and NZBW/F1 mice are two well-known murine models for lupus research that closely resemble human lupus illness. By reducing both Th1 and Th17 responses, Hou *et al*., (2012) demonstrated that artemisinin derivative, SM934, can significantly prolong life span and reduce glomerulonephritis in MRL/lpr mice (Hou *et al*., 2012). The therapeutic effects of SM934 include regulating the serum levels of pathogenic cytokines interferon- (IFN-) and interleukin-10 (IL-10), decreasing the secretion and deposition of pathogenic anti-dsDNA IgG autoantibodies in serum and kidneys, and alleviating renal injury (Shi *et al*., 2015). Artesunate is effective in the treatment of autoimmune and allergic illnesses such as allergic asthma, arthritis, dermatitis, hepatic fibrosis, pulmonary fibrosis, and myasthenia gravis. Artesunate's anti-inflammatory mechanism functions by inhibiting proinflammatory cytokines, namely inhibiting the synthesis of interleukin (IL)-1, IL-6, and IL-8 in TNF-stimulated rheumatoid arthritis fibroblast-like synoviocytes (RA FLS) via the NF-KB and phosphoinositide 3 kinases (PI3K) pathways (Xu *et al*., 2007). In combination treatment, it has been shown that artemether in conjunction with praziquantel can be used as an anti-schistosomal drug. In children, the combination reduces the prevalence and incidence of new schistosomiasis infections (Utzinger *et al*., 2003; Elmorshedy *et al*., 2016). More specifically, artemisone increased the removal of juvenile schistosomes in infected mice when encapsulated in a slow-release formulation (Zech *et al*., 2020).

1.4.3 Proposed mode of action of artemisinin and its derivatives as an anti-cancer treatment

The particular mechanism of action of artemisinin and its derivatives in terms of their anti-cancer activity has yet to be fully explored. Various mechanisms of action of artemisinin and its derivatives have been proposed, including reactive oxygen species (ROS) generation, topoisomerase inhibition, cell cycle arrest, induction of apoptosis, inhibition of angiogenesis, induction of ferroptosis, both alone and in combination treatment, and these are described in more detail below.

1.4.3.1 Free radical generation by the endoperoxide moiety

The C-radical theory has become one of the most widely accepted explanations for the anti-cancer activity of artemisinin and its derivatives and suggests a similar mode of action to that observed in malaria. In particular, it has been reported that toxic radicals, generated by the endoperoxide moiety of artemisinin via a ferrous iron-mediated reaction, are critical for killing cancer cells. It is thought that the carbon atom at the center of the peroxide bridge of artemisinin and its derivatives react with the ferrous iron [Fe (II)] atom to form free radicals or reactive ROS. ROS has a critical role in tumor cell death, by triggering apoptosis and oxidative DNA damage (Berdelle *et al*., 2011; Mercer *et al*., 2011).

Tumor cells are more vulnerable to ROS damage due to their intrinsically high levels of oxidative stress, as a result of increased metabolic activity (Hileman *et al*., 2004). For example, HeLa cervical cancer cells have been demonstrated to induce ROS at an earlier timepoint than when the cytotoxic effects appear after treatment with artesunate, implying that ROS may be one of the initiating events leading to cell death (Zyad *et al*., 2018). Cancer cells also require an abundant amount of iron compared to normal cells (Heath *et al*., 2013), and tumor cells are known to overexpress cell surface transferrin receptors (TfR), which could also account for the observation that cancer cells are more susceptible to ROS generation by artemisinin and its derivatives (Zyad *et al*., 2018).

1.4.3.2 Inhibition of topoisomerase by artemisinin derivatives

Topoisomerases (TOPOs) are important enzymes in DNA replication and transcription as they regulate the degree of supercoiling by generating transient breaks in the DNA strand, alleviating torsional strain (Wang, 1996). These enzymes are overexpressed in a variety of malignancies and are connected to a poor prognosis; thus, TOPOs have been identified as

targets for cancer therapy (Karachaliou *et al*., 2013; Pan *et al*., 2022). Examples of TOPO inhibitors that have been clinically investigated in cancer patients include the topoisomerase I (TOPO I) inhibitor camptothecin and the topoisomerase II (TOPO II) inhibitor etoposide (Ferraro *et al*., 2000). Camptothecin, while demonstrating potent activity in cancer cell lines, its capabilities are due to low solubility (Martino *et al*., 2017) and adverse side effects (Hsiang *et al*., 1985). On the other hand, etoposide is a well-tolerated, Food and Drug Administration (FDA) approved drug for small cell lung cancer and testicular cancer (Martin *et al*., 2020).

TOPO inhibition has been linked to the induction of DNA damage, a widely utilized mode of action in cancer therapies. *In silico* docking, experiments revealed that a range of novel artemisinin derivatives could potentially bind TOPO 1 (Wang, 1996). In a cancer model system using a transformed rat cell line (R6T24), these derivatives were also found to inhibit TOPO function and produce DNA damage by the use of TOPO decatenation and comet assays, respectively (Kadioglu *et al*., 2017).

1.4.3.3 Cell cycle arrest

Artemisinin and its derivatives have been demonstrated to inhibit tumor cell proliferation by disrupting the cell cycle, with G0/G1 and S phase arrest being the most widely reported of these effects (Jiang *et al*., 2012; Lin *et al*., 2016). The mechanism of action by which this occurs involves changes in the expression and activity of cell cycle regulatory proteins upon treatment with artemisinin and its derivatives. In particular, the downregulation of cyclins and the transcriptional activity of cyclin-dependent kinases (CDKs), by limiting the activity of the CDK promoter or increasing the activity of CDK inhibitors, has been linked to dihydroartemisinin-induced cell cycle arrest at the G0/G1 phase in the human hepatoma cell lines HepG2, Hep3B, and BEL-7404 (Kumar *et al*., 2019).

In addition, artemisinin has been reported to inhibit CDK-4 gene expression, resulting in cell cycle arrest in the LNCaP, PC3, and DU145 human prostate cancer cells (Abu-Izneid *et al*., 2020). Artesunate has been shown to cause cell cycle arrest in the G2/M phase MCF-7 and MDA-MB-231 breast cancer cells by upregulating the expression of Beclin1, an autophagy initiator, (Chen *et al*., 2014). Furthermore, it has been reported that artesunate may interfere with several genes that regulate the mitotic spindle checkpoint in the G2/M phase, namely, Bub3, Mad3, and Mad2 in carcinoma (HCT116), and glioma (U251) cancer cell lines (Steinbrück *et al*., 2010). The sensitivity of artemisinin drugs was also found to be associated

with the cell division cycle 25 homolog A (CDC25A) proteins in rat embryonic R12 cells (Li *et al*., 2016).

1.4.3.4 Induction of Apoptosis

The most common type of physiological cell death is apoptosis, or programmed cell death, in which the organism eliminates damaged single cells (Fiers *et al*., 1999; Bjelaković *et al*., 2005). This cellular process is mediated by a balance between the proapoptotic (Bax) and anti-apoptotic (Bcl2) family genes, as well as their effects on mitochondria (Hardwick and Soane, 2013). The release of cytochrome c is induced by a rise in the Bax/Bcl2 ratio, which is followed by caspase activation and cell death (Agarwal *et al*., 2004).

The degree of expression of Bcl2 and Bax genes in a cancer cell line has been linked to artemisinin sensitivity (Das, 2015). Artemisinin's apoptotic effects have been related to the activation of the intrinsic pathway in general. As a result, mitochondrial membrane damage is assumed to play a key part in the cell death cascade. Artemisinin and its derivatives cause apoptosis via altering the Bax/Bcl2 ratio (Crespo-Ortiz *et al*., 2012). In osteosarcoma cells, DHA and artesunate produced cytochrome c release, Bax overexpression, a rise in the Bax/Bcl2 ratio (Das, 2015), and activation of caspases 3 and 9. DHA also activates caspase 8 and lowers CDC25B, cyclin B1, and NF-$_{K}$B levels (Ji *et al*., 2011). Artesunate also depletes survivin in the same pathway, which has been linked to the apoptotic DHA response in lung cancer cells (Crespo-Ortiz *et al*., 2012).

1.4.3.5 Inhibition of Tumour Angiogenesis

Neoplastic vascularization is critical for tumor growth because it provides oxygen and nutrients to cancer cells. When tumor cells reach systemic blood circulation (Slezáková, S. and Ruda-Kucerova, 2017), which is common in aggressive and fast-growing tumors, it can lead to metastasis. The ability of tumor cells to synthesize angiogenic factors is critical, and anticancer treatment targets this ability (Zetter, 1998). Artemisinin and its derivatives have been shown to have antiangiogenic effects. For example, in nude mice injected subcutaneously with Matrigel pellets containing vascular endothelial growth factor (VEGF), TNFα, and heparin as angiogenic stimuli in a study involving Kaposi's sarcoma-IMM cell lines, artesunate, significantly reduced vascularization (Slezáková *et al*., 2017). In another study with multiple myeloma cells, artesunate was found to reduce VEGF expression and decrease angiopoietin 1 production (Sagar *et al*., 2006). In chronic myeloid leukemia K562 cells, artesunate

significantly reduced aortic sprouting, according to the number of new microvessels, in a time- and dose-dependent manner. (Zhou *et al*., 2007).

1.4.3.6 Induction of ferroptosis

In a study conducted by Lin *et al*., (2016), head and neck squamous cell carcinoma (HNSCC) cells were used to determine the mechanism of action of dihydroartemisinin based on the known antimalarial mode of action of artemisinin and its derivatives, including the involvement of Fe^{2+} ions, with ferroptosis as a proposed mode of cell death (Lin *et al*., 2016). Ferroptosis is a type of programmed cell death caused by the accumulation of lipid-reactive oxygen species in the presence of iron and the depletion of plasma membrane polyunsaturated fatty acids (Yang *et al*., 2014). The study reported increased ROS levels in HNSCC cells treated with dihydroartemisinin in a study (Lin *et al*., 2016).

On the other hand, deferoxamine, an iron chelator, prevented ROS induction and rescued the cytotoxicity, indicating that dihydroartemisinin requires iron to kill HNSCC cells. Changes in mitochondrial morphology, which are characteristic of ferroptosis cells, were also detected. As iron is required during DNA synthesis, as an essential cofactor of multiple DNA replication and repair proteins, tumor cells require high levels of iron to support their high rates of cell division (Lin *et al*., 2016). This greater dependence on iron may be responsible for dihydroartemisinin's specific impact on cancer versus healthy cells. Reduced levels of glutathione peroxidase 4 (GPx4) and reticular activating system (Ras) in dihydroartemisinin-treated HNSCC cells support the hypothesis of ferroptosis, as both GPx4 and Ras are essential regulators of ferroptosis (Lin *et al*., 2016).

1.4.3.7 Artemisinin in combination with other anticancer drugs

Combination chemotherapy is an alternative therapeutic strategy, which aims at maximizing efficacy and minimizing systemic toxicity, compared to monotherapy, by reducing the concentrations of individual drugs (Song *et al*., 2020). It has been reported that the addition of artemisinin and its derivatives to the standard treatment results in chemosensitization and may result in resistant cancer cell lines becoming responsive to such therapies (Crespo-Ortiz and Wei, 2012).

In particular, Wang *et al*., (2010) found that dihydroartemisinin and gemcitabine have a synergistic effect on pancreatic cancer cells. There was a significant increase in apoptosis, a decrease in Ki-67 index, and a decrease in NF-κB activity, as well as its linked gene products

such as c-myc, cyclin D1, Bcl-2, and Bcl-xL therapy as compared to dihydroartemisinin being the sole treatment. The principal mechanism through which dihydroartemisinin functions in synergy with gemcitabine against pancreatic cancer cells is thought to be the inhibition of gemcitabine-induced NF-$_K$B activation (Wang *et al*., 2010). In another study, dihydroartemisinin with gemcitabine, and artemisinin with gemcitabine, enhanced apoptosis in human hepatoma cells, including HepG2 (p53 wild-type), Huh-7, BEL-7404 (p53 mutant), and Hep3B cell lines. Similarly, both combinations inhibited tumor growth in animal models of human hepatoma carcinoma. When compared to artemisinin, dihydroartemisinin showed stronger, dose-dependent synergistic therapeutic benefits in all xenograft models (Slezáková and Ruda-Kucerova, 2017).

In mouse models of non-small cell lung cancer, a combination of dihydroartemisinin and standard chemotherapeutics such as cyclophosphamide and cisplatin were found to be highly effective. Compared to either therapy alone, the combination of high dosage dihydroartemisinin with cyclophosphamide and dihydroartemisinin with cisplatin resulted in a considerable reduction in tumor size. Furthermore, the combination of dihydroartemisinin and cyclophosphamide fully inhibited spontaneous pulmonary metastases (Zhou *et al*., 2010). In glioma cells, dihydroartemisinin enhanced the lethal action of temozolomide by producing reactive oxygen species. Dihydroartemisinin increased temozolomide-induced apoptosis and necrosis in a dose-dependent manner (Huang *et al*., 2008). These data show that artemisinin and its derivatives, particularly dihydroartemisinin, have chemosensitizing properties and could be used to treat chemoresistant carcinomas in the future.

1.4.3.8 Artemisinin and its derivatives treating TNBC

In a study conducted by Lang *et al*., (2019), *Artemisia's annual* herbal extract (precursor of artemisinin) was assessed against the MDA-MB-231 cell line (TNBC cells) when treated for 24 hours. *Artemisia annual* herbal was able to inhibit cell proliferation and induce apoptosis (Lang *et al*., 2019). In another study conducted by Zhou *et al*., (2022), DHA was able to inhibit cell proliferation in TNBC cell lines MDA-MB-231, MDA-MB-436, MDA-MB-468, and BT-549 in a concentration range of 0-50 μM for 24 hours in a dose-dependent manner using the 3-(4,5-dimethylthiazol-2-yl)-2H-tetrazolium bromide (MTT) assay (Zhou *et al*., 2021). Kajewole *et al*., (2020) investigated the toxicity of artemisinin and its derivatives in the HCC1937 cell line. It was discovered that WHN11 had the highest toxicity toward the cells and was able to inhibit cell growth (Kajewole *et al*., 2020).

Combination assays have also been conducted to observe how artemisinin and its derivatives work along with other drugs in treating TNBC. Zhou *et al*., (2021), also demonstrated that DHA combined with transferrin (TF), was able to inhibit cell growth of MDA-MB-231, MDA-MB-436, MDA-MB-468, and BT-549 cell lines in an MTT assay. After 24 hours of treatment, the IC_{50} values of MDA-MB-231 and MDA-MB-436 were 31.68 μM and 23.46 μM and the IC_{50} values of MDA-MB-468 and BT-549 were 4.49 μM and 5.86 μM (Zhou *et al*., 2021). This displays that artemisinin, and its derivatives can inhibit breast cancer cell growth *in vitro* and warrants further investigation into the therapeutic potential of such compounds.

Artemisinin derivatives display greatly enhanced activity in a range of diseases over the parent compound. Several studies demonstrate that artemisinin and its derivatives display anti-cancer activity, including a handful of reports of activity in TNBC cells, however, all these studies have been carried out *in vitro* and none have yet progressed to clinical trials (Bhaw-Luximon and Jhurry, 2017; Bu *et al*., 2021). The mode and mechanism of action of artemisinin and derivatives in cancer cells remain to be fully elucidated.

1.5 DNA binding as a mode of action for anti-cancer compounds

Several drugs either in advanced clinical studies or in current use as anti-cancer agents function by targeting DNA. In particular, by binding directly to the DNA, the drugs interfere with transcription or replication. Since cancer cells are highly proliferative, these processes are upregulated, making cancer cells more susceptible to agents which interfere with these DNA processes (Santos-de-Frutos and Djouder, 2021). The study of the interaction of small molecules with DNA is important for understanding the mechanisms of such interactions and for the development of novel treatments (Falese *et al*., 2021).

The most common drug-DNA interactions reported in the literature are external binding, intercalation, and groove binding (Rodrigues *et al*., 2021) which will be discussed in more detail below.

1.5.1. External binding

The external DNA binding mode occurs by electrostatic interaction between a small molecule and the DNA. Most cations, including the important cofactor Mg^{2+}, interact with the negatively charged backbone of DNA by external binding and thus some drugs consisting of metal complexes function by binding to DNA in this way. (Kelly *et al*., 1985). Examples of positively charged metal complexes that interact with DNA through external binding include Ru(II)

complexes, such as the DNA dye $[Ru(bpy)3]^{2+}$ (Joaqui-Joaqui *et al*., 2022). Another example that binds to DNA via external binding even though it is not a metal complex is artesunate (Kim *et al*., 2015). This mode of binding has been found to lessen charge-charge repulsion between ligand molecules and typically occurs when the ligand self-associates to create higher-order aggregates that may stack on the anionic DNA backbone (Sirajuddin *et al*., 2013).

1.5.2 Intercalation

Lerman first hypothesized the intercalation of planar organic molecules between DNA base pairs to explain the potent affinity of certain heterocyclic aromatic dyes, such as acridines, for DNA (Lerman, 1961). Significant *p*-electron overlap results from the intercalating action of planar heterocyclic molecules, which stack between neighboring DNA base pairs. Intercalators are molecules that stack parallel to the DNA backbone without creating covalent connections or severing hydrogen bonds between DNA bases. In a study conducted by Kadioglu *et al*., (2017), it was discovered artemisinin derivatives can bind to DNA via intercalation binding, thus inhibiting transcription (Kadioglu *et al*., 2017). Van der Waals, hydrogen bonding, hydrophobic and/or charge transfer are the only known forces that sustain the stability of the DNA-intercalator complex even more than DNA alone (Warring *et al*., 1994; Rehn and Pindur, 1996; Baginski *et al*., 1997; Shui *et al*., 2000; Martinez *et al*., 2005). In chemotherapeutic application, DNA intercalators are employed to prevent DNA replication in rapidly dividing cancer cells. Intercalators complex with DNA through a π-π stacking interaction, hence less susceptible to ionic strength in comparison to the two other binding types, groove, and external binding. The DNA must unwind significantly to create a gap between its base pairs for an intercalator to fit between them. Depending on the intercalators, the degree of unwinding varies. Comparing ethidium bromide to proflavine, the ethidium cation unwinds DNA by 26° and proflavine at about 17°. Intercalators are effective mutagens because these structural alterations can result in functional alterations, which frequently result in suppression of the transcription, replication, and DNA repair processes (Neidle and Abraham, 1984; Keck and Lippard, 1992).

1.5.3 Groove binding

Some small molecules interact with the minor groove of DNA through hydrogen bonds and van der Walls interactions. Minor groove binding molecules often consist of several aromatic rings joined by torsionally free bonds, such as pyrrole, furan (Sirajuddin and Badshah, 2013),

or artemisinin and its derivatives (Arafa, 2004). This type of binding has been observed in a study conducted by Maurya *et al*., (2020), where artemisinin and its derivatives were discovered to be minor groove binders. Drugs that bind to DNA through the minor groove typically have narrow, curved shapes which are isohelical to the curve of the minor groove and assist van der Waals interactions. These ligands can also form hydrogen bonds with bases, most frequently with the N_3 and O_2 of adenine and thymine. Groove binding molecules appear to be specific to adenine-thymine (AT) rich sequences. This preference in addition to the designed tendency for the electronegative pockets of AT sequences is probably due to better van der Waals contacts between the ligand and groove walls in this region since AT regions are narrower than GC groove regions and because of the steric hindrance in the latter, presented by the C_2 amino group of the guanine base (Mei and Barton, 1986). However, a few synthetic polyamides with a focus on the GC and CG regions of the grooves have been developed, such as lexitropsins and imidazole-pyrrole polyamides (Sirajuddin and Badshah, 2013).

1.6 Knowledge gap and Problem Statement

TNBC is a subtype of breast cancer that lacks expression of the ER-, PR^-, and $HER2^-$ receptors. This subtype represents a very aggressive form of the disease, is more frequently diagnosed in women of African descent, and there are no standardized targeted treatments due to the absence of the receptors mentioned above. Since TNBCs harbor high levels of *BRCA1/2* and *TP53* mutations, it has been suggested that these cells may be more susceptible to agents that cause DNA damage. One way to induce DNA damage specifically in cancer cells is to interfere with topoisomerase function. Topoisomerases relieve torsional strain during replication and transcription, both of which are upregulated in rapidly dividing cells, making cancer cells more susceptible to topoisomerase inhibition. Recent reports have examined whether artemisinin and its derivatives, originally used as antimalarials, could be repurposed as anti-cancer drugs, however, this research has not progressed to either animal models or clinical trials. There is limited data available regarding the mechanism of action of artemisinins in this disease and mechanisms likely differ between various derivatives due to the altering of functional groups. Recent studies have suggested that artemisinins may also function differently in different cancer types, with limited data available regarding the mechanism of action of artemisinins in TNBC. A recent, unpublished study demonstrates that a novel artemisinin derivative, WHN11, was highly toxic to TNBC cells *in vitro* and mediates its toxicity independently of ROS

induction by a dual mechanism involving both apoptosis and autophagy, however, the ability of this derivative to bind to DNA and interfere with DNA processes is unknown. While there have been some reports on the potential of artemisinin derivatives to both bind to DNA and act as potential topoisomerase inhibitors, the effect that the novel derivative WHN11 has on topoisomerase function, and its ability to cause DNA damage in TNBC cells remain to be explored.

1.7 Hypothesis

The novel artemisinin derivative WHN11 can interfere with topoisomerase function and cause DNA damage in TNBC cells.

1.8 Aim

This study aimed to determine the effects of artemisinin, the previously reported derivative artesunate, and the novel derivative WHN11 on topoisomerase function and to evaluate the compounds' potential in inducing DNA damage.

1.9 Objectives

- Determine the toxicity of artemisinin and its derivatives artesunate and WHN11 in TNBC and non-cancerous breast epithelial cells
- Determine whether artemisinin and its derivatives can bind to DNA directly (to potentially interfere with DNA processes)
- Determine the effect of artemisinin and its derivatives on topoisomerase function.
- Evaluate whether artemisinin and its derivatives cause DNA damage in TNBC cells.
- Determine whether artemisinin and its derivatives synergize in terms of cytotoxicity with known topoisomerase inhibitors in TNBC.

CHAPTER TWO

MATERIALS AND METHODS

2. Materials

2.1 Compounds

Artemisinin, artesunate, and WHN-11 were prepared and supplied by Prof Richard Haynes of the Centre of Excellence for Pharmaceutical Sciences, North-West University, South Africa. The compounds were dissolved in dimethyl sulfoxide (DMSO), made up to a stock concentration of 100 mM, and stored at -20 °C.

The following reagents were purchased from Sigma-Aldrich: methylene blue solution (catalog #: 03978), DMSO (catalog #: 41639), sodium hydroxide (catalog #: S5881), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (catalog #: 2236020), hydrocortisone (catalog #: H0888), hydrogen peroxide 30% (v/v) (catalog #: H1009), 7.5% (w/v) sodium bicarbonate (catalog #: S8761), insulin solution human (catalog #: 19278) ampicillin trihydrate (catalog #: A-1593), sodium dodecyl sulfate reagent (SDS) (catalog #: L4509), N,N, N',N' tetramethylethylenediamine (TEMED) (catalog #: T9281), resazurin sodium salt (catalog #: 62758-13-8), ammonium persulfate (catalog #: 09913), Ponceau S (catalog #: P3504), bromophenol blue (catalog #: B8026), 37:1 acrylamide/bis-acrylamide (acryl/bis) solution (catalog #: A6050), ammonium persulfate (APS) (catalog #: 09913), etoposide (catalog #: E1383), low gelling temperature agarose (catalog #: A4018), cis-diammineplatinum (II) dichloride (cisplatin) (catalog #: P4394), hydrochloric acid (catalog #: 258148), ethidium bromide (EtBr) (catalog #: E8751), β-mercaptoethanol, , cholera toxin from *Vibrio cholerae* (catalog #: C8052), hydrocortisone (catalog #: H0888), and Tris buffered saline (TBS) tablets (catalog #: T5030). Sodium chloride (catalog #: GLS GE8374) and Zymo Universal Quick DNA MiniPrep Kit (catalog #: D40685) were purchased from Inqaba Biotechnology Industries. Magnesium chloride (catalog #: SAAR4123000), glycine (catalog #: SAAR2676600EM), Tween-20 (catalog #: 8.22184.0500), glycerol (SAAR2676520lc), sodium lauryl sulphate (SAAR5823610EM) and glacial acetic acid (catalog #: SAAR1021000LC) were purchased from Merck group (now supplied by Sigma-Aldrich). Invitrogen ultra-pure calf thymus DNA (CT-DNA) solution (10 mg/mL, catalog #: 15-633-019), Invitrogen topoisomerase 1 (catalog #: 38-042-024), trypsin-ethylenediaminetetraacetic

acid disodium salt dihydrate (catalog #: 15400-045), propidium iodide (P1304MP) and 20 mM Hoescht 33342 solution (catalog # 62249) were purchased from Thermo Fisher Scientific. Phosphate buffer saline (PBS) tablets (18912-014), Dulbecco Modified Eagle Medium (DMEM) (catalog #: 21063045), GlutaMAX^{TM} (catalog #: 35050038) and nuclease-free water (catalog #: R0582) were purchased from LTC Tech. Phenol red-free Roswell Park Memorial Institute (RPMI) 1640 (catalog #: 11835105) and Dulbecco's Modified Eagle Medium/Nutrient Mixture Ham's F-12 (catalog #: 11320-033) were purchased from Gibco, the Zyppy plasmid Miniprep Kit (catalog #: D4020) was purchased from Zymo research, bovine serum albumin (BSA) (catalog #: GK4012) and camptothecin (catalog #: GA9966) were purchased from Glentham Life Sciences, Capricorn fetal bovine serum (FBS) (catalog #: FBS-GH2A) was purchased from Biocom Africa, BLOTTO (catalog #: SC-2325) was purchased from Santa Cruz Biotechnology, recombinant human epidermal growth factor (catalog #: 236-E-200) was purchased from WhiteSci, 6x Purple Gel loading Dye (catalog #: B7024S) and EcoR1 (catalog #: R0101S) was purchased from New England Biolabs.

2.2.1 Cell Lines and Culture Conditions

The Triple-Negative Breast Cancer cell line, HCC70 [*Homo sapiens* (ATCC-CRL-2315)] was obtained from the American Type Culture Collection (ATCC) and maintained in phenol red-free Rosewell Park Memorial Institute (RPMI) 1640 media which contained 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS), 1x Penicillin (100 U/mL), Streptomycin (100 µg/mL)-Amphotericin B (25 µg/mL) Solution (PSA), 1x Glutamax^{TM} and sodium bicarbonate 0.25% (w/v) solution. The non-cancerous breast epithelium cell line, MCF12A [*Homo sapiens* (ATCC-CRL-10782)] was obtained as a gift from Professor Anna Mart Engelbrecht (Cancer Research Group, Stellenbosch University) and was maintained in DMEM: Hams F12 (1:1 ratio) supplemented with 10% (v/v) FBS, 1x PSA, 10 µg/mL insulin, 100 ng/mL cholera toxin, 500 ng/mL hydrocortisone and 20 ng/mL Human Epidermal Growth Factor (hEGF). All cell lines were maintained in a humidified incubator at 37 ℃ and 9% CO_2.

2.2.2 Cytotoxicity

The resazurin assay was conducted according to the protocol previously described by Oderlino *et al*., (2018). The HCC70 cells and MCF12A cells were seeded at a cell density of 2 x 10^5 cells/mL in Costar clear 96 well plates at 37 °C and 9% CO_2 and allowed to adhere overnight. The cells were treated with DMSO vehicle control or artemisinin between concentrations of 0.32 and 500 µM for the parent compound. Artesunate and WHN11 had concentrations between 0.32 and 250 µM. After 96 hours of treatment of the cells, cell viability was determined by adding 20 µL of 0.54 mM resazurin sodium salt in phosphate-buffered saline (PBS) and incubating for an additional 4 hours. The reduction of resazurin to resorufin by viable cells was assessed by fluorescence readings (excitation 560 nm, emission 590 nm) in a Spectramax M3 plate reader (Molecular Devices, San Jose, CA, USA). Fluorescence readings were converted to percentage survival and the half-maximal inhibitory concentration (IC_{50}) of the compounds was determined by non-linear regression using GraphPad Prism software, version 6 (GraphPad Inc, San Diego, CA, USA).

2.2.3 Computational DNA docking studies

To gain insight into the potential of artemisinin, artesunate, or WHN11 to bind to B-DNA (PDB: 129D), molecular docking was performed using AutoDock4.2 (Freeware maintained by Centre for Computational Structural Biology, Scripps Institute, USA) according to Mbaba *et al*., (2020). The 3D structures of DNA and the drugs (artemisinin and artesunate) were downloaded from Protein Data Bank (PDB) and OpenBABEL GUI was used to convert artemisinin or artesunate from SMILE to PDB. The Hoechst 33342 dye was removed as it was bound to DNA and the 3D structure of WHN11 was obtained from Professor Richard Haynes of the Centre of Excellence for Pharmaceutical Sciences, North-West University, South Africa. From the PDB files (ligand or receptor), PDBQT files were created to allow docking to occur. This was conducted by opening the PDB file with the use of Autodock Vina, deleting the water molecules, adding polar hydrogens, computing charges, and saving the file as PDBQT. To determine the possible binding site and the binding affinity of drug–DNA interaction, AutoDock4.2 was employed and PyMOL (PyMOL2)-2.4.1 or Liplot+ v.1.4.5 was used to identify the interacting residues. The dimension of the three compounds of the grid size was set on x: 62, y: 66, and z: 126 planes. Genetic algorithm population size, number of evaluations of energy, and number of generations were used as parameters for docking. Liplot+ v.1.4.5 was used to visualize the molecular interactions.

2.2.4 Agarose Gel Electrophoresis-based Ethidium bromide Competition Assay

The abilities of the artemisinin compounds to interact with human genomic DNA (gDNA) isolated from HCC70 cells were assessed by ethidium bromide competition via agarose gel electrophoresis according to Gramni *et al*., (2019) with minor modifications. The HCC70 cells were washed with 5 mL of PBS. The cells were then lifted using 0.25 % (v/v) trypsin in 3 g/L EDTA and collected by centrifugation at 2000 rpm for 2 minutes. The supernatant was discarded and the gDNA was extracted using Zymo Universal Quick DNA MiniPrep Kit. In a 20 µL reaction volume, DMSO 0.2% (v/v) vehicle control, artemisinin or artesunate (50 or 200 µM), or WHN11 (10, 50, or 200 µM) or cisplatin positive control (200 µM) was incubated with 100 ng of gDNA in a water bath at 37 ℃ for 4 hours. Thereafter agarose gel electrophoresis was carried out using a 0.8% (w/v) agarose gel in 1×Tris-acetic acid EDTA (TAE, buffer components) for one hour at 90 V in 1×TAE, followed by staining with 0.5 µg/mL EtBr (dissolved in distilled water) visualization of the DNA was conducted under ultraviolet (UV) light in a ChemiDoc XRS System (BioRad). Densitometry analysis was conducted using ImageJ software, version 6 (1.42L; National Institutes of Health USA freeware, JACoP).

2.2.5 UV-Vis absorption spectroscopy

The A260 assay was performed in accordance with Mbaba *et al*., (2020). Artemisinin, artesunate, or WHN11 at concentrations of 10, 50, and 200 µM (in line with Mbaba *et al*., 2020) were incubated with 100 ng of CT-DNA in a total reaction volume of 100 µL in a 96-well UV-Vis plate for 15 minutes at temperatures ranging from 18-25 ℃ before the absorbance was read at wavelengths between 230 and 290 nm using a SpectraMax M3 microplate plate reader (Molecular Devices, San Jose, CA, USA). Controls included a 200 µM sample of the test compounds artemisinin, artesunate, and WHN11 lacking calf thymus DNA and a sample of CT-DNA alone (100 ng) in Milli-Q water, as well as CT-DNA incubated with 0.2% (v/v) DMSO vehicle control (corresponding to the highest compound concentration of 200 µM). The absorbance readings were analyzed and graphs were generated using GraphPad Prism software, version 6.

2.2.6 Competitive DNA binding assays

The ability of the compounds to competitively bind to DNA by intercalation and minor groove binding was investigated using methylene blue and Hoechst assays, respectively, according to Mbaba *et al*., (2020). Artemisinin, artesunate, or WHN11 at concentrations of 10, 50, and 200 µM (in line with Mbaba *et al*., 2020) were incubated with 100 ng of CT-DNA in a final volume of 100 µL Milli-Q-water in a black-walled clear bottomed 96-well plate for 15 minutes at temperatures ranging from 18-25 ℃. A solution of 15 µg/mL methylene blue or 10 µg/mL Hoechst 33342was added to the samples and incubated for 10 minutes in the dark before fluorescence spectra (excitation 665 nm, emission 650-750 nm for methylene blue, and excitation 350 nm, emission 400-600 nm for Hoechst 33342) were acquired. Cisplatin (200 µM) was employed as a positive control in the DNA intercalation study with methylene blue dye, whilst 0.2% (v/v) DMSO (equivalent to 200 µM of the compounds) served as a negative control in both assays. Samples containing either methylene blue or Hoechst 33342 dye and compound (200 µM) without DNA were included as additional controls. Fluorescence readings were analyzed, and graphs were generated using GraphPad Prism software, version 6.

2.2.7 Alkaline Comet Assay

The alkaline comet assay was performed according to Olive & Banath, (2006). HCC70 cells were seeded at a density of 2 x 10^5 cells/well in a 12-well plate overnight at 37º C and 9 % CO_2. Cells were treated with DMSO 0.2% (v/v) (negative control), hydrogen peroxide, or camptothecin (20 µM or 15 µM as positive controls) for 1 hour or the hit compounds, artemisinin, artesunate, or WHN11 (5, 20, 200 µM) for 2 hours at temperatures ranging from 18-25 ℃. Thereafter, cells were lifted using 0.25 % (v/v) trypsin in 3 g/L EDTA and collected by centrifugation at 1600 rpm for five minutes and the pellet was resuspended in ice-cold PBS to a cell density of 2 x 10^4 cells/mL. Low gelling temperature agarose [1 % (w/v)] was used to precoat 75 x 25 mm frosted slides and allowed to airdry to a thin film. The treated HCC70 cells were rapidly mixed with 1 % (w/v) low gelling temperature agarose, placed onto the agarose coated slides, and allowed to set for 2 minutes. Lysis was performed by submerging the slides in A1 Lysis Buffer [1.2 M NaCl; 100 mM Na_2EDTA; 0.1 % (w/v) sodium lauryl sarcosinate; 0.26 M NaOH] in the dark overnight at 4 °C. Following lysis, the slides were submerged in A2 Buffer rinse and electrophoresis solution [30 mM NaOH; 2 mM Na_2EDTA] for 20 minutes, repeated three times. Electrophoresis was performed in an A2 solution for 25 minutes at 20 V and 40 mA. The slides were then rinsed with distilled deionized (dd) water, stained with 10

ug/mL propidium iodide (PI) for 20 minutes, and rinsed again with ddH_2O to remove excess stain. The cells were viewed using an Olympus BX43 (Olympus Corporation, Japan) optical fluorescence microscope, and images were taken with an Olympus DP74 digital camera using cellSens Entry Software. The cells were captured using a U-MWG wide green filter cube with an excitation wavelength of 510 nm, an emission wavelength of 590 nm, a beam splitter wavelength of 570 nm, and viewed at 20 X magnification. A minimum of fifty images were taken per treatment and analyzed using the Image J Plugin, OpenComet version 1.53e. The formula (OM) [OM = Tail length x Tail% DNA] was used to measure the olive moment. GraphPad version 6 was used to plot the data obtained from excel after analyzing the images. The Mann-Whitney test was used to obtain statistics in GraphPad version 6 where each compound was relatively compared to the vehicle control, DMSO, and the results were displayed using the Whisker box plot.

2.2.8 Topoisomerase inhibition Analysis

Topoisomerase inhibition analysis was conducted according to Kadioglu *et al*., 2017 with modifications. The reaction mixtures contained 250 ng of pCDNA-CL8D-His CYP2A6 plasmid DNA (a kind gift from Dr. Laura Dingle, Biomedical Biotechnology Research Unit, Department of Biochemistry and Microbiology, Rhodes University), diluted 1 x reaction buffer (50 mM Tris HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA and 30 µg/mL BSA), two units of TOPO 1 enzyme, DMSO vehicle control, positive cisplatin positive control (methylene blue assay) or test compounds (as above), made up to a final volume of 10 µL in nuclease-free water. The reaction mixtures were incubated for 2 hours at 37 °C. A restriction digest sample was included as a control for linearized plasmid which contained pCDNA-CL8D-His CYP2A6 plasmid (250 ng), 1 µL of 10 x universal NEB CutSmart buffer, 8 units/mL of the NEB restriction enzyme (EcoR1) and the difference was made up with nuclease-free water to achieve a total volume of 10 µL, and incubated in a heating block at 37 °C overnight. The entire Topoisomerase 1 reaction mixture or 6 µL of the restriction digest was added to 2 µL of 6 × loading dye and loaded onto a 0.6% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). TAE buffer was used for electrophoresis of the gel at 90 V for 1 hour. The gel was then stained with 50 µg/mL of EtBr for 20 minutes, de-stained for 20 minutes with distilled water, and viewed with a ChemiDoc XRS System (Bio-Rad Laboratories) with Image Lab Software (Bio-Rad Laboratories).

2.2.9 Combination assay for the analysis of synergistic relationships between compounds

A combination assay for the analysis of synergistic relations between compounds was conducted according to Chou and Talalay, (1983) with minor modifications. Synergistic relationships between the artemisinin compounds and known topoisomerase inhibitors were investigated by the combination of two compounds in constant ratios (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100). HCC70 cells were seeded overnight at a density of 2 x 10^5 cells per well in Costar clear 96-well plates at 37 °C and 9% CO_2. Cells were treated with compounds for 96 hours and subjected to a resazurin assay as previously described in section 2.5 (cytotoxicity assay). Combination indexes (CI) were developed from the constant ratio of combined drugs using CompuSyn software, version 1.0. CI was defined as synergistic (CI < 1), additive (CI = 1), or antagonistic (CI > 1) from a graph representing the CI versus Fa (fraction of cells affected where 0 represents 100% survival/0% cell death and 1 represents 0% survival/100% cell death).

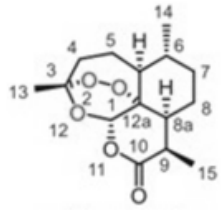
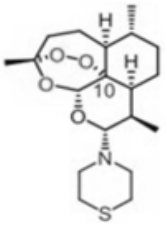
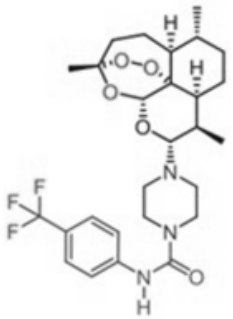
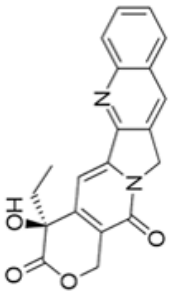
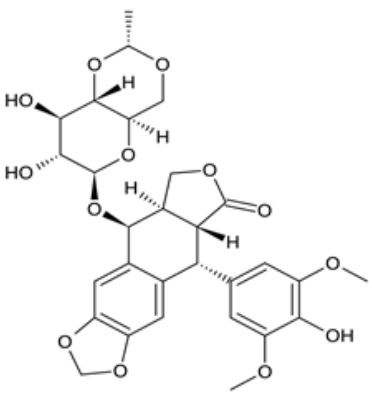
CHAPTER THREE

RESULTS

3.1 Sensitivity profiles of triple-negative breast cancer and non-cancerous breast epithelial cells to artemisinin and selected derivatives

Artemisinin and its derivatives were screened against HCC70 triple-negative breast cancer and MCF12A non-tumorigenic breast epithelial cell lines to determine the half-maximal inhibitory concentrations (IC_{50}) of the compounds using the resazurin assay (Table 3.1). Artemisinin is an established anti-malarial agent (Gao *et al*., 2018), and is modified at carbon 10 (C10) to produce derivatives (Woodrow *et al*, 2005). Artesunate is a semi-synthetic derivative of artemisinin and is synthesized from dihydroartemisinin (DHA) by reaction with succinic anhydride (Morris *et al*., 2011). The novel WHN11 contains piperazine carboxamide and trifluoromethyl-benzene groups that are bound to artemisinin (Kajewole *et al*., 2020). In this assay, the fluorescent resazurin dye (blue) was reduced by oxidoreductase enzymes to a fluorescent pink product, resorufin, as a measure of the metabolic activity of viable cells (Präbst *et al*., 2017). The structures of the artemisinin compounds are displayed in Table 3.1. Among the compounds, WHN11 displayed the highest toxicity, displaying IC_{50} values of 3.20 ± 0.51 μM in HCC70 and 8.35 ± 0.92 μM in MCF12A cells, followed by artesunate with IC_{50} values of 25.48 ± 1.40 μM in the HCC70 cell line and 87.53 ± 1.94 μM in the MCF12A cell line. Artemisinin was markedly less toxic than its derivatives, demonstrating IC_{50} values of 214.70 ± 2.33 μM in the HCC70 cell line and 298.30 ± 2.47 μM in the MCF12A cell line. Artemisinin and its derivatives displayed greater toxicity against HCC70 TNBC cells as compared to MCF12A non-cancerous cells. This was indicated by selectivity indexes (SI), calculated by dividing the IC_{50} of the compound in the MCF12A cell line by the IC_{50} in the HCC70 cell line, of 1.39, 3.44, and 2.61 for artemisinin, artesunate, and WHN11, respectively. The topoisomerase inhibitors camptothecin and etoposide were included for comparison as clinically utilized chemotherapeutic agents (Ferraro *et al.*, 2000). Camptothecin displayed an IC_{50} of 0.05 ± 1.30 μM in the HCC70 cell line and an IC_{50} value of 0.04 ± 1.40 μM in the MCF12A cell line, indicating that the compound was highly toxic, in the nanomolar range, to both cancer and non-cancer cell lines. Although camptothecin is an approved drug, the SI value of 0.8 indicates that the compound was more toxic to the non-cancerous breast epithelial cells than the TNBC cells. Etoposide was used as a second control, and it was less toxic to the cells compared to camptothecin displaying IC_{50} values of 6.00 ± 0.79 μM in the HCC70 cell line and 99.00 ± 2.00 μM in the MCF12A cell line.

Table 3.1: Cytotoxicity screening of artemisinin and its derivatives artesunate and WHN11 against the HCC70 TNBC and MCF12A non-cancerous breast epithelial cell lines

Name of compound	Structure	IC_{50} (µM)	
		HCC70	MCF12A
Artemisinin		214.70 ± 2.33 SI: 1.39	298.30 ± 2.47
Artesunate		25.48 ± 1.41 SI: 3.44	87.53 ± 1.94
WHN11		3.20 ± 0.51 SI: 2.61	8.35 ± 0.92
Camptothecin		0.05 ± 1.30 SI: 0.80	0.04 ± 1.40
Etoposide		6.00 ± 0.79 SI: 16.50	99.00 ± 2.00

*SI: selectivity index, TNBC = triple-negative breast cancer, IC_{50} = half maximal inhibitory concentration, n=3

3.2 Assessment of DNA binding abilities of artemisinin compounds

In this study, we carried out an extensive investigation of the ability of artemisinin and its derivatives to bind directly to DNA as a potential mode of action and to attempt to explain the differences in cytotoxicity between the parent compound and its derivatives. Cytotoxicity may be related to DNA binding as the binding of a molecule to DNA may affect DNA replication and transcription, processes that are upregulated in rapidly dividing cancer cells (Cepeda *et al*., 2007; Ahmad, 2010).

3.2.1 DNA binding analysis *in silico*

Computational docking studies were conducted to predict whether artemisinin and its derivatives can interact with B-DNA (PDB: 129D) *in silico*. Molecular docking is a tool used to understand potential interactions between the drug and DNA in terms of their binding site, location, and binding mode (Maurya *et al*., 2020). The B-DNA "receptor" structure was derived from a co-crystallized complex with the Hoechst 33342, but the Hoechst 33342 was removed before conducting molecular studies. Auto Dock tools 1.5.6 were used for *in silico* simulations and the potential interactions were observed using PyMOL2 and Discovery Studio 2020. The results are displayed as surface images and stick models in Figure 3.1. Figures, A, C, and E are three-dimensional models that display the surface view of the DNA upon interaction with artemisinin (A), artesunate (C), or WHN11 (E), where the ligands (artemisinin, artesunate, or WHN11, shown in black) were situated in the minor groove of the DNA (shown in grey). Figures 3.1B, D and F are zoomed-in images of the DNA-compound interaction in a stick model representation. The ligands (shown in black) were bound to DNA (shown in grey) for artemisinin, artesunate, and WHN11 through hydrogen bonds (shown as dashed yellow lines). All three compounds formed four potential hydrogen bonds to DNA. Artemisinin formed potential hydrogen bonds to the DNA, at guanine and cytosine residues, at positions G4, C21, G22, and C23 with bond distances of 1.176, 1.540, 2.410, 3.965 Å, with binding energies of -7.3, -7.1, -6.8 and -6.8 kcal/mol, respectively (Figure 3.1B). Artesunate formed potential hydrogen bonds with the DNA, at adenine and thymine residues, at positions A6, T7, T19, and T20, with bond distances of 1.619, 2.023, 2.298, 3.722 Å, with binding energies of -6.1, -6.3, -6.5 and -6.5 kcal/mol, respectively (Figure 3.1 D). Finally, the novel artemisinin derivative WHN11 also displayed potential hydrogen bonds to the DNA guanine and cytosine residues, at positions C3, G4, C21, and G22 with bond distances of 3.539, 5.118, 6.361, 13.137 Å, with binding energies of -8.9 -8.8, -8.5 and -8.5 kcal/mol, respectively (Figure 3.1F).

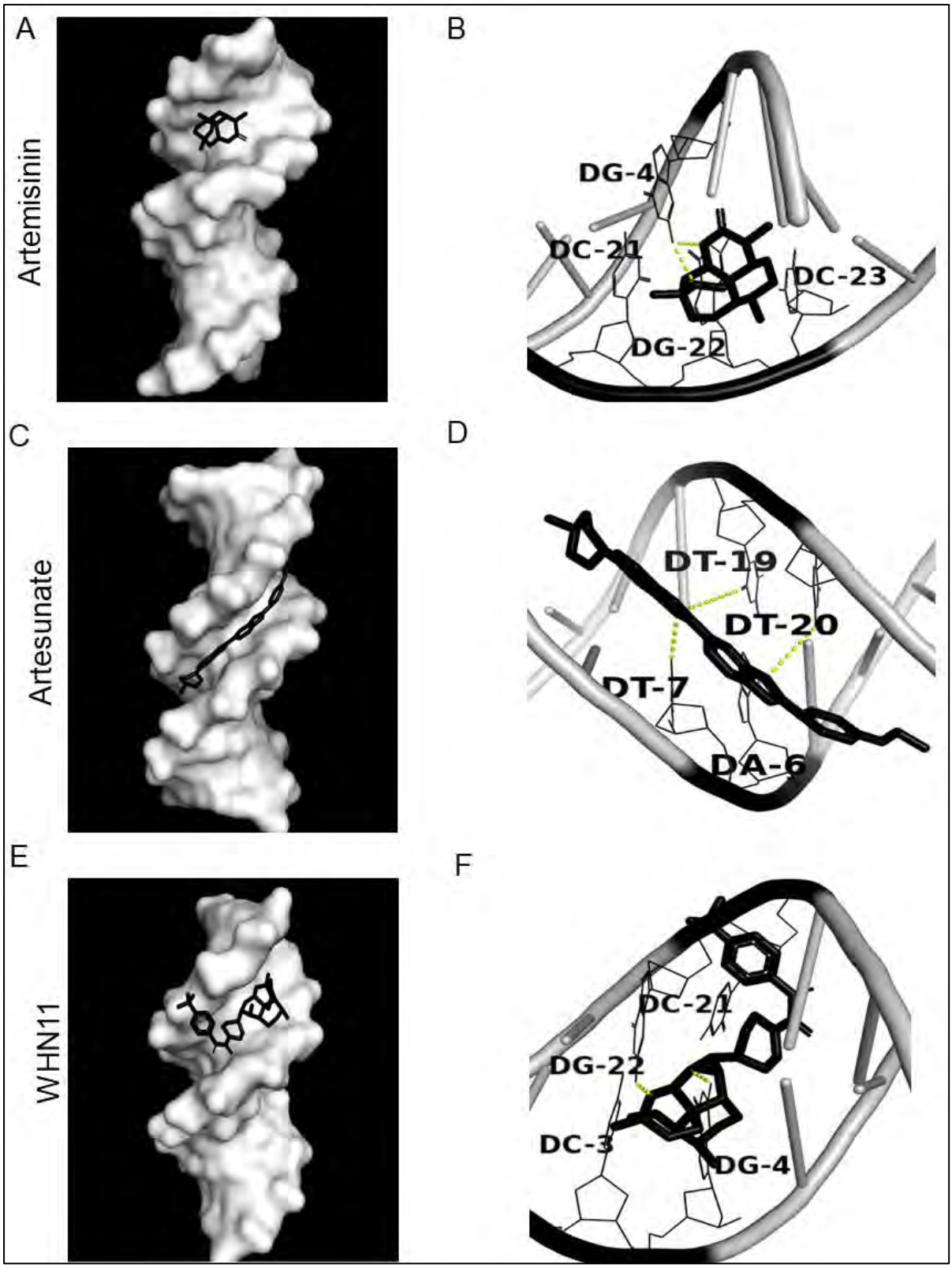


Figure 3.1: Computational docking analysis for the *in silico* prediction of DNA binding capabilities of artemisinin and its derivatives. The images were obtained through Discovery Studio 2.5 and PyMOL2 A, C, and E: Three-dimensional surface view with artemisinin (A), artesunate (C), and WHN11 (E) docked in the DNA minor groove. B, D, and F: zoomed-in stick models of artemisinin (B), artesunate (D), and WHN11 (F) bound to specific residues of the DNA. The DNA sugar-phosphate backbone is shown as a three-dimensional stick model in grey while the ligand of the three compounds is shown in black. Hydrogen bonds are indicated by broken yellow lines. DG = deoxyguanine, DC = dexoycytosine, DA = deoxyadenosine, and DT = deoxythymine.

3.2.2 Binding to genomic DNA by ethidium bromide competition

Agarose gel electrophoresis (AGE) was used to determine the ability of artemisinin and its derivatives to interact with genomic DNA (gDNA) extracted from HCC70 cells (Figure 3.2). Ethidium bromide staining was used in AGE as it binds to DNA by intercalating between the base pairs of the DNA (Sigmon and Larcom, 1996). In this assay, the ability of the compounds to bind gDNA was assessed by a decrease in band intensity on the agarose gel because of the compounds competing with ethidium bromide (Figure 3.2A). Cisplatin, a known DNA intercalator (Dasari *et al*., 2017) was used as a positive control (second lane) to displace the ethidium bromide, which yielded a reduced band intensity compared to the DMSO vehicle control (first lane). As seen from the third to the ninth lane of the gel, artemisinin or artesunate at concentrations of 50 and 200 µM or WHN11 at 10, 50 and 200 µM were not able to displace the ethidium bromide as the band intensity was not reduced and was like that of DMSO vehicle control. This suggested that there was no interaction between the compounds at different concentrations with gDNA. Densitometry analysis was carried out (Figure 3.2B) using GraphPad version 6 to assess statistical significance revealing that cisplatin ($P<0.05$, *) was the only compound that was able to compete with and displace ethidium bromide and bind to gDNA via intercalation as demonstrated by the significant decrease in band intensity compared to DMSO vehicle control.

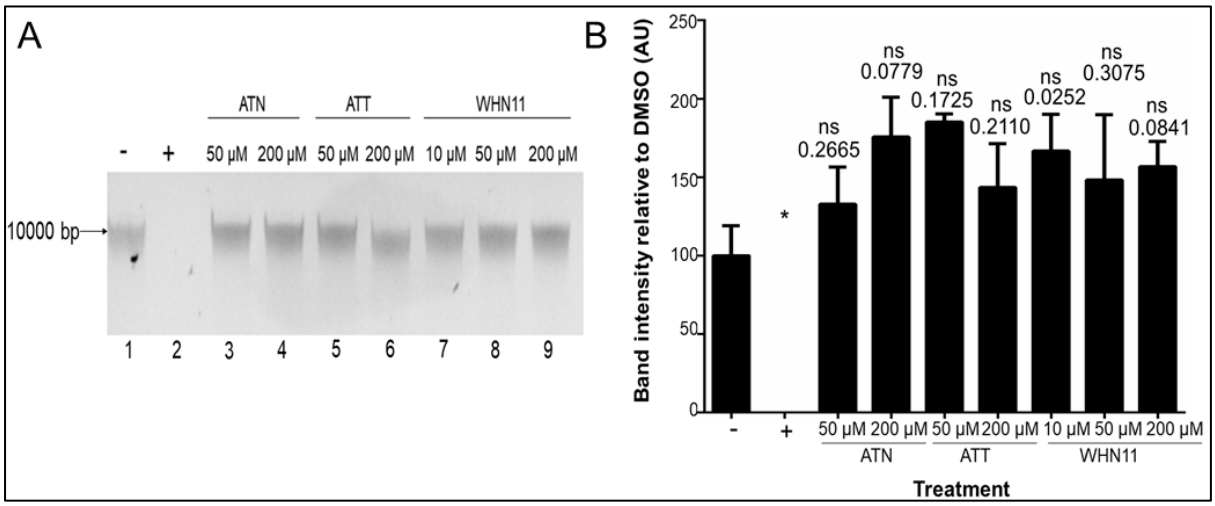


Figure 3.2: Assessment of binding of artemisinin and its derivatives to human genomic DNA by agarose gel electrophoresis. A) 0.8% (w/v) agarose gel stained with 0.5 µg/µl ethidium bromide containing genomic DNA isolated from HCC70 cells and incubated with artemisinin and its derivatives. (-): DMSO control 0.2% (v/v), (+): Cisplatin control (200 µM), ATN: Artemisinin, ATT: Artesunate B) Densitometry analysis of agarose gel electrophoresis for triplicate DNA binding experiments/agarose gels using Image J (NIH) and plotted using GraphPad 6 Prism. The Mann-Whitney test was used to determine the statistical significance of each treatment in comparison to the DMSO vehicle control, 2: Cisplatin (200 µM; $p<0.005$), 3: Artemisinin (50 µM), 4: Artemisinin (200 µM), 5: Artesunate (50 µM), 6: Artesunate (200 µM), 7: WHN11 (10 µM) and 8: WHN11 (50 µM), and 9: WHN11 (200 µM)

3.2.3 UV-Vis absorption spectroscopy

Following on from the AGE studies using gDNA, a more sensitive and quantitative, UV-Vis spectroscopic assay was employed to assess the binding of the artemisinin compounds to DNA, which has the potential to better detect small changes in absorbance upon binding of the compounds to DNA. The study was conducted whereby artemisinin and its derivatives were incubated with CT-DNA and the absorbance was recorded in the UV range (Sirajuddin *et al*., 2013). In this assay, an increase in absorbance at 260 nm, or hyperchromic shift, is witnessed when a molecule binds to CT-DNA via electrostatic interaction, while a hypochromic effect and red shift are observed when a compound binds by intercalation (Sirajuddin *et al*., 2013). The hyperchromic effect is caused by the CT-DNA becoming uncoiled and more nucleotide bases being exposed because of the binding of the compound (Sirajuddin *et al*., 2013). Artemisinin, artesunate, and WHN11 at the highest concentration of 200 µM in the absence of CT-DNA (shown in red) were added to the assay as controls to confirm that the compounds alone did not absorb light in the tested range of wavelengths. As demonstrated in Figure 3.3A-C, artemisinin, artesunate, and WHN11 alone did not produce an absorbance peak in the tested range and thus do not interfere with the assay. Artemisinin, artesunate, or WHN11 at concentrations of 10, 50, and 200 µM shown in purple, orange and black, respectively (Figure 3.3A-C) was incubated with CT-DNA and the absorbance was recorded at wavelengths from 230-300 nm and compared to samples containing the DMSO vehicle control [0.2% (v/v)], equivalent to that in the 200 µM sample of the compounds, displayed in blue) or CT-DNA alone (displayed in green). Artemisinin at all concentrations did not appear to bind to CT-DNA as all the absorbance peaks at 260 nm for the DNA incubated with artemisinin at the different concentrations were equivalent to that of the DMSO vehicle control or CT-DNA sample alone and no red shift was observed. On the other hand, when artesunate (Figure 3.3B) at all three concentrations was added to the DNA, the absorbance peaks at 260 nm shifted above that of the DMSO vehicle control (displayed in blue) and CT-DNA (displayed in green), suggesting that artesunate was binding to CT-DNA via electrostatic interaction causing a hyperchromic shift as nucleotide bases were exposed. This effect was, however, not observed to be dose-dependent. The results for incubation of WHN11 with CT-DNA (Figure 3.3C) were like that of artemisinin, as the three different concentrations of WHN11 did not affect the absorbance peaks of the DNA compared to that of the DMSO vehicle control and CT-DNA alone, suggesting that WHN11 was not binding to CT-DNA. This suggests that the compound does not interact with CT-DNA through intercalation.

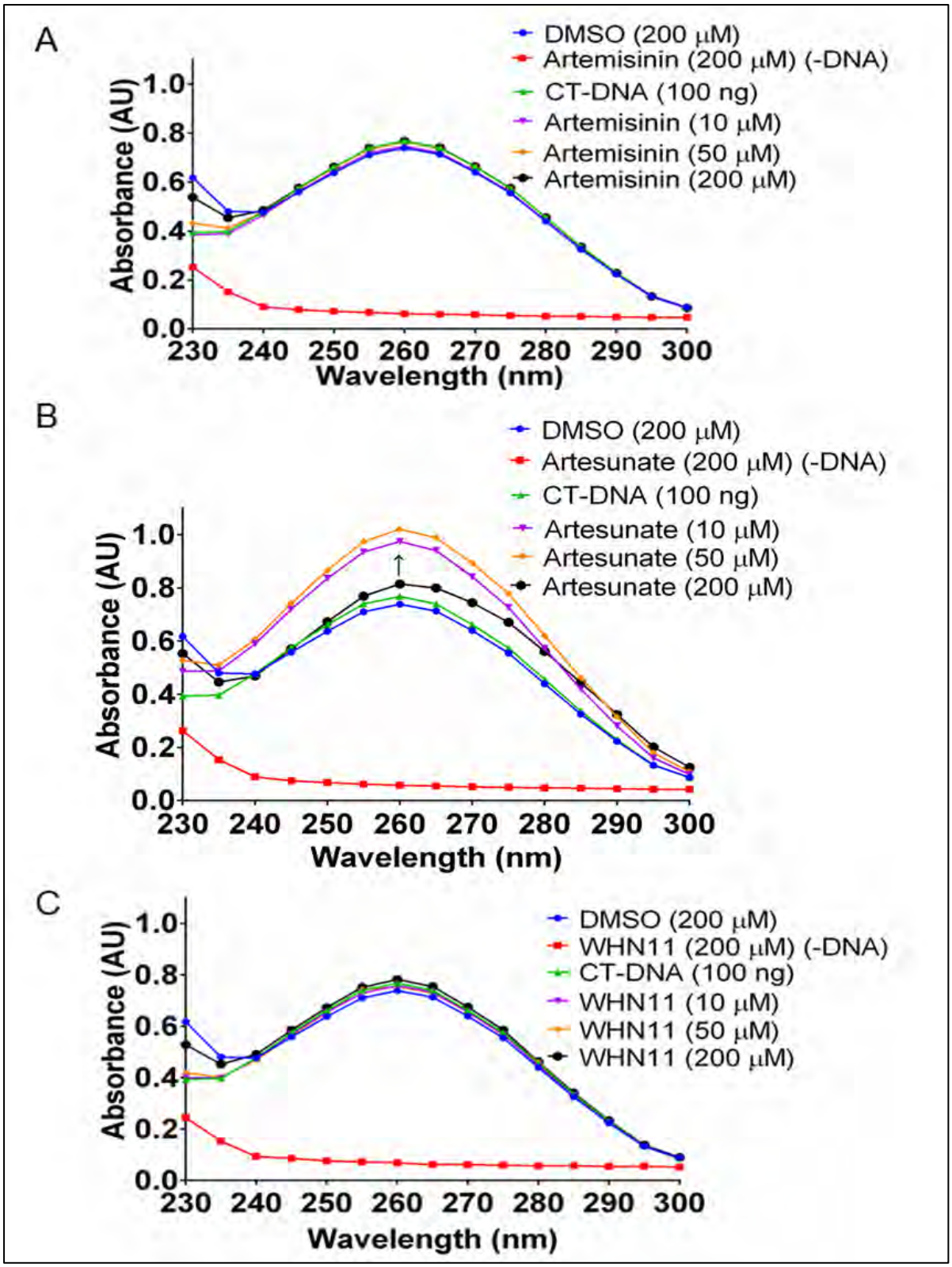


Figure 3.3: Assessment of the ability of artemisinin and its derivatives to interact with CT-DNA at different compound concentrations. All the compounds are incubated with CT-DNA unless stated otherwise (-DNA). A-C) Changes in absorbance at 260 nm of 100 ng CT-DNA upon addition of artemisinin (A), artesunate (B), or WHN11 (C) at varying concentrations (10, 50, and 200 μM). DMSO was used as a negative control at a concentration [0.2% (v/v)] equivalent to the highest compound concentration (200 μM).

3.2.4 Methylene Blue DNA Intercalation Assay

Next, the methylene blue assay was used as a specific assay to determine whether artemisinin, artesunate, and WHN11 were DNA intercalators. Methylene blue is known to display a strong fluorescence emission at 660-670 nm, which decreases when interacting with DNA (Vardevanyan *et al*., 2013). A strong DNA intercalator increases the fluorescence emission of methylene blue as it competes with the dye for DNA, displaces the bound dye molecules, and increases their concentration in the medium. Cisplatin, dissolved in an aqueous solution, was used as a positive control as it is a known DNA intercalator able to form covalent bonds with guanine bases thus interfering with the base pairing between DNA strands (Mbaba *et al*., 2020). As depicted in Figure 3.4A-C, 200 μM cisplatin (shown in blue) incubated with CT-DNA and methylene blue indeed increased the fluorescence peak at 665 nm above that of the methylene blue and CT-DNA alone control (shown in purple), validating the assay. DMSO either with (in red) or without CT-DNA (in green), was used as vehicle control for the assay. Artemisinin, artesunate, and WHN11 alone (200 μM), without CT-DNA (displayed in orange), were also included as controls. The controls that lacked CT-DNA but contained methylene blue specified the maximal absorbance of methylene blue in the unbound state. The DMSO with the CT-DNA sample did display an increase in fluorescence over that of the CT-DNA alone sample, suggesting that there was a displacement of methylene blue from the CT-DNA and that the 0.2% (v/v) DMSO may be interfering with the assay. This interference would not apply to the cisplatin positive control since it is dissolved in water and not DMSO. Nonetheless, the artemisinin, artesunate, and WHN11 at concentrations 10, 50, and 200 μM (black, brown, and dark blue) samples displayed lowered fluorescence readings at 665 nm compared to that of CT-DNA control with methylene blue and the DMSO control, suggesting that artemisinin and its derivatives do not compete with methylene blue for DNA interaction and are therefore unlikely to be DNA intercalators.

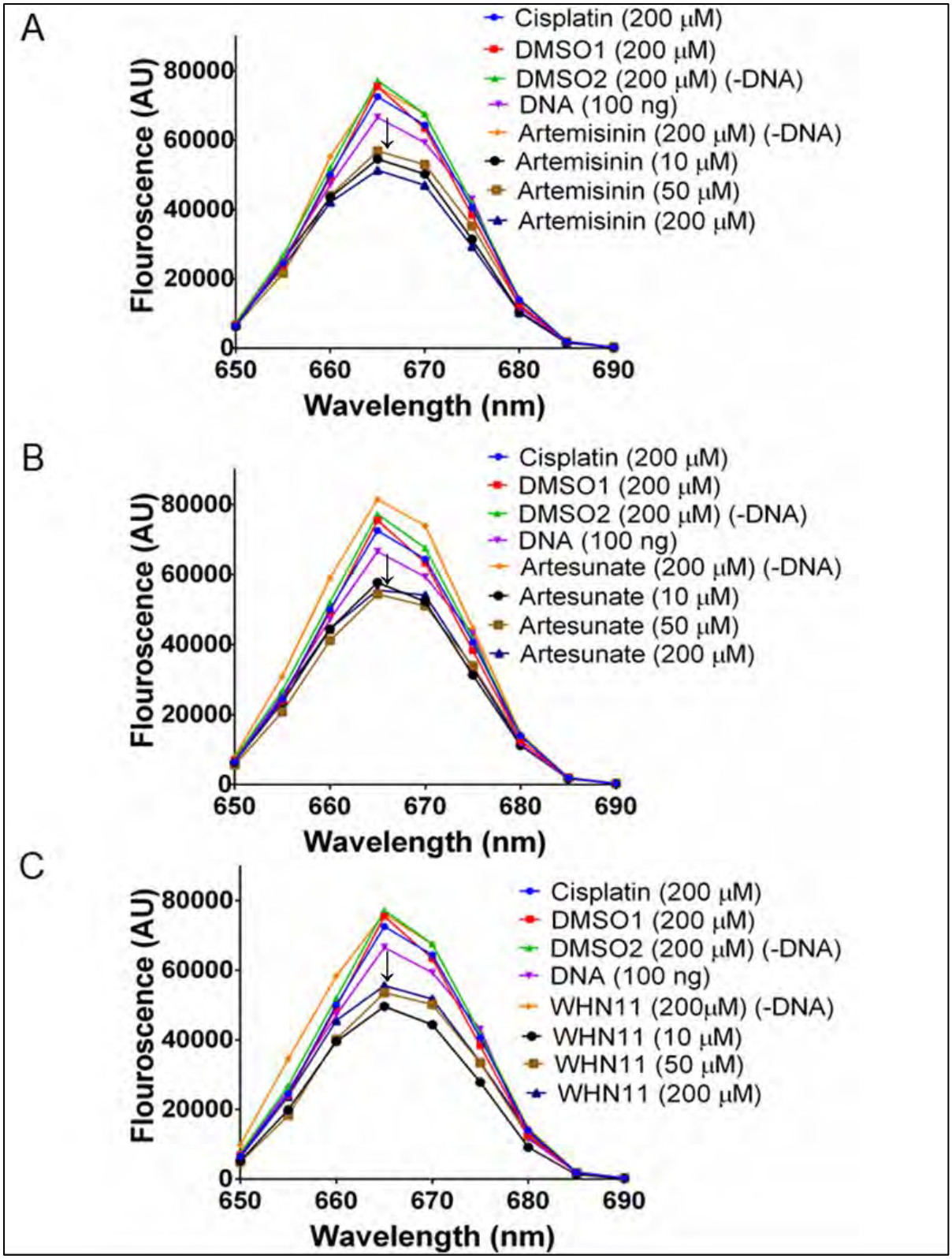


Figure 3.4: Methylene blue assay used to assess whether artemisinin, artesunate, and WHN11 are DNA intercalators. All the compounds are incubated with DNA unless stated otherwise (-DNA). A-C) results of methylene blue assay demonstrating fluorescence intensity of methylene blue upon addition of artemisinin and its derivatives at different concentrations (10, 50, and 200 μM) together with CT-DNA of 100 ng. All samples contain the methylene blue dye and controls without CT-DNA are indicated by (-DNA). DMSO was used as a negative control at a concentration [0.2% (v/v)] equivalent to the highest compound concentration (200 μM). Cisplatin (200 μM) was used as a positive control as a known DNA intercalator. AU: arbitrary units.

3.2.5 Hoechst 33342 exclusion assay for minor groove binding analysis

Finally, an investigation of the CT-DNA groove binding ability of the artemisinin compounds was conducted using a Hoechst 33342 exclusion assay, as the latter is a known minor groove binder dye (Bucevičius *et al*., 2018). The Hoechst dye exhibits a strong fluorescence emission at 460-485 nm upon interaction with CT-DNA, but not in its unbound, free state (Sandhu *et al*., 1985). When a competitive minor groove binder is present, the emission is reduced as the compound competes with the DNA-bound dye molecules and decreases the fluorescence of the Hoechst bound to the DNA. In Figure 3.5A-C, 0.2% (v/v) DMSO, equivalent to the amount of DMSO contained in the highest concentration of the compounds (200 μM) used in the assay (displayed in blue), was used as the vehicle control and contained Hoechst and CT-DNA. This control displayed the highest fluorescence peak as compared to all other samples. DMSO alone, without CT-DNA (displayed red), did not produce a fluorescence peak as expected since there is no DNA for the Hoescht to bind to in this sample. Artemisinin, artesunate, or WHN11 at the highest concentration of 200 μM [displayed in green, not seen as it overlapped by DMSO2 (displayed in red)] without CT-DNA were included as negative controls to assess any background fluorescence that may interfere with the assay. The compounds (green) or DMSO alone (red), without DNA, had the lowest fluorescence reading and did thus not interfere with the assay. Upon addition of artemisinin, artesunate, or WHN11 to CT-DNA at concentrations of 10, 50, and 200 μM (displayed in purple, orange and black, respectively), the fluorescence peak was drastically reduced compared to that observed for the DMSO control (displayed in blue), indicating that the compounds were able to displace the Hoechst dye molecules from the CT-DNA minor groove and that the artemisinin and its derivatives may be minor groove binders.

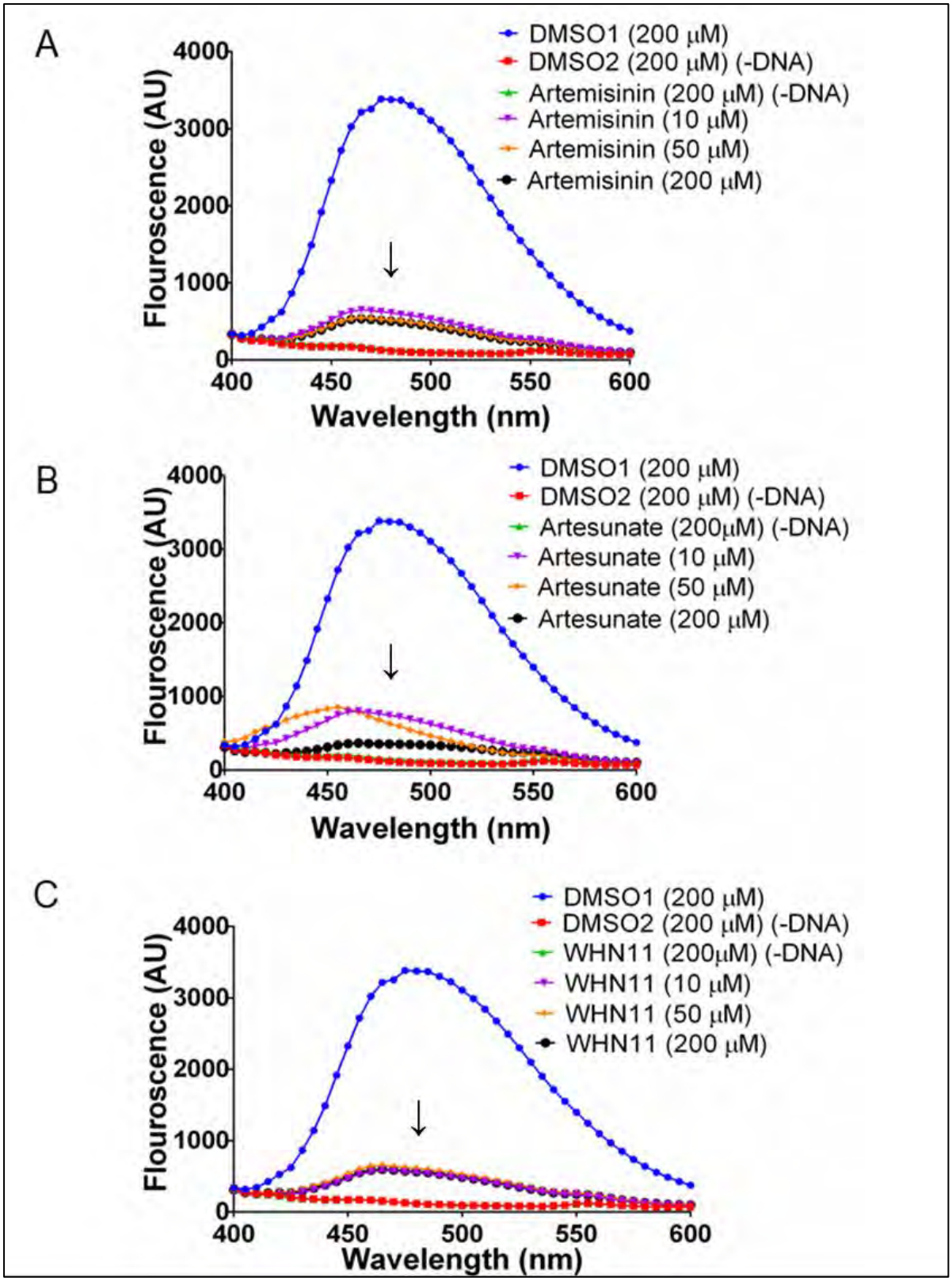


Figure 3.5: **Hoechst 33342 assay used to assess whether artemisinin, artesunate, or WHN11 are DNA minor groove binders**. A-C) results of the Hoechst assay demonstrating changes in the fluorescence intensity at 400 – 600 nm of DNA-bound Hoescht 33342 upon addition of artemisinin and its derivatives at different concentrations (10, 50, and 200 μM). CT-DNA was used at 100 ng in all cases. All samples contain Hoescht 33342 dye and controls without CT-DNA are indicated by (-DNA). DMSO was used as a negative control at a concentration [0.2% (v/v)] equivalent to the highest compound concentration (200 μM). AU: arbitrary units.

3.3 Investigation of DNA damage induction by artemisinin compounds

To assess whether the potential minor DNA groove binding observed by the compounds was ultimately associated with DNA damage, a comet assay was carried out, which measures the extent of DNA damage in the form of double-strand breaks, DNA single-strand breaks, and alkali-labile lesions (Speit and Hartmann, 1999) in cells. The technique is carried out on intact cells that are embedded in agarose on a microscopic slide, electrophoresed, and lysed by high salt concentrations followed by DNA staining with propidium iodide. In this assay, the damaged DNA migrates slower in the agarose gel, causing a smear or comet behind the nucleus upon electrophoresis of intact treated cells (Collins, 2004; Nandhakumar *et al*., 2011). As shown in Figure 3.6A, HCC70 cells treated with DMSO 0.2% (v/v) as the negative control, displayed a compact nuclear DNA staining pattern with no significant comet detected, indicating little to no DNA damage induced. Hydrogen peroxide (H_2O_2) or camptothecin (15 μM) were employed as positive controls for the assay. In these controls, the treated cells displayed clear comets of diffuse propidium iodide staining extending from the nucleus (comet tail), indicating extensive DNA damage induction. Artemisinin, artesunate, and WHN11 were also assessed for the ability to induce DNA damage at concentrations related to their IC_{50} value, as well as at 200 μM in line with the DNA binding experiments (in the case of artesunate and WHN11), against HCC70 cells (Table 3.1). Of these compounds, only artemisinin (200 μM) and artesunate (20 μM) appeared able to cause DNA damage as the propidium iodide staining extends from the nucleus in a clear comet not seen for WHN11-treated cells. Figure 3.6B displays the Olive tail Moment quantification of the comets, carried out for at least 50 images of individual nuclei for each treatment. The Olive Moment is calculated as the product of the tail length and the proportion of the total DNA in the tail (Mozaffarieh *et al*., 2008). From the graph in B, the Olive Moment differs significantly ($p<0.0001$) between the DMSO vehicle control and both positive controls, H_2O_2 (20 μM) or camptothecin (15 μM). Artemisinin (200 μM) caused significant DNA damage that was evidenced by a highly statistically significant change in Olive Moment value ($p<0.0001$) compared to the DMSO vehicle control, and this effect was also observed for artesunate at 20 μM ($p<0.01$), but not at 200 μM, while no significant increase in Olive Moments was observed upon treatment with WHN11 at either 5, 15, or 200 μM (Figure 3.6A and B).

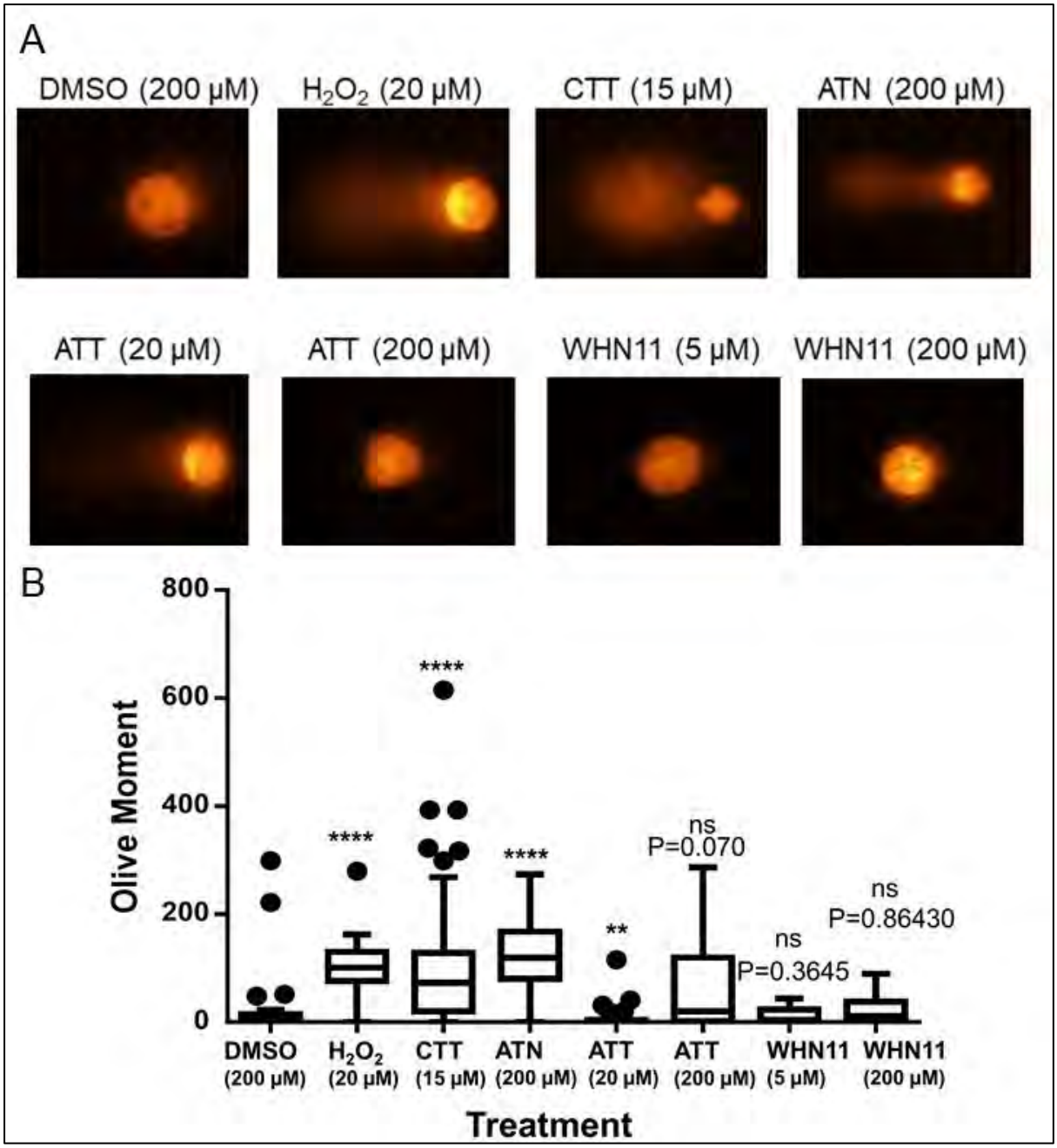


Figure 3.6: Assessment of DNA damage induction by artemisinin and derivatives using the comet assay. The circles in dark black represent the outliers for each compound. A) Representative fluorescence microscopy images of propidium iodide nuclei of HCC70 cells treated with artemisinin, artesunate, or WHN11. Camptothecin (CTT) and H_2O_2 were used as positive controls and DMSO 0.2% (v/v) was used as a negative control. The cells were treated for 2 hours with either DMSO vehicle control, artemisinin (ATN, 200 µM) artesunate (ATT, 20 or 200 µM), or WHN11 (5 µM, 15 or 200 µM) or for 1 hour with camptothecin (15 µM) or H_2O_2 (15 µM) before electrophoresis on 1% (w/v) low gelling temperature agarose gels and staining with 10 µg/ml propidium iodide. The fluorescence emission was captured using a U-MWG wide green filter cube with an excitation wavelength of 510 nm, an emission wavelength of 590 nm, and a beam-splitter wavelength of 570 nm and viewed at 20 X magnification. B) Box and whisker plot showing quantification of DNA damage according to Olive Moment from multiple images per treatment. OpenComet software (an Image PLUGIN) was used to quantify the Olive Moment (OM) [OM = Tail length x Tail% DNA]. The Mann-Whitney test was used to determine the statistical significance of each treatment in comparison to the DMSO vehicle control. ****$p<0.0001$, **$p<0.01$, $n \geq 50$.

3.4 Analysis of the effect of artemisinin and its derivatives on topoisomerases

To characterize the mechanism behind the observed DNA damage induction, by the artemisinin parent compound, the effect of the artemisinin compounds on topoisomerase enzyme activity and their ability to synergize with known topoisomerase inhibitors was assessed. Topoisomerase inhibition has been linked to DNA damage induction. This is due to the inhibition of the enzyme's function, namely, to cause transient single or double-stranded breaks during replication and transcription to relieve torsional strain on the DNA molecule (Yang *et al*., 2014; Vann, 2021). When this is inhibited, the DNA can be damaged during replication and transcription by excessive torsional strain (Puc *et al*., 2017).

3.4.1 Effects on topoisomerase function

Artemisinin, artesunate, and WHN11 were analyzed for their ability to inhibit TOPO 1 activity in terms of the nicking of supercoiled plasmid DNA as assessed by agarose gel electrophoresis (Figure 3.2). The artemisinin compounds were incubated with pCDNA-CL8D-HisCYP2A6 plasmid in the presence of TOPO 1 isolated from the calf thymus. In this assay, TOPO activity on supercoiled DNA was observed as an upwards shift in the position of the DNA band on the agarose gel, representing the nicking of the plasmid, while inhibition of the enzyme results in a downwards shift in the DNA band, towards the position of its supercoiled form. The first lane of the agarose gel in Figure 3.7 shows the pCDNA-CL8D-HisCYP2A6 plasmid occurring in the supercoiled conformation. In the second lane of the gel, the treatment of the plasmid with TOPO 1 caused an upward shift in the position of the band in the gel, consistent with the nicking of the supercoiled plasmid DNA and indicating that the TOPO 1 enzyme was active. In the third lane, restriction digestion of the plasmid with the single cutter EcoRI is displayed and the position of the (single) band is consistent with the expected plasmid size of 6937bp for the linearized plasmid. The restriction digest served to confirm the plasmid size and that the lower band in lane one corresponded to the supercoiled pCDNA-CL8D-HisCYP2A6 plasmid since it appeared lower down on the gel than the linearized plasmid, however, it would be expected that nicked/relaxed plasmid (in lane 2) would appear above this linearized plasmid on the gel. The remainder of the lanes include samples containing pCDNA-CL8D-HisCYP2A6 plasmid DNA incubated with TOPO 1 and the various compounds. DMSO [0.2% (v/v) corresponding to 200 μM of the artemisinin compounds] was included as the vehicle control, while camptothecin was employed as a positive control as it is a chemotherapy drug known to inhibit TOPO I (Wall *et al*., 1986). In the figure below, in the fourth lane, DMSO treatment of

the enzyme caused a very slight downward shift in the plasmid band position on the agarose gel compared to the untreated sample containing TOPO in the second lane. In the fifth lane, camptothecin treatment resulted in a minor downward shift in the plasmid band compared to that of DMSO treatment to generate a second band slightly lower down on the gel. Although the lower band did not correspond to the position of the supercoiled conformation observed for the plasmid alone (first lane), indicating complete TOPO inhibition, the shift in the band suggested that camptothecin may have caused a degree of inhibition of the enzyme. Treatment of TOPO 1 with artemisinin or its derivatives (last three lanes) caused a very slight downward shift in the position of the plasmid DNA band on the gel compared to both the untreated samples (second and third lane) and the DMSO vehicle control (fourth lane) suggesting potential minor TOPO 1 inhibition by the artemisinins, in particular WHN11, to a lesser extent than that observed for camptothecin.

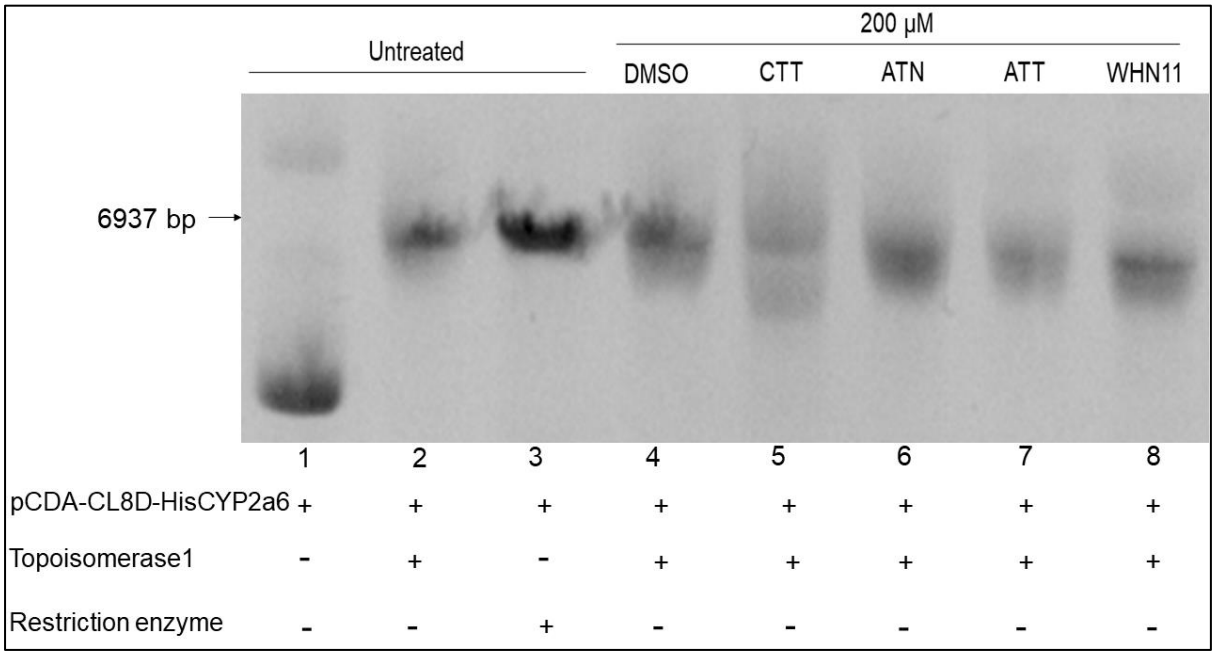
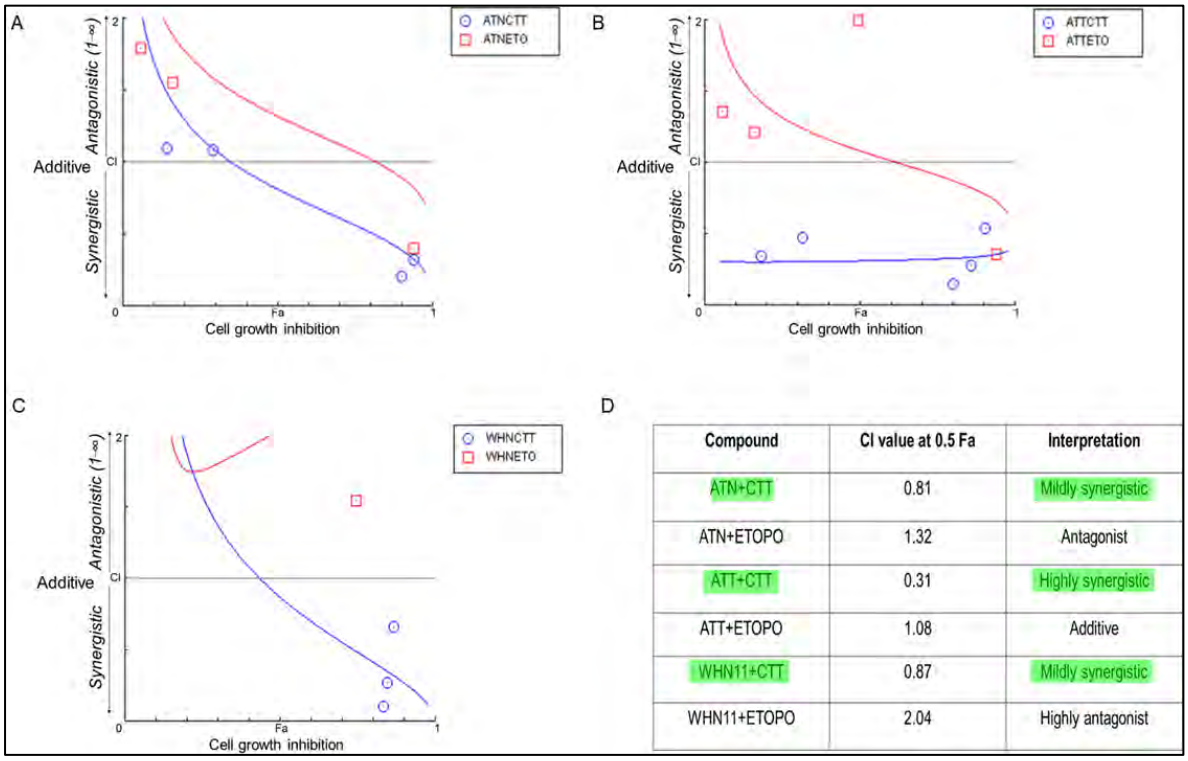


Figure 3.7: Effect of Artemisinin, artesunate, and WHN11 on topoisomerase I function. Ethidium bromide-stained 0.6% (w/v) agarose gel was used to analyze DNA single-strand nicking of plasmid DNA by topoisomerase I. An amount of 2 units of topoisomerase 1 enzyme (from calf thymus) and 250ng of pCDNA-CL8D-His CYP2A6 plasmid were incubated with topoisomerase 1 at 37 ºC for 2 hours. The restriction digest was incubated overnight at 37 °C having 250 ng of pCDNA-CL8D-His CYP2A6 plasmid, and 0.254 units of EcoR1 restriction enzyme. Lane 1: pCDNA-CL8D-His CYP2A6 plasmid without topoisomerase 1, Lane 2: pCDNA-CL8D-His CYP2A6 plasmid with topoisomerase 1, Lane 3: restriction digest (Eco R1), Lane 4: DMSO: 1% (v/v). corresponding to 200 µM of the artemisinin compounds as vehicle control. Lane 5: CTT: Camptothecin (200 µM), Lane 6: ATN: Artemisinin (200 µM), Lane 6: ATT: Artesunate (200 µM), and Lane 7: WHN11 (200 µM).

3.4.2 Synergy studies with known topoisomerase inhibitors

To investigate a potential indirect effect of the artemisinins on TOPO function, synergy cytotoxicity assays were carried out with the artemisinin compounds and TOPO inhibitors, camptothecin (TOPO I inhibitor), and etoposide (TOPO II inhibitor). CompuSyn software was used to determine combination index (CI) values with the corresponding fraction affected for each of the drug combinations according to the Chou-Talalay method (Chou, 2010; Greenshields *et al*., 2019). Combination indexes are divided into three categories, where the interpretation is that the combination is synergistic when the CI value is less than 1 (CI < 1), antagonistic when the CI value is greater than 1 (CI > 1), or additive when the CI value is equal to 1 (CI = 1) (Zhang *et al*., 2016). The fraction affected (Fa) refers to the proportion of dead cells as a fraction between 0.1 and 1, where, at a Fa of 0, all the cells are alive (viable or metabolically active as assessed by resazurin assay) and, at a Fa of 1, all of the cells are dead (non-viable or not metabolically active). A Fa of 0.5 corresponds to half of the cells being dead and half being alive and correlates with a typical IC_{50} value. Figure 3.8A-C depicts graphs displaying the Fa versus CI for all the combinations of the artemisinins with either camptothecin (shown in blue) or etoposide (shown in red). As seen in Figure 3.8A, the individual combination points and the trendline generated for the ATN:CTT combination (artemisinin and camptothecin) lie on either side of the additive cut-off point (CI = 1), with more points falling in the synergistic portion (CI < 1) as the Fa value increases. For the ATN:ETOPO (artemisinin and etoposide) combination, the majority of the combination points, as well as the trendline, fall in the antagonist section (CI > 1). The summary table (Figure 3.8 D) indicates a CI value (at Fa of 0.5) of 0.81 for ATN:CTT and 1.32 for ATN:ETOPO, suggesting that the combination of artemisinin with camptothecin is mildly synergistic, while the combination of artemisinin with etoposide is antagonistic. In Figure 3.8B, where artesunate was combined with the topoisomerase inhibitors, for the ATT:CTT (artesunate and camptothecin) combination, all of the combinations and the entire trendline fall in the synergistic region (CI < 1), while the ATT:ETOPO (artesunate and etoposide) combination showed the majority of the combination points and the trendline in the antagonistic region, showing synergistic behaviour only at a Fa of 0.9 (90% cell death). This is supported in the summary table in Figure 3.8D, where the ATT:CTT combination displayed a CI value at Fa = 0.5 of 0.31, indicating a highly synergistic relationship between artesunate and camptothecin, while the ATT:ETOPO combination displayed a CI value at Fa = 0.5 of 1.08, indicating an additive relationship between artesunate and etoposide. In Figure 3.8C, for the combination of

the novel artemisinin derivative WHN11 with the topoisomerase inhibitors, for the WHN11:CTT (WHN11 and camptothecin) combination, the majority of the points fell in the synergistic region, particularly at Fa values greater than 0.8, while, for the WHN11:ETOPO (WHN11 and etoposide) combination, all of the points and the trendline lie well above the CI = 1 additive cut-off, in the antagonistic region. The summary table in Figure 3.8D reveals a CI value at Fa = 0.5 of 0.87, for the WHN11:CTT combination, indicating a mildly synergistic relationship between WHN11 and camptothecin, while the WHN11:ETOPO combination displayed a CI value at Fa = 0.5 of 2.14, indicating a high antagonist relationship between WHN11 and etoposide.



Compound	CI value at 0.5 Fa	Interpretation
ATN+CTT	0.81	Mildly synergistic
ATN+ETOPO	1.32	Antagonist
ATT+CTT	0.31	Highly synergistic
ATT+ETOPO	1.08	Additive
WHN11+CTT	0.87	Mildly synergistic
WHN11+ETOPO	2.04	Highly antagonist

Figure 3.8: Compusyn drug synergy analysis for the combination of the artemisinin compounds with the topoisomerase inhibitors camptothecin or etoposide. A-C) Combination index (CI) versus Fraction affected (Fa) graphs for the combination of artemisinin, artesunate, or WHN11 with camptothecin (in blue) or etoposide (in red) generated using Compusyn software version 1. The HCC70 cells were treated for 96 hours with the compounds having constant ratios (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100) as well as single treatments and subjected to a resazurin assay to determine cell viability. D) Summary table of results for the combination of artemisinins with the topoisomerase inhibitors, showing the CI values at Fa = 0.5, together with the interpretation of the data as indicative of a synergistic or antagonistic combination

CHAPTER FOUR

DISCUSSION

Triple-negative breast cancer (TNBC) is an aggressive form of the disease that is more frequently diagnosed in women of African descent (Dietze *et al*., 2015). Currently, there are no effective targeted therapies for triple-negative breast cancer as the disease lacks the hormone receptors, estrogen receptor, progesterone receptor, as well as the human epidermal growth factor receptor 2, making the current therapies designed against these receptors ineffective and this results in prognosis being poor (Rakha *et al*., 2007). This means that novel therapies are required for this subtype of the disease. Artemisinin has been established as an anti-malarial agent and has been under investigation for anti-cancer properties (Ho *et al*., 2014; Liu *et al*., 2019). Several synthetic derivatives of artemisinin have been described to date (Woodely *et al*., 2021).

In this study, artemisinin and the previously described derivative artesunate, as well as a novel derivative, WHN11, were screened for cytotoxicity against triple-negative breast cancer and non-cancerous cells. The compounds were further assessed for their capability to bind to DNA as this could allow the compound to directly regulate cell growth and cell division, inhibit topoisomerase function, and initiate DNA damage. Artesunate is derived from DHA, and it is reacted with succinic acid anhydride (Morris *et al*., 2011). WHN11 differs from the parent compound in that it consists of piperazine carboxamide and trifluoromethyl-benzene groups that are bound to artemisinin (Kajewole *et al*., 2020).

4.1 The novel artemisinin WHN11 is highly cytotoxic against TNBC and non-cancerous breast epithelial cells

In the current study, the ability of artesunate and WHN11, in comparison to the parent compound artemisinin, to inhibit the growth of TNBC and non-cancerous breast epithelial cells were assessed following 96 hours of treatment in a resazurin assay. Artemisinin displayed very low toxicity in both the HCC70 cell line (IC_{50} = of 214.70 ± 2.33 μM) and the MCF12A non-cancerous cell line (IC_{50} = 298.3 ± 2.47 μM). The latter cell line is immortalized and transformed and is used as a control for normal breast epithelial cells. It is possible, however, that the additives (EGF, hydrocortisone, etc) can interfere with the assay by giving the cells a growth advantage, thus resulting in lower cytotoxicity of compounds in this cell line. In a previous study, Kumari *et al*., (2017), reported that artemisinin, at a concentration of 100 μM, was able to reduce cell viability to below 50% in hormone receptor-positive MCF7 and

hormone receptor-negative MDA-MB-231 cell lines (Kumari *et al*., 2017). Efferth *et al*., (2004), reported that artemisinin was able to inhibit cell growth in the CCRF-CEM leukemia cell line and exhibited an IC_{50} value of 11.5 µM in these cells (Efferth *et al*., 2004). In a study conducted by Tilaoui *et al*., (2014), assessing the cytotoxicity of artemisinin using the MTT assay, artemisinin induced cytotoxicity in P815 (murine mastocytoma) and BSR (kidney adenocarcinoma of a hamster) cell lines, displaying IC_{50} values of 12 µM and 52 µM, respectively, after 72 hours of treatment (Tilaoui *et al*., 2014). In a previous study by Zhang *et al*., (2014), it was discovered that artemisinin was able to inhibit cell growth after 30 hours of treatment in a dose-dependent manner in gastric cell lines (AGS, MKN74) at a concentration range of 0 to 5 µM. Tong and colleagues (2016) also found that dihydroartemisinin significantly suppressed cell growth in lung cancer cell line (A549) where artemisinin yielded an IC_{50} value of 20 µM at a concentration range of 0 to 200 µM after 48 hours of treatment with minimal effect on the BEAS-2B (normal human bronchial epithelial cells) (Tong *et al*., 2016). These previous studies thus demonstrate significantly higher toxicity of artemisinin in a range of cancer cell lines and indicate that the compound is less effective against the HCC70 TNBC cell line, derived from an African American woman in comparison to other breast cancer cell lines. This could be due to the different genotypes of these cells, which could harbour unique driver mutations not found in cell lines from Caucasian women and further studies in other cell lines derived from women of African descent could be carried out to explore this. It is worth noting that most commercially available cell lines are derived from Caucasian women, bringing into question their relevance to drug discovery in the African context and this is demonstrated in this study by the reduced toxicity of artemisinin in the HCC70 cell line.

Artesunate was found to be only moderately toxic toward the HCC70 TNBC cell line (IC_{50} = 25.48 ± 1.4 µM) and displayed lower toxicity toward the MCF12A non-cancerous breast epithelial cell line (IC_{50} = 87.53 ± 1.94 µM), reflecting a degree of selectivity for the cancer cell line over the non-cancerous cell line. This is also because cancer cells take up more iron as compared to normal cells which makes cancer cells more susceptible to artesunate (Lai *et al*., 2005). Xu *et al*., (2011), assessed the toxicity of artesunate towards the HOS (human osteosarcoma) cell line using the MTT assay. It was found that artesunate was able to inhibit HOS cell proliferation, with an IC_{50} value of 52.8 µM after 48 hours of treatment (Xu *et al*., 2011). In a previous study by Våtsveen *et al*., (2018), artesunate was toxic to the B-cell lymphomas cell lines (BL-41, Raji, Ramos, Rec-1, Namalwa, ULA, SU-DHL-6, Oci-Ly-18, Oci-Ly-2, SU-DHL-4, U-2932, Will-2, Mino, JeKo-1, Granta-519, Ros-50, K-422, and

DoHH2 lines) after 72-hour treatment using the CellTiterGlow assay. The IC_{50} concentration range for all lymphoma cell lines was 0.189 to 3.72 µM. This proposed artesunate is a promising anti-cancer drug for clinical trials used to treat lymphomas (Våtsveen *et al*., 2018). Kim and Park (2020) tested artesunate to inhibit cell proliferation with the use of an MTT assay. Within 48 hours of the cells being treated with the compound, artesunate significantly inhibited cell proliferation (IC_{50} value of 10 µM) of colon cancer cells (HCT116 cell line) at a concentration range of 0 and 160 µM. The authors suggested that artesunate was a promising compound for further studies in colon cancer (Kim and Park, 2020). Observing previous studies and comparing them to our study, artesunate was highly toxic on the HCC70 cell line with an IC_{50} value half that of previous studies displaying its effectiveness except on the HCT116 cell line where the IC_{50} of the HCT116 cell line was double that on the HCC70 cell line.

In this study, it was found that WHN11 was more toxic compared to the parent compound, artemisinin, and the previously described derivative artesunate, inhibiting cell viability in the HCC70 cell line at a low micromolar concentration (IC_{50} = 3.20 ± 0.51 µM), however, the compound was also highly toxic to the MCF12A cell line (IC_{50} = 8.35 ± 0.92 µM). This toxicity of WHN11 may be attributed to the piperazine carboxamide and trifluoromethyl-benzene groups, which have been reported to enhance anti-cancer activities (Zhang *et al*., 2021). The piperazine group has been reported to have the ability to intercalate with DNA, initiate apoptosis through oxidative stress, and cause cell cycle arrest (Walayat *et al*., 2019). On the other hand, in a study conducted by Kim *et al*., (2019), it was discovered that the anti-cancer activity of trifluoromethyl-benzene-containing compounds involved the initiation of apoptosis and the inhibition of lactate dehydrogenase (Kim *et al*., 2019). In a previous study, screening of a series of artemisinin derivatives revealed that the addition of fluorides, benzyl groups, and units of artemisinin forming dimers increased the cytotoxic activity of the compounds (Kajewole *et al*., 2020). The addition of fluorine in therapeutic drugs enhances pharmacokinetic and physicochemical properties (Shah and Westwell, 2007). Benzyl groups are known to enhance the toxicity of compounds hence their frequent use in medicinal chemistry (Mahalingam *et al*., 2010; Zhou *et al*. 2012)

Comparing the results of artemisinin and artesunate with that of literature, it was found that the two compounds were less toxic in our study. This may be due to differences in the assay used as well as cell lines. To the best of our knowledge, this is the first study where artemisinin, artesunate, and WHN11 were assessed in HCC70 in terms of cytotoxicity. It is possible that artemisinin and its derivatives were less toxic to the HCC70 cell line due to its potentially more

aggressive nature as a TNBC line derived from an African-American woman. The concentration ranges used in this study were in line with those used by other researchers thus it is unlikely that the concentration range of the drug was one of the possible reasons why the two drugs had a minimal effect (Kajewole *et al*., 2020). Another possible reason was the use of the resazurin assay compared to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. While both assays measure mitochondrial NAD(P)H reductase activity of mitochondrial enzymes (Gonzalez and Tarloff, 2001; Lavogina *et al*., 2022), the resazurin assay differs from the MTT assay since it assesses metabolic activity in living cells with the fluorescent product being secreted, whereas the MTT assay (Stockertt *et al*., 2018) determines cellular viability based on the ability of cells to transform water-soluble dyes into insoluble formazan that requires solubilization and thus rupture and death of the cells. The resazurin assay is widely regarded as the more robust and sensitive of the two assays (Chakansin *et al*., 2022).

Artemisinin and its derivatives were subsequently assessed for their ability to bind to and damage DNA and inhibit topoisomerase function to explain the differences in their cytotoxicity.

4.2 Artemisinin and its derivatives artesunate and WHN11 are DNA minor groove binders

Although artemisinin and its derivatives have been used as anti-malarial agents for several years, their mode of action in this and other diseases, including cancer, is not fully understood. Recent studies have suggested several modes of action, largely linked to the highly reactive endoperoxide bridge present in the compound, such as the induction of oxidative stress, induction of apoptosis, inhibition of angiogenesis, the arrest of the cell cycle at stages G0 or G1, and ferroptosis (Das, 2015; Augustin *et al*., 2020). The work described here forms part of a broader study within our laboratory in terms of the screening and mode of action analysis of WHN11 and other novel artemisinin derivatives. In the present study described herein, an assessment of the binding capability of artemisinin, artesunate, and WHN11 to DNA, both *in silico* and *in vitro*, was carried out in an order to gain insight into the mode of action of the novel derivative WHN11. This study explored whether WHN11 may be more effective than its parent compound in terms of the ability to bind to and damage DNA and interfere with topoisomerase function, to explain the greater toxicity observed for WHN11. The ability of

compounds to bind to DNA can result in interference with DNA processes, such as topoisomerase inhibition, which can lead to DNA damage and cell death.

In silico docking studies suggested that artemisinin, artesunate, and WHN11 were capable of binding to the DNA in the minor groove and it was observed that WHN11 had a higher binding affinity in comparison to artemisinin and artesunate. The binding affinities obtained in the study are in line with the moderate binding range (-6.9 to -9.5 kcal/mol) (Gurung *et al*., 2021). It was also observed that WHN11 had long hydrogen bonds of which the maximum length of a hydrogen bond has been reported to be 3.0 Å (Bučko *et al*., 2004). To verify the possible binding of artemisinin and its derivatives to DNA *in vitro*, several assays were conducted. Using genomic DNA extracted from TNBC cells, ethidium bromide competition was carried out for the compounds and the results from this assay suggested no significant binding nor DNA displacement suggesting that artemisinin, artesunate, or WHN11 are not DNA intercalators. On the other hand, cisplatin was used as a positive control as it is a DNA intercalator and successfully displaced the ethidium bromide from the DNA, as expected, validating the experimental procedure. Cisplatin is an approved cancer drug, and its mechanism of action has been attributed to its ability to link with purine bases on DNA, interfering with DNA repair systems, and causing DNA damage leading to apoptosis in cancer cells (Dasari and Tchounwou, 2014).

In UV-Vis spectroscopy studies it was revealed that artesunate, but not artemisinin or WHN11, was able to bind to CT-DNA, likely via electrostatic interaction, and that none of the compounds displayed the hypochromic and red shift characteristic of DNA intercalators, in agreement with the AGE EtBr competition results. The ability of artesunate to bind to CT-DNA but not gDNA could be due to the AT-rich nature of the CT-DNA (Maurya *et al*., 2020), and this agrees with the docking studies, which identified potential interactions between artesunate and adenine and thymine residues of the DNA, whereas artemisinin and WHN11 potentially interacted with cytosine and guanine residues. Since the DNA of malaria parasite is AT-rich (Bulloch and Ralph, 2022), this direct binding of artesunate to AT-rich DNA demonstrated *in vitro* may contribute towards its reported greater anti-malarial action than artemisinin (Uddin *et al*., 2021), WHN11 demonstrated potential interaction with adenine, guanine, and cytosine residues. The ability of artesunate to bind to CT-DNA and not gDNA could also be due to the nature of the DNA sample, where commercially available CT-DNA is supplied as fragmented DNA, whereas gDNA is made up of much larger and more compacted DNA molecules, which could impact the access of the compounds to the DNA.

More specific assays, in terms of potential mode of DNA binding, were employed, namely the methylene blue assay competition assay for assessment of DNA intercalation and the Hoescht 33342 exclusion assay to assess minor groove binding. The methylene blue assay revealed that neither artemisinin nor its derivatives were able to displace methylene blue, suggesting that they were not able to intercalate into the CT-DNA, in agreement with the UV-Vis studies. On the other hand, artemisinin and its derivatives appeared to be able to bind to the minor groove as assessed in the Hoechst exclusion assay, which correlates with what was predicted *in silico* through molecular docking studies. While the *in silico* studies predicted a greater binding affinity for WHN-11, compared to that of artemisinin and artesunate, all three compounds appeared to be equally able to displace the Hoescht 33342 dye, resulting in a drastically reduced fluorescence signal upon incubation with the CT-DNA in the assay. The ability to detect the binding of artemisinin and WHN-11 to CT-DNA in the Hoescht assay but not the UV-Vis assay may be due to the nature of the assay and the interaction detected. In the UV-Vis assay, if a compound binds to DNA by intercalation, a hypochromic effect and red shift are observed, whereas electrostatic interaction is indicated by a hyperchromic effect (Jabeen *et al*., 2019). This indicates that artemisinin and WHN11 act specifically as DNA groove binders and not intercalators While screening and mechanistic analysis of WHN11 have been carried out in our laboratory (Kajewole, 2020), this is the first time the ability of the novel derivative to bind to DNA has been assessed.

In a study conducted by Maurya *et al*., (2020), artemisinin and its derivatives, artemisinic acid, and dihydro-artemisinic acid, were assessed for their ability to interact with DNA using molecular docking, UV-Vis spectroscopy, cyclic voltammetry, viscosity, melting temperature measurements, and circular dichroism, all of which led the authors to conclude that the compounds were minor groove binders and did not interact with the DNA via intercalation, which is in agreement with the results from this study (Maurya *et al*., 2020). In another study, Slavkovic *et al*., (2022) investigated the potential of artemisinin to bind to aptamer DNA and reported that artemisinin was able to bind to aptamer as well as other DNA structures that had a three-way junction and duplex DNA but not to single-stranded DNA or RNA. DNA binding was assessed through isothermal titration calorimetry and verified with nuclear magnetic resonance spectroscopy and the authors concluded that the compound requires the endoperoxide bridge to bind to the DNA (Slavkovic *et al*., 2022).

The study conducted herein was in correlation with known conducted studies assessing the ability of artemisinin and its derivatives to bind to DNA. In our study as well as that of Maurya

et al., (2020), several assays were conducted to determine the mode of binding of artemisinin, but all concluded with artemisinin and its derivatives binding to DNA through the minor groove. This was verified in our study with the Hoechst assay which assesses the mode of binding through minor groove binding, and it was shown that artemisinin and its derivatives are minor groove binders.

4.3 Artemisinin and artesunate but not WHN11 induce DNA damage in TNBC cells

The most accepted of the proposed mechanisms of action of artemisinin involves damage to macromolecules because of the generation of free radicals upon cleavage of the endoperoxide bridge in the presence of haem iron (Berdelle *et al*., 2011; Mercer *et al*., 2011; Kong *et al*., 2012). Considering this, together with the results from this study regarding the ability of artemisinin and its derivatives to bind to DNA and potentially inhibit TOPO I activity, the potential of these compounds to cause DNA damage was assessed in a comet assay. In this assay, like the positive controls, camptothecin and hydrogen peroxide, artemisinin at 200 µM was able to cause significant DNA damage in comparison to the DMSO control. On the other hand, while DNA damage was detected for artesunate 20 µM, this was not observed at the higher concentration of 200 µM. The lack of dose dependency for the artesunate treatment could be due to excessive cell death at 200 µM since this concentration is nearly 10-fold higher than the IC_{50} of the compound, although the treatment times differed, with the cytotoxicity assay being conducted after 96 hours and the comet assay after 2 hours of treatment. Previous studies have suggested that 70% cell viability should be maintained in cells before carrying out a comet assay to exclude false positives in the assay due to chromosomal fragmentation in dead cells (Li *et al*., 2018).

Unlike artemisinin and artesunate, WHN11 was unable to induce DNA damage in HCC70 TNBC cells, either when treatment was carried out at a concentration in line with the IC_{50} of the compound or at a high concentration of 200 µM. Previous work in our laboratory indicated that artemisinin, but not WHN11, at concentrations in line with their IC_{50} values, generated significant ROS levels in TNBC cells as assessed by DCFDA assay, increased the expression of the ROS reporter gene NQ01, and demonstrated reduced toxicity in the presence of a free radical scavenger (Kajewole, 2021). This could explain the inability to detect DNA damage in WHN11-treated cells in this study at a concentration equivalent to the IC_{50} value if this damage is a result of ROS induction. In a study conducted by Kadioglu *et al*., (2017), artemisinin derivatives CMK-0398, WWLL-022, WWLL-1098, WWLL-013, and CMK-0298 were tested

for their ability to cause DNA damage in the R6T24 transformed rat6 cell line using a comet assay. It was found that the artemisinin compounds caused DNA damage at concentrations ranging from 0.5 to 1 μM, with CMK-0398 and CMK-0298 causing the greatest level of DNA damage to R6T24 cells compared to the other artemisinin derivatives. In a similar study, Li and colleagues (2018), found that artemisinin at 500 μM as well as artesunate at 150 μM caused DNA damage in human bronchial epithelial (HBE) and A549 human lung adenocarcinoma cells after 24 hours of treatment, with the compounds causing DNA damage in A549 cells to a greater extent as compared to the HBE cells in the alkaline comet assay. The results conclude that artemisinin and its derivatives cause DNA damage in cancerous cells proposing this as one of the modes of action of these compounds.

4.4 Artemisinin and its derivatives may interfere with topoisomerase I function and display drug synergism in combination with topoisomerase I inhibitors

Topoisomerases (TOPOs) are enzymes that create temporary single (TOPO I) or double-strand breaks (TOPO II) to relieve torsional strain during replication and transcription (Champoux, 2001). Inhibition of TOPO is of importance as a therapeutic strategy as it interferes with DNA replication, promotes DNA damage, and initiates cell arrest in cancerous cells (Liu *et al*., 2019). DNA binding can be linked to topoisomerase inhibition if a compound binding to the DNA somehow prevents TOPOs from binding to the DNA to fulfill their function. In the current study, artemisinin, artesunate, and WHN11 were investigated for their ability to inhibit TOPO I activity by incubation with supercoiled plasmid DNA and subsequent assessment of nicking of the DNA using agarose gel electrophoresis. A restriction digest served to confirm the plasmid size and that the lower band on the gel representing plasmid DNA alone, without TOPO I, corresponded to the supercoiled plasmid since it appeared lower down on the gel than the linearized plasmid. It would be expected that a plasmid nicked by TOPO I would appear above this linearized plasmid on the gel, however, this was not observed, and plasmid DNA exposed to TOPO I appeared at a similar position in the gel to the linearized plasmid. The low agarose concentration of 0.6% gel might have made it more difficult to distinguish between nicked and linear plasmid or that excessive nicking could have resulted in a plasmid conformation more like the linearised form. A clear change in band position upon the addition of TOPO indicated that the enzyme was active and causing an effect on plasmid conformation. The assay revealed that artemisinin, artesunate, and novel WHN11, although appearing to have some inhibitory effect on TOPO I when compared to the DMSO control, were not able to fully inhibit the TOPO activity, causing only a minor downward shift in the position of the DNA

bands on the agarose gel, towards that of the supercoiled conformation. In a study conducted by Kadioglu *et al*., (2017), artemisinin and 40 derivatives were assessed using *in silico* molecular docking with human TOPO I to detect possible TOPO inhibitors. The authors reported that five of the artemisinin derivatives, WWLL-013, WWLL-1098, CMK0298, CMK0398, and WWLL-022, displayed binding energies like those of the known inhibitor camptothecin, and further analysis was conducted *in vitro* to verify possible inhibition of TOPO. In a decatenation assay, it was found that two artemisinin derivatives, CMK-0398, and CMK-0298, were able to inhibit TOPO I, provided that the concentration was higher than 1 µg/mL. The two compounds also displayed intercalating properties like that of ethidium bromide suggesting that they can simultaneously bind to DNA as an intercalator and inhibit TOPO I (Kadioglu *et al*., 2017). Youns and colleagues, (2009) investigated the potential of artesunate as a TOPO IIα inhibitor. Artesunate was able to downregulate the expression of TOPO II and this was verified by the real-time polymerase chain reaction (RT-PCR) and western blot. A TOPO II relaxation assay was conducted where a known TOPO II inhibitor, etoposide, was employed. A concentration range of 50-100 µM for etoposide and artesunate was used and it was noted that artesunate inhibited the relaxation of the supercoiled plasmid DNA in a dose-dependent manner indicating that artesunate was able to act as a TOPO II inhibitor (Youns *et al*., 2009). Comparing the results of this study with those of literature, it is noted that artemisinin and its derivatives can inhibit TOPO function. To some extent, this proposes artemisinin, artesunate, and WHN11 as potential inhibitors of TOPO.

To assess the therapeutic potential of dual treatment with the artemisinins and TOPO inhibitory drugs, combination assays were carried out using camptothecin (TOPO I inhibitor) and etoposide (TOPO II inhibitor) (Ferraro *et al*., 2000). Combination therapy is a promising strategy to overcome the systemic toxicity of chemotherapeutic agents by administration of lower doses of individual compounds and has the potential to overcome resistance to monotherapy (Song *et al*., 2020). Camptothecin has shown significant activity in cancer cell lines, but its poor solubility has caused problems in clinical trials (Martino *et al*., 2017). Etoposide, on the other hand, is a well-tolerated medication licensed by the Food and Drug Administration (FDA) for small cell lung cancer and testicular cancer (Martin *et al*., 2020). In this study, it was found that artemisinin, artesunate, and WHN11 acted synergistically when combined with camptothecin, however, their relationship was either additive (artemisinin) or antagonistic (artesunate and WHN11) when used in combination with etoposide. This suggests a potential therapeutic benefit to combination therapy with artemisinins and camptothecin, in

which artemisinins may sensitize cancer cells to TOPO 1 inhibition. Aside from the potential clinical benefits of combination therapy, it has been reported that compounds that act on targets of a highly similar function may be synergistic (Ren *et al*., 2020), which gives potential insights into the mechanism of action of the compounds as influencing proteins involved in ensuring DNA integrity, although this would need to be verified experimentally. Combination therapy with artemisinin and its derivatives in cancer cells has previously been assessed, were a combination of DHA and sodium butyrate, a histone deacetylase inhibitor, in MOLT4 acute lymphoblastic leukemia cells indicated that the compounds acted synergistically and that the combination had little effect on normal cells (Mancuso *et al*., 2021). Efferth, (2017), assessed the effect of the combination treatment of artesunate and erlotinib, an inhibitor of epidermal growth factor receptor tyrosine kinase (Burris *et al*, 2005) used to treat non-small cell lung and pancreatic cancer in the clinic, in a range of glioma cell lines, revealing a synergistic relationship between artesunate and the chemotherapeutic agent in some, but not all, of the cell lines. The results obtained were in correlation with that of literature, proposing artemisinin and its derivatives as potential cancer inhibitors in combination treatment with known cancer drugs, in particular showing that artemisinin and its derivatives may sensitize cancer cells to camptothecin treatment.

4.5. Conclusion

In this study, cytotoxicity assays revealed that WHN11 was more potent than artemisinin and artesunate towards TNBC cells *in vitro* as it had the lowest IC_{50}, in the low micromolar range, followed by artesunate, with artemisinin having the highest IC_{50} value. WHN11 was, however, similarly toxic to non-cancerous breast epithelial cells. Upon assessing their ability to bind to DNA, all three compounds were found to be minor groove binders. The drugs were also found to act synergistically in combination with camptothecin while demonstrating either additive or antagonistic behaviour when combined with etoposide. Evaluation of the potential of the compounds to inhibit TOPO 1 enzyme activity in terms of the ability to nick supercoiled plasmid DNA revealed that there was a slight inhibition of the decatenation activity of the enzyme by the compounds, however, this was to a lesser extent than that observed for the positive control, camptothecin a known TOPO I inhibitor. Inhibition of topoisomerase leads to the damage of DNA and this was indeed observed in TNBC cells treated with artemisinin, and artesunate but not WHN11. It is, therefore, possible that DNA damage may not be the primary

mode of action of WHN11, which contrasts with the parent compound, although further studies are required to verify this. It is possible that DNA binding by WHN11 could be linked to its TOPO I inhibition, such that DNA binding is competitive between WHN11 and TOPO I.

4.6 Future work

Following on from this study, different assays assessing DNA damage induction, including γH2AX detection and a TUNEL assay, could be employed to validate the present findings. In addition, the effect of the compounds on TOPO II function could also be determined. The assessment of competitive binding between the artemisinins and TOPOs to DNA could then be carried out to provide information on a potential mechanism of inhibition of the enzymes by blocking DNA binding. As articulated above, this work formed part of a larger study within our group, during which proteomic analysis was carried out and revealed several potential target proteins, for example, cytochrome c oxidase assembly factor 7 (COA7), vacuolar protein sorting-associated protein 37B (VPS37B), Ras-related protein Rap-2b (RAP2B), Rac GTPase-activating protein 1 (RACGAP1), growth arrest and DNA damage-inducible proteins-interacting protein 1 (GADD45GIP1), cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 2 inhibitor C (CDKN2C), some of which are oncogenes, and regulate diverse processes including vesicular trafficking, mitochondrial electron transport, and cell cycle progression. It is thus possible that WHN11 binds promiscuously to several targets within the cell. Further studies could focus on validating the proteomics analysis in terms of assessing the effect of the compounds on the expression and function of these proteins and their cellular pathways to ascertain any potential off-target effects. Finally, *in vivo* studies in mouse models would add great value to this work, to determine if the effects seen in cell lines translate to effectiveness at the level of a tumor in a whole organism and would give an indication of any adverse toxic effects.

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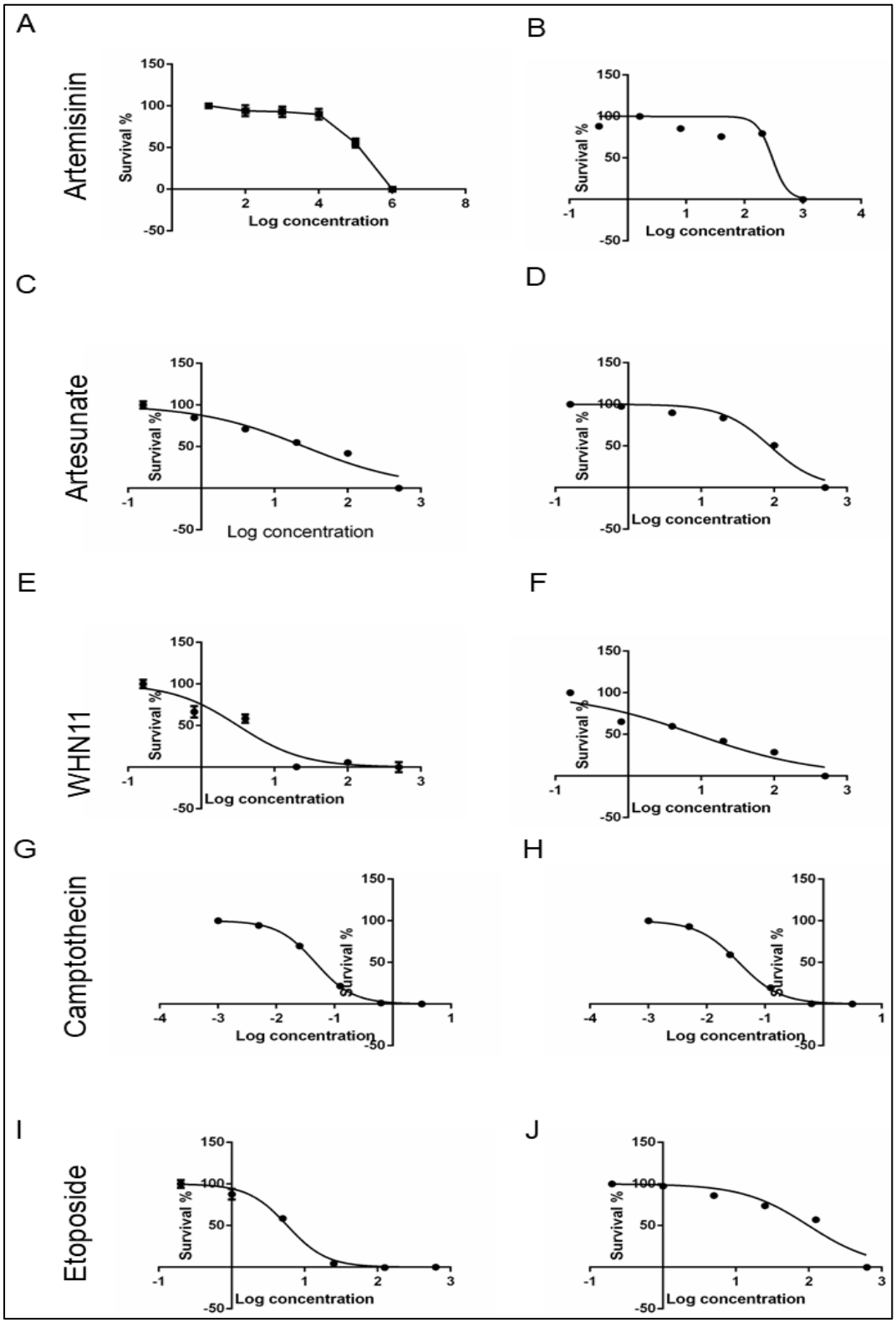
Supplementary

Supplementary Figure S1



S1: Cytotoxicity screening of artemisinin and its derivatives artesunate and WHN11 against the HCC70 TNBC (figures: A, C, E, G and I) and MCF12A (figures: B, D, F, H, and J) non-cancerous breast epithelial cell lines

Supplementary Table S1

Table displaying the combination index (CI) values that are in accordance with Fa = 0.5 for TNBC cell treated with artemisinin and camptothecin/etoposide.

Fa = 0.5		Dose (μM)		
Drug/combo	**CI value**	**Artemisinin**	**Camptothecin**	**Etoposide**
Artemisinin		1493.47		
Camptothecin			0.11331	
Etoposide				22.8962
ATNCTT	0.81247	322.429	0.06760	
ATNETO	1.31968	709.516		19.3381
Average CI value	**1.0661**			

Supplementary Table S2

Table displaying the combination index (CI) values that are in accordance with Fa = 0.5 for TNBC cell treated with artesunate and camptothecin/etoposide.

Fa = 0.5		Dose (μM)		
Drug/combo	**CI value**	**Artesunate**	**Camptothecin**	**Etoposide**
Artesunate		176.563		
Camptothecin			0.18702	
Etoposide				22.8962
ATTCTT	0.31177	12.6955	0.04486	
ATTETO	1.08307	42.0988		19.3389
Average CI value	**0.69742**			

Supplementary Table S3

Table displaying the combination index (CI) values that are in accordance with Fa = 0.5 for TNBC cell treated with WHN11 and camptothecin/etoposide.

Fa = 0.5		Dose (μM)		
Drug/combo	**CI value**	**WHN11**	**Camptothecin**	**Etoposide**
WHN11		60176.7		
Camptothecin			0.11331	
Etoposide				22.8962
WHN11CTT	0.87020	6.90096	0.09859	
WHN11ETO	2.04366	25.1878		46.7826
Average CI value	**1.45693**			

Supplementary Table S4

Table displaying the combination index (CI) values that are in accordance with Fa = 0.50, 0.75 and 0.90 for TNBC cell treated with artemisinin, artesunate or WHN11 and camptothecin/etoposide.

Drug			
Fraction affected	Average CI value		
	Artemisinin	Artesunate	WHN11
Fa = 0.50	1.0661	0.6974	1.4570
Fa = 0.75	0.8181	0.6183	1.4507
Fa = 0.90	0.6408	0.5632	1.5640