Effect of chemotherapeutics on ovarian function in women with breast cancer

by

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SUMMARY

This PhD thesis evaluated the effect of a clinically relevant combination of chemotherapeutics; Doxorubicin (Dox) and 4-Cyc on cytotoxicity and ROS production by human breast and ovarian cancer-derived cell lines *in vitro.*

Breast cancer is the number one malignancy among women accounting for more than 400,000 deaths each year globally. The incidence of breast cancer is higher in western societies and in postmenopausal women, but in Asian countries is increasingly reported in young premenopausal women; around 25% of all breast cancer patients in Asia are premenopausal women ≤ 35 years old.

The combination of Dox and cyclophosphamide (AC) is one of the most widespread anthracycline-based adjuvant regimens used for early-stage and advanced breast cancer in both premenopausal and postmenopausal women. In early trials treatment with AC resulted in improved survival and recurrence compared to CMF; Furthermore, AC was considered less toxic than CMF which justifies the use of AC regimen in premenopausal patients. Nonetheless, AC-induced adverse effects such as peripheral neuropathy, anaemia and cardiac failure are described for breast cancer patients. Moreover, AC induces premature ovarian failure in young breast cancer patients. Approximately 35% of breast cancer patients treated with AC report amenorrhea a year after completion of chemotherapy. Studies suggest that both Dox and cyclophosphamide target proliferating cells in the ovary by interfering with DNA replication and by inducing ROS production, but there are currently no reports describing the cytotoxic effects on ovarian granulosa cells of the combined Dox and cyclophosphamide regimen used to treat breast cancer patients.

The administration of Vitamin E lowered Dox-induced ROS damage in animal studies and reduced Dox toxicity without reducing its effectiveness as

chemotherapeutic agent. Therefore, it was hypothesized that the addition of alpha and gamma tocopherol to the AC regimen would decrease chemotherapeutic-induced ROS and therefore, will reduce cytotoxicity on ovarian granulosa cells. But due to the anticancer effect that gamma tocopherol demonstrated against breast cancer cells in both *in vitro* and *in vivo* studies, it was hypothesised that γToc, but not αToc, would potentiate the cytotoxic activity of the combined Dox and 4-Cyc regimen *in vitro.*

The cytotoxic effects of clinical relevant doses of Dox and 4-Cyc as single agents and as combined AC regimen were tested on breast cancer (MCF-7) and ovarian granulosa (KGN) cells. Dox was cytotoxic to both MCF-7 and KGN cells, but no effect on KGN cell viability was observed after exposure to 4-Cyc. Only a long exposure (72h) at the highest concentration (2.5μM) of 4-Cyc was able to significantly reduced the numbers of viable MCF-7 cells. The combination of Dox and 4-Cyc caused the same cytotoxicity to MCF-7 breast cancer cells *in vitro* as Dox alone, suggesting that the concentration of 4-Cyc chosen for this study was too low for this *in vitro* study.

The numbers of MCF-7 or KGN cell viability were not affected after exposure to αToc, but γ-Toc was significantly more cytotoxic to MCF-7 cells than to KGN cells. Similarly γToc combined to chemotherapeutics increased the cytotoxicity of the combined Dox and 4-Cyc regiment against MCF-7 cells but did not increase nor reduce the cytotoxicity of Dox and 4-cyc towards the KGN cells. This study showed that the addition of γToc to the combined of Dox and 4-Cyc regimen has the potential to increase the *in vitro* efficacy against breast cancer cells without affecting viable ovarian granulosa cells.

The effective concentration of chemotherapeutics and γToc that killed 25% (EC25) of MCF-7 breast cancer cells were determined to evaluate the potential synergistic effect between Dox and 4-Cyc. T47D breast cancer cells, OVCAR-3 ovarian cancer cells and COV434 granulosa cancer cells were exposed to MCF-7 derived EC25 values of Dox and 4-Cyc. 4-Cyc significantly decreased the cell viability of T47D, OVCAR-3 and COV434 to 71±2.2%, 60±14% and 45±8.6% of the media controls, respectively. Similarly, MCF-7 EC25 values of Dox significantly reduced T47D, OVCAR-3 and COV434 cell viability to 66±5.6%, 74±5% and 57±1.9% respectively. MCF-7 derived EC25 values of γToc significantly decreased breast cancer cell viability, but had no effect on ovarian granulosa cells. Combining EC25 values of Dox with EC25 values of 4-Cyc had no synergistic effect. No significant differences in cell viability were observed between cells exposed to Doxorubicin or 4-Cyc and cells exposed to the combination of Doxorubicin and 4-Cyc, in any of the cell lines tested. Similarly, EC25 values of γToc did not affect cytotoxicity of the combined Doxorubicin and 4-Cyc in any of the cell lines.

The effect of chemotherapeutics and antioxidants on ROS production by human breast and ovarian cancer-derived cell lines was tested. Exposure to 4-Cyc induced more ROS faster than exposure to Dox, except in COV434 cells, in which both agents generated the same response. Similar to the results from the cell viability assays, exposure to the combination of Doxorubicin and 4-Cyc did not increase the amount of ROS compared to each agent alone, except in COV434 cells where levels of ROS were slightly higher than after exposure to each chemotherapeutic alone. Exposure to γToc did not stimulate ROS generation by human breast and ovarian cancer-derived cell lines; but the addition of γToc to the combination of Doxorubicin and 4-Cyc significantly decreased the amount of ROS

produced in COV434 granulosa cancer cells, even though the numbers of viable cells were not affected.

Apoptosis induction was measured in human breast and ovarian cancer-derived cell lines. Dox caused apoptosis, as observed by TUNEL and DAPI positive results, in 14±2% and 11±3% in MCF-7 and T47D cells, respectively. Similarly, 4-Cyc – related apoptosis percentages were between 10-25% in all cell lines. No caspase -3, -7 activity was observed in the MCF-7 human breast cancer cell line which was expected as MCF-7 cells are deficient in caspase -3 expression; however the absence of caspase -3, -7 activity in T47D cells after 24h exposure to Dox or 4-Cyc suggest that that the optimal time period to measure caspase -3, -7 in T47D cells is different than for the ovarian cell lines.

In ovarian cancer-derived cells, the combination of Dox and 4-Cyc caused a significant caspase -3, -7 activation, but luminescence values were not significantly different compared to the exposure to 4-Cyc alone. Similarly, percentage of apoptotic cells after exposure to the combination of Dox and 4-Cyc was not significantly different to the percentage of dead cells after treatment with either Dox or 4-Cyc as single agents as shown by both TUNEL and DAPI results. Adding α or γToc to the combination of Dox and 4-Cyc had no effect on Caspase -3, -7 activity or apoptosis induction in any of the cell lines tested compared to the combination of Dox and 4-Cyc.

From the results of PhD project it can be concluded that the combination of Dox and 4-yc did not potentiate the *in vitro* cytotoxic effect of each agent alone against breast cancer cells. γToc was found to be cytotoxic towards the breast cancer cell lines but had no effect against COV434 or KGN granulosa cells. It was also concluded d that the antioxidant properties of γToc reduced chemotherapy-

related ROS generation and probably supported estrogen hormone production by the KGN cells, suggesting that γToc may have the potential to be developed into a fertility preservation therapy for premenopausal breast cancer patient.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text

Daniela Figueroa

“Science is but an image of the truth.”

Francis Bacon

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NOTE ON THE STRUCTURE AND FORMAT OF THE THESIS

Sections 3.1, 4.1 and 4.2 consist of published papers and/or papers submitted for publication during the candidature. Where papers have multiple authorship, written permission of co-authors has been granted.

LIST OF PUBLICATIONS

Peer-reviewed published articles

1. Asaduzzaman, M, Figueroa, D & Young, F 2018, 'Ovarian follicle disaggregation to assess granulosa cell viability', International Journal of Clinical Medicine, vol. 9, pp. 377-399.
2. Figueroa, D, Asaduzzaman, M & Young, F 2018, 'Real time monitoring and quantification of reactive oxygen species in breast cancer cell line MCF-7 by 2′, 7′–dichlorofluorescindiacetate (DCFDA) assay', Journal of pharmacological and toxicological methods, vol. 94, pp. 26-33.
3. Alhalili, Z, Shapter, J, Figueroa, D, Sanderson, B 2018, 'Specific Targeting of Breast Cancer Cells with Antibodies Conjugated Gold Nanoparticles', Drug Delivery Letters, vol. 8(3), pp. 217-225.
4. Figueroa, D, Asaduzzaman, M & Young, F 2019, Effect of chemotherapeutics and γTocopherol on MCF-7 breast adenocarcinoma and KGN ovarian carcinoma cell lines *in vitro'*, Biomed Research International, vol. 18, pp. 1-13.

Articles submitted for publication

1. Figueroa D & Young, F 2019 'Gamma tocopherol reduced chemotherapeutic-induced ROS in an ovarian granulosa cell line, but not in breast cancer cell lines *in vitro* ', Antioxidants.

GLOSSARY OF TERMS

αToc:	Alpha Tocopherol
γToc:	Gamma Tocopherol
4-Cyc:	4-hydroperoxycyclophosphamide
AC:	Doxorubicin (Adriamycin) + cyclophosphamide
AMH:	Anti-Mullerian Hormone
ANOVA:	Analysis of Variance
ATCC:	America Type Culture Collection
BSA:	Bovine Serum Albumin
cAMP:	Cyclic Adenosine Monophosphate
CoV:	Coefficients of Variation
CV:	Crystal Violet
DAPI:	4′,6-Diamidino-2-phenylindole dihydrochloride
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	Dimethylsulfoxide
DNA:	Deoxyribonucleic Acid
Dox:	Doxorubicin
E2:	Estradiol
EC:	Effective Concentration
ECM:	Extracellular Matrix
EDTA:	Ethylene Diamine Tetra-Acetic Acid
EIA:	Enzyme-Linked Immunoassay
ER	Estrogen receptor
ELISA:	Enzyme-Linked Immunosorbent Assay

EtOH: Ethanol

FCS: Foetal Calf Serum

FSH: Follicle Stimulating Hormone

GC: Granulosa Cell

HBSS: Hanks Balanced Salt Solution

HER2: human epidermal growth factor receptor 2

HR: Hormone receptor

ITS: Insulin, Transferrin and Selenium

IVF: *In Vitro* Fertilization

LH: Luteinizing Hormone

mRNA: Messenger Ribonucleic acid

NaCl: Sodium chloride

NO: Nitric Oxide

P4: Progesterone

P450: Family of oxidase cytochrome enzymes

PARP: Poly-ADP-Ribose Polymerase

PBS: Phosphate Buffered Saline

PCOS: Polycystic Ovary Syndrome

ROS: Reactive Oxygen Species

SD: Standard Deviation

SDS: Sodium dodecyl sulphate

SSC: Sodium chloride and sodium citrate solution

TB: Trypan Blue

TGF: Transforming Growth Factor

TUNEL: Terminal dUTP Nick End-Labelling

1. CHAPTER I: LITERATURE REVIEW

1.1 About breast cancer

1.1.1 Incidence & frequency of breast cancer

Breast cancer is the most common malignancy among women worldwide (DeSantis, Ma et al. 2017, Siegel, Miller et al. 2017), is the second leading cause of cancer-related death after lung cancer (Siegel, Miller et al. 2017), and the first cause of death in breast cancer patients under 45 years of age (Assi, Khoury et al. 2013). There are more than 1 million new cases of breast cancer and 400,000 deaths each year globally (Siegel, Miller et al. 2017). The incidence of breast cancer is higher in western societies because of early menarche, null parity or late pregnancy, short lactation periods, delayed menopause, exposure to exogenous estrogen (McPherson, Steel et al. 2000). In 2012, 15,050 females were diagnosed with breast cancer in Australia, and it is estimated that 18,235 new cases of cancer will be diagnosed in 2018 (Ferlay, Soerjomataram et al. 2015, AIHW 2018). In the US around 252,710 new cases of invasive breast cancer and 63,410 cases of *in situ* breast carcinoma were expected to be diagnosed in 2017; and 40,610 women were expected to die from breast cancer in the same year (DeSantis, Ma et al. 2017).

1.1.2 Breast cancer patients ages

In the Western world breast cancer is largely associated with postmenopausal women, but in Asian countries is increasingly reported in young premenopausal women (Agarwal, Pradeep et al. 2007, Son, Dominici et al. 2015). In 2012, the risk of

diagnosis for breast cancer in premenopausal Australian women younger than age 50 years was 2.2%, or 1 in 46 (Ferlay, Soerjomataram et al. 2015). In the United States, 20% of all breast cancers are diagnosed in women younger than 50 years and is the most common malignant disease in adolescent and young adult women (15-39 years of age) (Johnson, Chien et al. 2013). In 2008, the risk of developing cancer before the age of 40 was 1 in 173, and in 20-39 year old women, the incidence of breast cancer increased by 2% per year between 1978 and 2008 (Johnson, Chien et al. 2013). In Africa, 70% of breast cancer patients are aged 50 or less, and most of the cases occur in premenopausal women (Sighoko, Kamaté et al. 2013). In a large-scale epidemiological investigation in China, a total of 320 cases of breast cancer were identified, out of which 60% were diagnosed before menopause (Yu, Jia et al. 2012). In another study, breast cancer patients in developing Asian countries were found to be on average a decade younger than their equivalents in developed Asian and western countries; with a prevalence of 25% among patients of 35 years of age (Fan, Goss et al. 2015).

1.2 Different types of breast cancer

In the past, breast cancer was perceived as a single disease with varying histological features (Rakha, Reis-Filho et al. 2010). The clinical histopathological assessment includes the histological grade or degree of differentiation and the histological type or growth pattern of the tumours (Elston and Ellis 2002)

From a histological perspective, breast cancer can be generally classified as *in situ* carcinoma and invasive carcinoma (Malhotra, Zhao et al. 2010, Weigelt, Geyer et al. 2010) (Fig 1.1a). *In situ* carcinoma can be further categorized as either

ductal or lobular and ductal carcinoma *in situ* (DCIS) is the most common. It originates from the milk ducts and comprises a mixed group of tumours (Malhotra, Zhao et al. 2010). Lobular carcinoma *in situ* (LCIS) is characterized by a diffuse growth pattern, and it originates in the milk-producing lobules (Malhotra, Zhao et al. 2010).

Invasive carcinoma are also differentiated into histological subtypes including infiltrating ductal, invasive lobular, ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas (Weigelt, Geyer et al. 2010). Similar to *in situ* carcinoma; infiltrating ductal carcinoma (IDC) is the most common invasive subtype, accounting for 70-80% of all invasive malignancies (Li, Uribe et al. 2005). IDC is classified based on the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index into well-differentiated (grade 1), moderately differentiated (grade 2) or poorly differentiated (grade 3) (Lester, Bose et al. 2009).

A major drawback of the histological classification is that it fails to show the much broader heterogeneity of breast cancer by grouping tumours with different biological and molecular profile (Rakha, Reis-Filho et al. 2010). The histological appearance of breast tumours is not enough to explain the underlying biological events implicated in the development and progression of cancer. Furthermore, tumours with similar clinical and histological presentations may behave differently and may need a different treatment approach (Clarke, Keegan et al. 2012). Therefore, the relevance and clinical utility of the histopathological classification is quite modest (Viale 2012).

More recent studies have focused on refining breast cancer classification for predicting prognosis as well as the potential response to treatment (Vuong, Simpson et al. 2014). Apart from the histological subtypes, analyses of gene-expression show

breast cancer can also be classified based on Estrogen-receptor (ER), progesterone receptor (PR) (often named together as hormone receptor (HR)) and human growth factor receptor (HER2) status (Zhang, Man et al. 2014).

A more detailed biological classification has been derived by incorporating the molecular characteristics to the conventional breast cancer classification system (Weigelt, Geyer et al. 2010). The employment of microarray-based gene expression profiling allowed the description of the intrinsic breast cancer subtypes based on the HR status (Weigelt, Geyer et al. 2010)

There are two predominantly HR-positive molecular breast cancer subtypes: luminal A and luminal B; and two HR-negative subtypes: human epidermal growth factor receptor 2 (HER2) – enriched and basal-like/triple negative (Yersal and Barutca 2014). The molecular subtypes are categorized by the gene expression involved in luminal epithelial differentiation (ER and PR genes); proliferation, HER2 pathway, and basal differentiation (Yersal and Barutca 2014, Zhang, Man et al. 2014)

HR-positive tumours are the most common type of breast cancer, reported in 60-70% of cases (Blows, Driver et al. 2010). Luminal A and B tumours activate HR-responsive genes that encode for specific proteins associated with luminal epithelial cells (Blows, Driver et al. 2010). The luminal A subtype usually has a good prognosis and it can be distinguished by its low histological grade, low degree of nuclear pleomorphism, and include special histological types (*i.e.*, tubular, invasive cribriform, mucinous and lobular) (Park, Lee et al. 2017). Luminal A can be distinguished by the high expression of luminal epithelial genes and low proliferation; and can be classified as ER-positive or progesterone-receptor positive (PR-positive) but HER2- negative. The luminal B subtype is ER-positive or PR-positive and HER-

2- positive (Sorlie, Tibshirani et al. 2003), it comprises 15-20% of all breast cancers and has a more aggressive phenotype, higher histological grade, proliferative index and usually poor prognosis (Blows, Driver et al. 2010).

Among HR-negative tumours, HER2- enriched has been reported in 18-25% of human breast cancers (Owens, Horten et al. 2004). Morphologically, these tumours are highly proliferative and, in most cases, have high histological and nuclear grade. HER2 positivity confers more aggressive biological and poor prognosis (Sagara, Takada et al. 2018). Basal-like/triple negative subtype is classified as HR-negative and HER2-negative, and it can be distinguished by the high expression of basal stratified epithelial cytokeratins, and the expression of proliferation-related genes (Sagara, Takada et al. 2018). Basal-like cancers have a high histological and nuclear grade, most of these tumours are infiltrating ductal tumours with solid growth pattern, aggressive behaviour and increased rate of metastasis (Fulford, Reis-Filho et al. 2007).

Normal breast-like subtype accounts for 5-10% of all breast cancers (Yersal and Barutca 2014) They are poorly characterized and are classified as HR-negative and HER2-negative (Yersal and Barutca 2014). However, these tumours differentiate from basal-like carcinoma because they lack expression of CK5 and EGFR (Pillai, Tay et al. 2012)

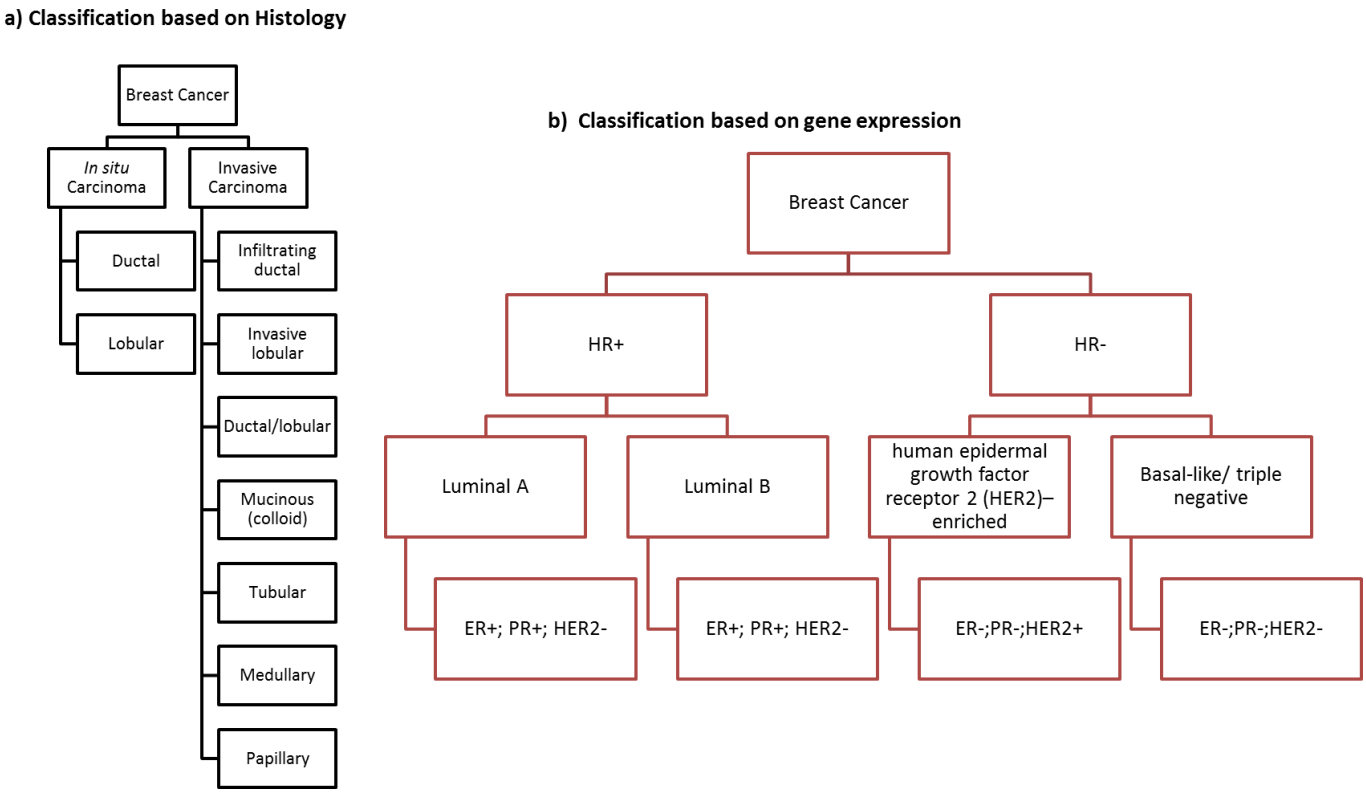


Figure 1.1 Classification of Breast cancer based on: a) Histological subtypes and, b) HR and HER2 gene expression.

The age incidence rates for all types of breast cancer show a bimodal distribution consisting of early-onset (near age 50) and late-onset (near age 70) peaks (Weigelt, Geyer et al. 2010). Luminal A and luminal B cases also have bimodal age distributions with the highest incidences near age 50 and 70 (Kurian, Fish et al. 2010). Clarke et al. (2012) highlighted that the luminal A subtype is the most common type of breast cancer across all ages, including women between 20-44 years of age (Clarke, Keegan et al. 2012) and approximately two thirds of breast

tumours in women ≤40 years are identified as ER or PR-positive (Clarke, Keegan et al. 2012).

In the case of ER-negative tumours, the basal-like subtype has shown an increased rate early in life, with an early-onset mode at age 52 years (Keegan, DeRouen et al. 2012). Similar results were observed in HER2-enriched phenotypes (Telli, Chang et al. 2011).

Shoemaker et al., (2018) calculated breast cancer incidence rates and trends among women aged 20-49 years from the National Program of Cancer Registries and Surveillance, Epidemiology, and End Results Program data. It was found that HR+/HER2- or luminal A represented 58% of all cases and it was the most common subtypes among all cases, followed by basal-like and Luminal B (Fig 1.2) (Shoemaker, White et al. 2018) Incidence rates also varied by race/ethnicity. In women aged 20-34 years, black women had higher rates of Luminal A subtype compared to other ethnic groups; similarly, white women had significantly higher rates of Luminal than Asian and Hispanics (Shoemaker, White et al. 2018).

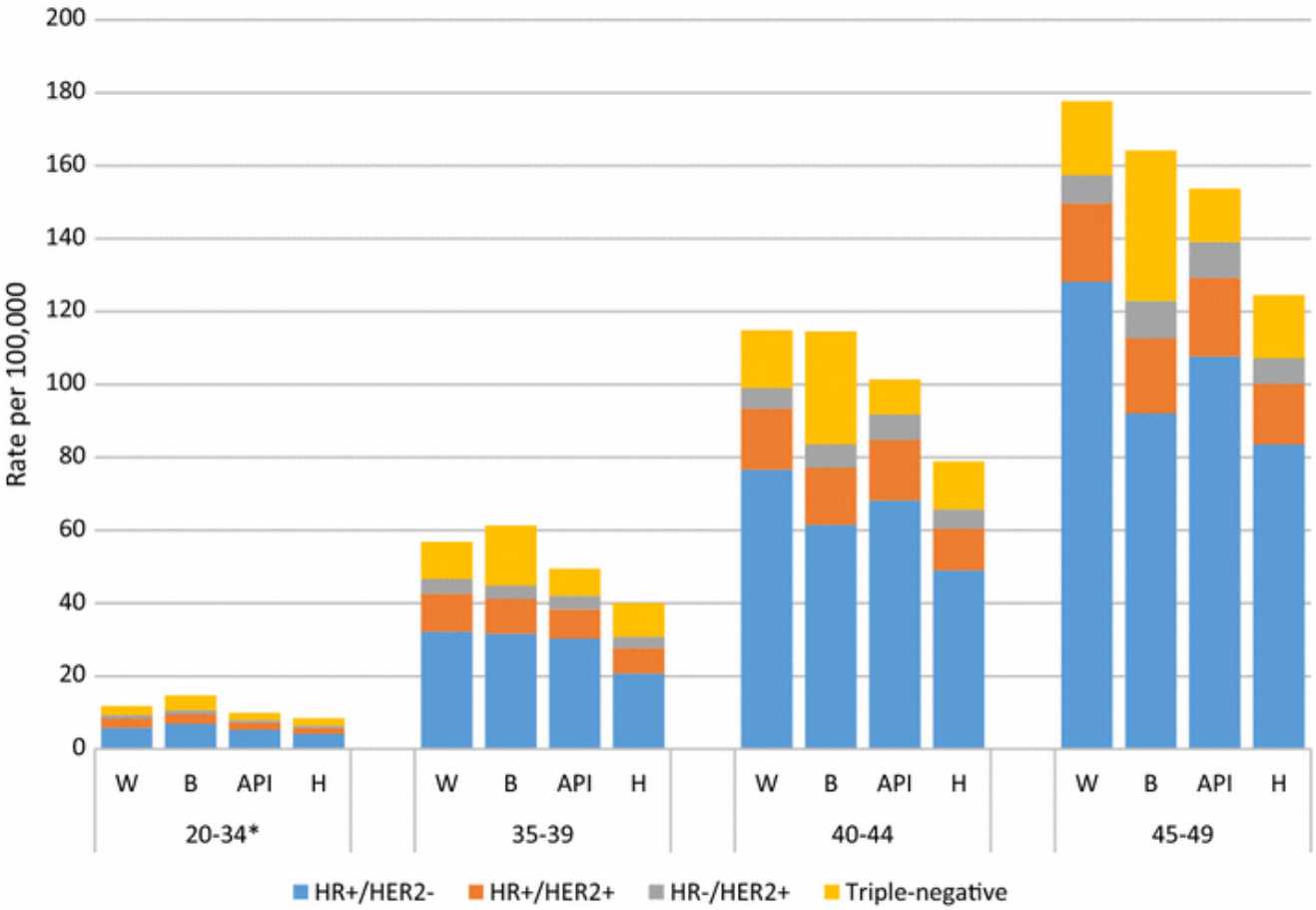


Figure 1.2. Female invasive breast cancer incidence by: tumour subtype, race/ethnicity, and age group, NPCR/SEER 2011–2013.

W: white, B: black, API: Asian or Pacific Islander, AIAN: American Indian or Alaska Native, H: Hispanic. *Rates are age adjusted to the 2000 US Standard Population (Shoemaker, White et al. 2018).

1.3 Risks factors

Numerous factors are associated with a risk for developing breast cancer including genetic predisposition, diet, physical activity, body mass index (BMI), and hormone replacement therapy (HRT) (Terry et al., 2002, Tjønneland, 2008).

The risk for breast cancer may be higher in women with a family history of BC (Lynch, Watson et al. 1988). Nearly 10% of all breast cancer cases in Western countries are associated to a genetic predisposition (McPherson, Steel et al. 2000).

Women aged 25 to 29 years with family history of breast cancer are more likely to be diagnosed with benign breast cancer (relative risk [RR], 1.96; 95% CI, 1.55-2.47) (Webb, Byrne et al. 2002). Women with previous benign breast disease have a four to five times higher risk of developing breast cancer than women with no previous history (McPherson, Steel et al. 2000). Breast cancer susceptibility is generally inherited as an autosomal dominant, several genes have been related to an increased risk to develop the disease including breast cancer gene one (BRC1) and breast cancer gene two (BRC2); however, the total amount of genes involved in breast cancer is still unknown (Rizzolo, Silvestri et al. 2011)

Dietary fat intake is also correlated to the incidence of breast cancer (Lubin, Burns et al. 1981, Howe, Hirohata et al. 1990) but the true link between fat intake and breast cancer has not been yet elucidated (Greenwald, Sherwood et al. 1997, Boeke, Eliassen et al. 2014). Similarly, different studies suggest that there is a relationship between vitamin E and Breast cancer (Rohan, Howe et al. 1993, Kline, Yu et al. 2004, Lee, Ju et al. 2009) Epidemiologic studies studying the correlation between vitamin E and breast cancer concluded that dietary vitamin E may offer a modest protection from developing breast cancer; however, there was no evidence that further supplementation with vitamin E conferred any additional protection against the malignancy (Stanner, Hughes et al. 2004)

1.4 Treatment for breast cancer

The selection of treatment depends on the stage of the disease, the hormone-receptor status and other characteristics of the individual patient (Wendy and Graham 2007); In most cases more than one type of treatment is needed and

usually chemotherapy is administered either as neoadjuvant (before surgery) or as adjuvant (after surgery) therapy.

Combined chemotherapy, a treatment modality that combines two or more therapeutic agents, is a foundation of cancer therapy (Blagosklonny 2004, Yap, Omlin et al. 2013). It was originally hypothesized that combination therapy regimens 1) should administer agents with synergistic effect at the same time and at the maximum possible cumulative dose (Pritchard, Lauffenburger et al. 2012, Bayat Mokhtari, Homayouni et al. 2017), and 2) drugs selected for combination therapy should have non-overlapping mechanisms of action, to address tumour cell heterogeneity, minimize the probability of therapeutic resistance, and reduce the occurrence of side effects (Pritchard, Lauffenburger et al. 2012). For breast cancer patients, combined chemotherapy provided higher rates of response and longer progression to recurrence in comparison to single agent chemotherapy (O'Shaughnessy, Miles et al. 2002, Sledge, Neuberg et al. 2003). Although there is ongoing debate about the increase in toxicity and survival benefit, adjuvant therapy with two or more chemotherapeutics is widely used in the treatment of ER- and/or PR-positive breast cancer (Park, Lee et al. 2017). Adjuvant treatment with chemotherapy is associated with a 35% reduction of recurrence in patients younger than 50 years (Abe, Abe et al. 1998, Chew 2001). Early studies of adjuvant therapy suggested that regimens combining cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) could improve survival for operable breast cancer patients (Bonadonna, Brusamolino et al. 1976). More recent studies suggest that anthracycline-based adjuvant therapy is more beneficial than non-anthracycline-based chemotherapy (Chew 2001, Anampa, Makower et al. 2015). Patients treated with six cycles of docetaxel 75 mg/m^2, doxorubicin 50 mg/m^2 and cyclophosphamide

500 mg/m^2 (TAC), showed a five year recurrence-free survival rate of 88% and similarly, six adjuvant cycles of doxorubicin 60 mg/m^2 and cyclophosphamide 600 mg/m^2, showed a five year recurrence-free survival rate of 87% and overall survival in 94% of the patients (Martin 2006, van Rossum, Kok et al. 2018).

On the other hand, neoadjuvant chemotherapy is increasingly being used as a treatment for patients with locally advanced breast cancer and for patients with large operable tumours or positive, metastatic lymph nodes (Straver, Rutgers et al. 2010). Compared to adjuvant therapy, neoadjuvant chemotherapy offers the advantage of monitoring treatment effect in terms of pathologic complete response (pCR) (von Minckwitz, Kümmel et al. 2008). Neoadjuvant therapy for 24 weeks with an anthracycline-cyclophosphamide followed by a taxane has the best results with higher pCR rates in patients with Luminal A subtype (Von Minckwitz and Fontanella 2013).

1.5 Doxorubicin and cyclophosphamide (AC) chemotherapy for treating breast cancer

The combination of doxorubicin and cyclophosphamide (AC) is one of the most widespread anthracycline-based adjuvant regimens used for early-stage and advanced breast cancer (Nabholtz, Falkson et al. 2003, Jones, Walsh et al. 2009, Early Breast Cancer Trialists' Collaborative 2012). This combination was first tested by the National Surgical Adjuvant Breast and Bowel Project (NSABP) as an alternative to thedox cyclophosphamide, methotrexate and 5-fluorouracil (CMF) regimen (Rampurwala, Rocque et al. 2014). 4 cycles of adjuvant doxorubicin and cyclophosphamide proved to be as effective as 6 cycles of CMF in women with

node-positive breast cancer (Fisher, Brown et al. 1990) and a meta-analysis performed by the early breast cancer trialists collaborative group found that the combination of doxorubicin and cyclophosphamide resulted in improved survival and recurrence, compared to the standard CMF (Early Breast Cancer Trialists' Collaborative 2012). Findings from the NSABP trials also supported the efficacy of doxorubicin and cyclophosphamide (AC) in patients with node-positive disease with ER-positive tumours, compared to the CMF regimen (Rampurwala, Rocque et al. 2014). Furthermore, AC was considered less toxic than CMF which justifies the use of AC regimen in either premenopausal or postmenopausal women with breast cancer with/without positive axillary nodes, and in patients with ER-negative or ER-positive tumours (Sayer, Kath et al. 2002)

Standard AC therapy commonly involves the administration of 60mg/m^2 of doxorubicin followed by 600mg/m^2 of cyclophosphamide intravenously (Dees, O'reilly et al. 2000, Jones, Savin et al. 2006), which results in plasma concentrations of 1.8±0.4μM doxorubicin 24h post-infusion (Swenson, Bolcsak et al. 2003), and serum concentrations of 4-hydroxycyclophosphamide ranging from 0.23-1.08 μg/h/m in the 24h period post-administration. In previous studies, the serum concentrations of 4-hydroxycyclophosphamide increased to approximately 0.5ug/mL (0.02uM) 2-4h after the *in vivo* administration of cyclophosphamide (Struck, Alberts et al. 1987).

The treatment of early breast cancer with AC reduced breast cancer mortality rates by 20-25% (Early Breast Cancer Trialists' Collaborative 2012). Similarly, 2-year recurrence rates were halved, and the 8-year recurrence rate was reduced by one-third (Early Breast Cancer Trialists' Collaborative 2012). On the other hand, patients with large primary or locally advanced breast cancer have only a partial clinical response to AC treatment, ranging from 61-79% (Fisher, Brown et al. 1997, Bear,

Anderson et al. 2003, Evans, Yellowlees et al. 2005) while complete clinical response rates range from 17-35% (Fisher, Brown et al. 1997, Bear, Anderson et al. 2003, Evans, Yellowlees et al. 2005).In phase III randomised trials, an AC regimen showed a response rate of only 50% in advanced cancer patients, and complete remission in 7% (Nabholtz, Falkson et al. 2003).

For premenopausal women with Luminal A (HR-positive; HER2- negative) breast cancer, treatment modalities have been extrapolated from postmenopausal patients' data (Bardia and Hurvitz 2018). But this appears suboptimal because several studies found that younger women had considerably worse survival than older women aged 40-49 (De Camargo Cancela, Comber et al. 2016). Younger women with HR-positive tumours have a worse prognosis than older women and they are still often under-represented in clinical trials (Bardia and Hurvitz 2018)

Premenopausal patients frequently present with a different tumour biology and clinical response profiles (Bardia and Hurvitz 2018). Compared to postmenopausal patients, premenopausal women often have a more advanced cancer stage at diagnosis (Assi, Khoury et al. 2013) often a consequence of the lack of routine screening in this age cohort (Zabicki, Colbert et al. 2006). Premenopausal women are more likely to have positive axillary lymph nodes (Anders, Hsu et al. 2008); and more aggressive breast cancer subtype (Assi, Khoury et al. 2013) which requires more intensive treatment approaches (Partridge 2013). Young premenopausal patients therefore have poorer clinical outcomes; with increased risk of recurrence, which is 1.5 times higher than in postmenopausal women (Assi, Khoury et al. 2013, Bardia and Hurvitz 2018).

1.5.1 Adverse off-target toxicity of AC chemotherapy

Anthracyclines such as doxorubicin can be cardio-toxic to cancer patients and lead to the development of potentially fatal congestive heart failure (Zhang, Liu et al. 2012). Despite numerous studies (Swain, Whaley et al. 2003, Takemura and Fujiwara 2007, Chatterjee, Zhang et al. 2010), the precise mechanism for doxorubicin-induced cardiotoxicity is not completely understood (Zhang, Shi et al. 2009). It is believed ROS-mediated oxidative stress plays a pivotal role cardiomyocyte death, as doxorubicin treatment increases oxidative stress and affects calcium homeostasis (Zhang, Shi et al. 2009). High doses of doxorubicin have also caused hepatic, haematological and testicular toxicity (Mohan, Kamble et al. 2010, Damodar, Smitha et al. 2014), as well as damage to the DNA of pancreatic β-cells (Heart, Karandrea et al. 2016).

The administration of cyclophosphamide is associated with bone marrow suppression and dose-dependent leukopenia, thrombocytopenia and anaemia (Emadi, Jones et al. 2009). Cardiotoxicity has also been reported as a toxic effect of high doses of cyclophosphamide (Santos, Sensenbrenner et al. 1972) and bladder toxicity usually occurs in the form of haemorrhagic cystitis (Stillwell and Benson 1988). Jonge et al. (2006) found that high doses of cyclophosphamide correlated to an increased risk of hepatic venoocclusive disease, and Mc Donald et al. (2003); found that exposure to high levels of the active metabolite carboxyethylphosphoramide mustard were related to hepatic toxicity. Nerve damage, nausea, vomiting and reversible alopecia are also common adverse effects of cyclophosphamide (Emadi, Jones et al. 2009, Jayachandran, Chandrasekhara et al. 2009).

Different studies have evaluated the toxicity of AC in breast cancer patients (Vriens, Aarts et al. 2013, van Rossum, Kok et al. 2018). In a randomised study, out of 317 patients that received adjuvant cycles of doxorubicin 60mg/m^2 and cyclophosphamide 600 mg/m^2 every 2 weeks; 18.9% developed anaemia, 6.4% diarrhoea and 4.6% peripheral neuropathy (van Rossum, Kok et al. 2018). Two patients suffered severe toxic effects: one developed acute myeloid leukaemia, and another patient had cardiac failure (van Rossum, Kok et al. 2018). In a different study, the most common side effect in breast cancer patients treated with AC, was neutropenic fever (23%) followed by nausea and fatigue (Vriens, Aarts et al. 2013).
Off-target toxicity towards the ovary has only recently been recognised as another adverse effect (refs).
Patients with locally advanced breast cancer who were treated with AC had grade 1 – 2 nausea, vomiting and anorexia (Moon et al 2013).

1.5.2 Doxorubicin chemistry and mechanisms of action

Doxorubicin is a nonselective class I anthracycline antibiotic first isolated from *Streptomyces peucetius var. caesius* in the 1970's (Thorn, Oshiro et al. 2011) and is regarded as one of the most effective FDA-approved chemotherapeutic drugs (Sullivan 2008). Its ability to interfere with enzymes required for DNA replication and to therefore combat rapid cell proliferation (Tacar, Sriamornsak et al. 2012) has resulted in doxorubicin being used widely to treat several types of cancers including breast, lung, gastric, ovarian, and thyroid (Weiss 1992, Cortes-Funes and Coronado 2007).

Doxorubicin possesses aglyconic moieties with tetracyclic ring, quinine-hydroquinone groups and a methoxy substituent short side chain followed by the carbonyl group (Tacar, Sriamornsak et al. 2012). It also has sugar components bound to one of the rings by a glycosidic bond. This is comprised of a 3-amino-2,3,4–trideoxy-L-fucosyl moiety (Fig 1.3) (Hilmer, Cogger et al. 2004).

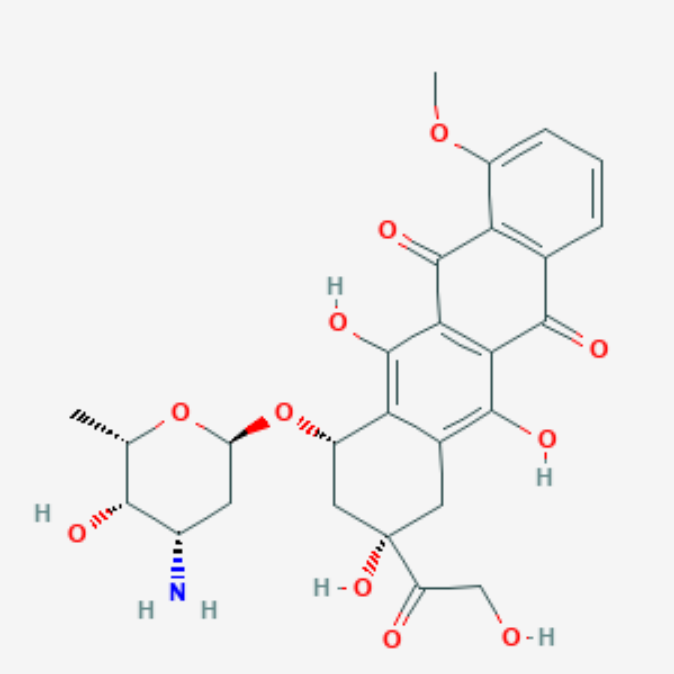


Figure 1.3 Chemical structure of doxorubicin

(PubChem 2019)

Doxorubicin causes cell death by binding to DNA-associated topoisomerase enzymes I and II (Buchholz, Stivers et al. 2002). Apoptosis is triggered when cells fail to repair breaks in DNA, and cellular growth is inhibited at phases G_1 and G_2 (Tacar, Sriamornsak et al. 2012). Doxorubicin can also intercalate into the DNA double helix, inhibit DNA and RNA polymerases and prevent DNA replication and RNA transcription (Tacar, Sriamornsak et al. 2012). Another mechanism of action

involves the generation of free radicals which cause damage to cellular membranes and DNA, inhibition of macromolecule production, DNA unwinding and increases in alkylation (Gewirtz 1999).

Doxorubicin diffuses into cells because it has a high binding affinity to the 20S proteasome subunit in the cytoplasm (Fig 1.4) (Tacar, Sriamornsak et al. 2012). The doxorubicin-proteasome complexes are then translocated into the nucleus (Kiyomiya, Matsuo et al. 2001) where the complex is dissociated, allowing doxorubicin to bind to the DNA (Hilmer, Cogger et al. 2004). Doxorubicin can also undergo a one-electron reduction to produce a DOX-semiquinone radical. This is mediated by several oxidoreductases, including mitochondrial NADH dehydrogenase, NAD(P)H dehydrogenase, xanthine oxidase and nitric oxide synthase (Vasquez-Vivar, Martasek et al. 1997, Miyamoto, Koh et al. 2003, Pawlowska, Tarasiuk et al. 2003). Re-oxidation of the DOX-semiquinone radical back to doxorubicin releases reactive oxygen species (ROS), which causes oxidative stress and triggers apoptosis and ultimately cell death (Fig 1.4) (Minotti, Recalcati et al. 1998).

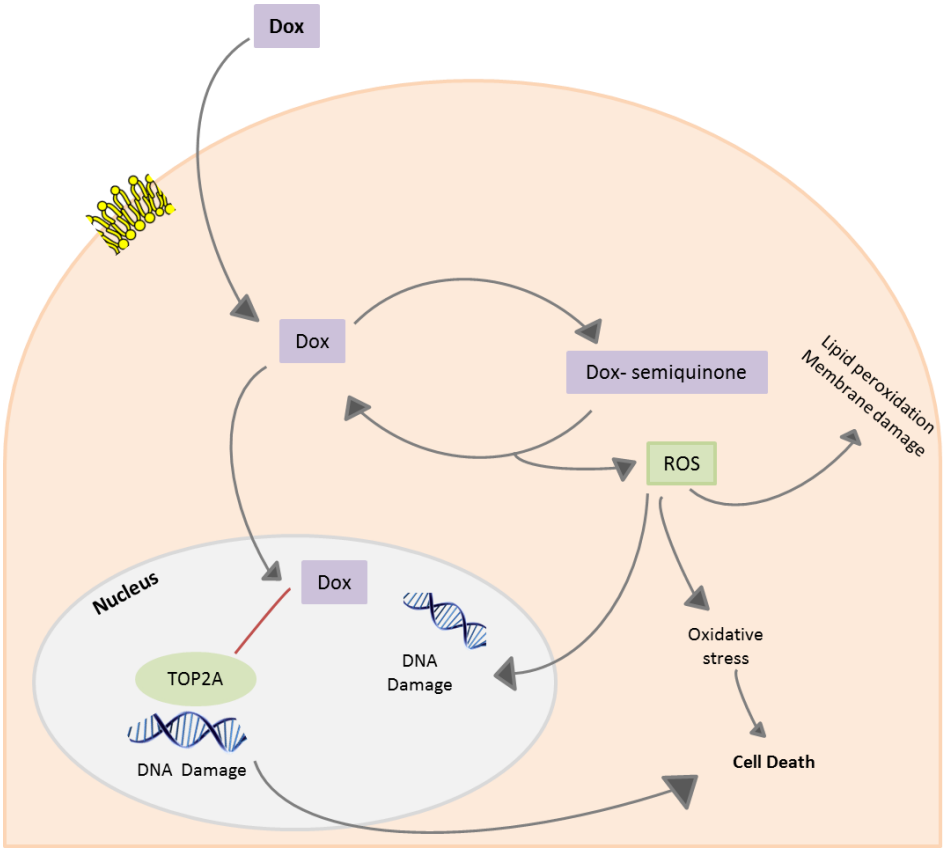


Figure 1.4. Representation of Doxorubicin pathway in a cancer cell.

Adapted from Thorn, Oshiro et al. (2011).

Doxorubicin can also cause cell death by binding to mitochondrial DNA (Ashley and Poulton 2009). It has been suggested that doxorubicin can bind to plasma proteins which triggers a cascade of enzymatic reactions that causes the reduction of doxorubicin and the formation of more ROS (Tacar, Sriamornsak et al. 2012). As well as causing apoptosis doxorubicin also induces autophagy and necrosis by hyperactivation of PARP-1 which in turn depletes both Nicotinamide adenine dinucleotide (NAD^+) and Adenosine Triphosphate (ATP); resulting in energy failure and cell death (Tacar, Sriamornsak et al. 2012).The specific pathway to doxorubicin-induced cell death varies depending on the type of cancer cell,

concentration of the drug and treatment duration (Tacar, Sriamornsak et al. 2012).

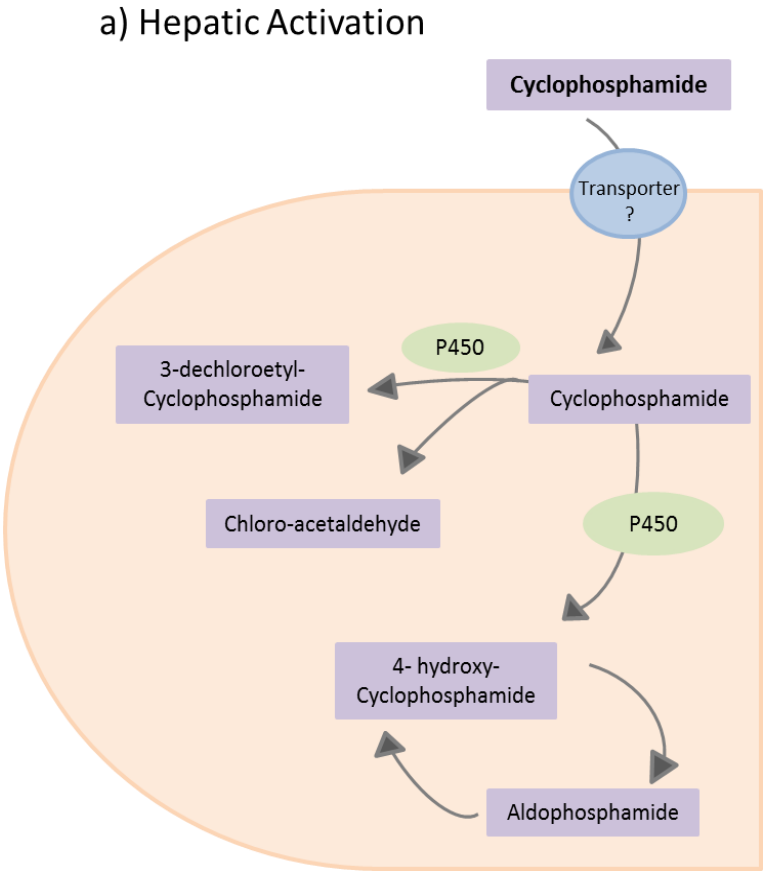
Studies on the pharmacokinetics of Doxorubicin have shown that after intravenous infusion, the distribution half-life is approximately 3-5min, which suggests that Doxorubicin is rapidly taken by the cells (Zheng, Pavlidis et al. 2006). The terminal half-life of Doxorubicin is 24-36h indicating that elimination from the tissues takes much longer than the uptake (Zheng, Pavlidis et al. 2006).

1.5.3 Cyclophosphamide chemistry and mechanisms of action

Cyclophosphamide is an alkylating agent that has been used as an anticancer agent for over 50 years (Emadi, Jones et al. 2009). After early clinical trials cyclophosphamide became the eighth cytotoxic agent approved by the FDA. It was the most effective agent against 33 tumours among 1,000 drugs and antibiotics (Brock and Wilmanns 1958). Cyclophosphamide is an inactive prodrug that requires hepatic activation (Emadi, Jones et al. 2009). The main active compound, phosphoramide mustard, causes intra-strand and inter-strand cross-linking of DNA, which disrupts cell division (Dong, Barsky et al. 1995).

Hepatic activation begins with hydroxylation of cyclophosphamide by a series of cytochrome P450 (Fig 1.5a) to form 4-hydroxycyclophosphamide and aldophosphamide. These coexist and diffuse freely out of hepatic cells into circulation and ultimately into other cells. Intracellular aldophosphamide is metabolized into phosphoramide mustard and acrolein (Fig 1.5b) (Boddy and Yule 2000, Emadi, Jones et al. 2009). Apart from the hydroxylation of cyclophosphamide a second mechanism involves the oxidation of cyclophosphamide and the production of neurotoxic chloroacetaldehyde (Whirl-Carrillo, Mcdonagh et al. 2012).

Phosphoramide mustard interferes with DNA replication (Dong, Barsky et al. 1995) and acrolein, increases ROS production (Luo and Shi 2004, Mythili, Sudharsan et al. 2004, Liu, Li et al. 2012).



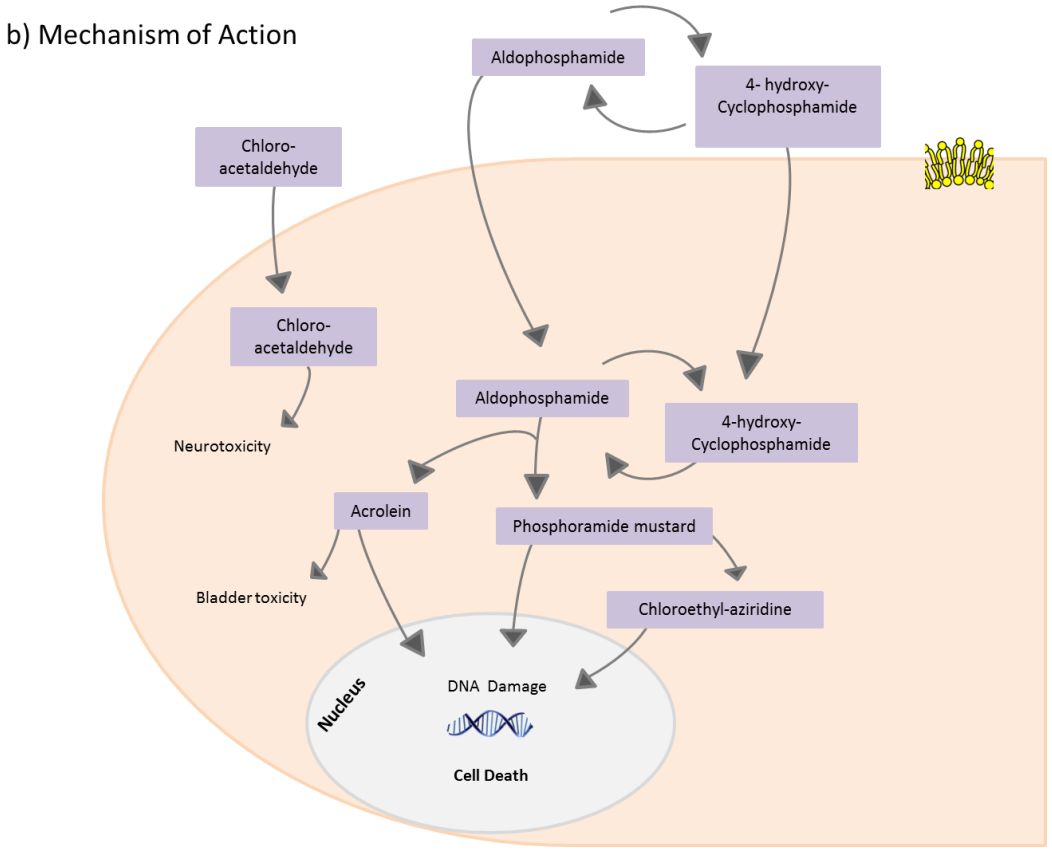


Figure 1.5. Representation of cyclophosphamide pathway.

A) Model human liver cell showing metabolism of cyclophosphamide; B) Model non-tissue-specific cancer cell displaying cyclophosphamide pathway. Adapted from (Whirl-Carrillo, Mcdonagh et al. 2012).

The pharmacokinetics of cyclophosphamide are unclear, due to the complex, multistep metabolic processes and the production of unstable intermediate molecules (Emadi, Jones et al. 2009). Different studies have found that the plasma levels of cyclophosphamide are not predictive of toxicity or tumour response and that the levels of the active metabolites (4-hydroxycyclophosphamide and aldophosphamide) are better predictors of response to therapy (Anderson, Chen et al. 1996, Chen, Kennedy et al. 1997, Nieto, Xu et al. 1999).

Because the parent cyclophosphamide molecule has no anti-cancer activity and requires hepatic activation, a synthetic compound, 4-hydroperoxycyclophosphamide (4HPCYP) and its metabolite 4-hydroxycyclophosphamide have been used to examine the effects of cyclophosphamide *in vitro* (Ozer, Cowens et al. 1982, Dees, O'reilly et al. 2000) and *in vivo* (Teicher, Holden et al. 1996, Yuksel, Bildik et al. 2015).

1.6 Follicle development in the ovary

In woman, the ovaries contain follicles made up of an oocyte, surrounded by granulosa cells, and outer layers of theca cells (McGee and Hsueh 2000). The process of ovarian follicle development, folliculogenesis, starts when quiescent primordial follicles are activated to grow (Fig 1.4) (Gougeon 1996). At birth, the ovary contains millions of primordial follicles (Gougeon 1996). After puberty elevated levels of follicle-stimulating hormone (FSH) induce the recruitment and development of activated primary follicles (Fig 1.6) (McGee and Hsueh 2000).

Figure 1.6 Life history of ovarian follicles.

Every woman is born with a fixed number of primordial follicles, which are maintained in a resting pool. Some of these quiescent follicles are recruited before and throughout reproductive life (initial recruitment). Follicles grow into primary, secondary and antral stages. At the antral stage, most follicle undergo atresia and a few of them, under optimal hormonal stimulation that occurs after puberty, are rescued (cyclic recruitment) to reach the preovulatory stage. Eventually, depletion of the resting pool leads to ovarian follicle exhaustion and menopause (McGee and Hsueh 2000).

Follicle development is divided into two distinct stages based on responsiveness to the gonadotropins, FSH and LH (Matsuda, Inoue et al. 2012, Bertoldo, Bernard et al. 2013, Raju, Chavan et al. 2013). During the first stage, which takes over 120 days (Gougeon 1996), intraovarian and/or other unknown factors activate some primordial follicles to grow (McGee and Hsueh 2000). In this stage, primordial follicles grow in size from 30-40µm to pre-antral follicles of 100-200µm

(Gougeon 1986, Griffin, Emery et al. 2006). The single layer of granulosa cells present in the primordial follicles begins to proliferate into several layers of cuboidal granulosa cells that surround the oocyte (Gougeon 2004). Precursor theca cells are recruited from surrounding stroma, a basement membrane forms around the follicle (Erickson, Magoffin et al. 1985), and the oocyte resumes meiosis (Gougeon 2004).

During the second stage, FSH and LH play important roles in follicle growth (Matsuda, Inoue et al. 2012, Bertoldo, Bernard et al. 2013, Raju, Chavan et al. 2013). Further development results in the formation of a follicular fluid-filled antrum (Rodgers and Irving-Rodgers 2010) and follicles increase in size from 200µm to 2-5mm in the early stages, and to 20mm by the time of ovulation (Gougeon 1986). FSH induces the proliferation and differentiation of granulosa cells which as a result are capable of producing estrogen from the aromatization of androgens secreted by theca cells (Dorrington, Moon et al. 1975, Hillier, Whitelaw et al. 1994, Magoffin 2005).

Increasing estrogen concentrations combined with high FSH levels promotes further proliferation of granulosa cells and increase sensitivity of the follicle to FSH by increasing the number of FSH receptors (McGee and Hsueh 2000). This sets a negative feedback on the hypothalamus-pituitary axis which limits FSH secretion. After this only follicles with increased numbers of FSH receptors continue developing, while other follicles in the growing pool undergo atresia (Fauser and Van Heusden 1997). The dominant follicle continues to grow as a result of granulosa cells acquiring LH receptors (Fauser and Van Heusden 1997, Sullivan, Stewart-Akers et al. 1999, Vegetti and Alagna 2006); which continue to secrete large quantities of estrogen (Zeleznik and Hillier 1984, Zelinski-Wooten, Hess et al. 1994).

1.6.1 Role of AMH

Anti Müllerian hormone (AMH), also referred to as Müllerian inhibiting substance (MIS), is a growth factor involved in early stages of folliculogenesis (Grootegoed, Baarends et al. 1994, Baarends, Uilenbroek et al. 1995). During mouse and rat female foetal development ovarian AMH activity cannot be detected, although AMH mRNA expression is present in ovarian granulosa cells 4 days after birth, which coincides with the initiation of primary follicle growth (Ueno, kuroda et al. 1989, Durlinger, Gruijters et al. 2002). Rajpert-De Meyts et al. (1999) studied AMH production in 135 women. Ovarian tissues of patients aged from 6 weeks post birth to 38 years of age were collected to examine the expression of AMH. AMH could not be detected in early ovarian samples (9-34 weeks), but tissues from 36-weeks' post birth indicated a production of AMH (Rajpert-De Meyts, Jørgensen et al. 1999).

AMH levels in women decrease after birth, and start to increase after two years of age, reach a peak in the mid-20s and then begin to decline with the increasing age until menopause (Kelsey, Anderson et al. 2011, Seifer, Baker et al. 2011).Serum levels of AMH do not vary significantly during the menstrual cycle (Hehenkamp, Looman et al. 2006, La Marca, Stabile et al. 2006), and production of AMH is primarily by primary, secondary and small antral follicles (Weenen, Laven et al. 2004, Hanna, Pellatt et al. 2006).

Studies in AMH knockout (AMHKO) mice indicate that AMH has an inhibitory effect on initial follicle recruitment (Weenen, Laven et al. 2004). In AMHKO mice, depletion of the primordial follicle pool is accelerated and this results in premature cessation of the estrus cycle and reduced reproductive lifespan (Durlinger, Kramer et al. 1999). This data supports the conclusion that AMH regulates the rate of follicle activation (Weenen, Laven et al. 2004).

Recently, AMH levels have been used to predict ovarian follicle reserve (Anders, Marcom et al. 2008, Anderson and Cameron 2011). Serum concentrations of AMH before and after chemotherapy have been evaluated in breast cancer patients (Anderson, Pfeiffer et al. 2006, Oktay, Oktem et al. 2006, Singh, Muttukrishna et al. 2007). In a multivariate study, AMH remained a significant predictor of ongoing ovarian function (Anderson and Cameron 2011); AMH decreased rapidly during treatment, and recovery of menstruation thereafter varied with risk of gonadotoxicity (Anderson and Cameron 2011).

1.6.2 General effects of chemotherapy on the ovary: 'follicle burn out'

Chemotherapy drugs have different mechanisms of action, and therefore can cause damage to the ovary in different ways (Morgan, Anderson et al. 2012), although most chemotherapeutic agents target dividing cells such as the granulosa cells in developing follicles (Raz, Fisch et al. 2002, Devine, Perreault et al. 2012, Mahajan 2015). The follicle 'burn-out' theory proposes that chemotherapy-induced premature ovarian failure occurs through the reduction of the primordial pool of follicles consequent to the loss of activated, growing follicles (Meirow, Biederman et al. 2010). Since chemotherapy causes apoptosis in granulosa cells, which synthesise AMH (Durlinger, Kramer et al. 1999), and because AMH is believed to regulate the rate of primordial follicle activation (Visser, de Jong et al. 2006, Kollmann, Bersinger et al. 2015) depletion of AMH caused by chemotherapy-induced death of granulosa cells, results in an increased recruitment of quiescent primordial follicles into the growing pool (Baarends, Uilenbroek et al. 1995, Stubbs, Hardy et al. 2005, Woodard and Bolcun-Filas 2016). Repeated cycles of chemotherapy may also

reduce the reserve of dormant follicles as well as the cohort of active growing follicles and result in premature ovarian failure.

1.7 Doxorubicin, cyclophosphamide and the ovary

1.7.1 Doxorubicin, cyclophosphamide and ovarian follicles

In animal studies, doxorubicin alone and cyclophosphamide alone caused a significant loss of ovarian follicles (Yucebilgin, Terek et al. 2004, Morgan, Anderson et al. 2012) and it has been hypothesised that each drug targets the more metabolically active granulosa cells (Morgan, Anderson et al. 2012).

1.7.2 Doxorubicin in animal models

Doxorubicin caused apoptosis in mature murine oocytes (Perez, Knudson et al. 1997, Jurisicova, Lee et al. 2006). Mice administered 7.5 or 10 mg/kg Dox IP had increased levels of ovarian apoptosis and significantly less primordial and secondary follicles 1 month post-treatment (Ben-Aharon, Bar-Joseph et al. 2010). Roti et al (2012) administered a high dose of Dox (20mg/kg) to 4-week-old mice. 2h later Dox was found in the ovarian stromal cells. The accumulation of doxorubicin was related to the location of follicles in the ovary; soon after administration Dox was found in the larger centrally located follicles soon after administration, and after 6h in the primary and primordial follicles located in the periphery. Granulosa cells were more susceptible to the apoptotic effect of Dox than the stromal cells, and DNA fragmentation was detected in the oocyte 10 hours post administration (Roti,

Leisman et al. 2012). In another *in vitro* study, human ovarian cortical pieces were exposed to different doses of Doxorubicin. After 24h doxorubicin caused dose-dependent double-strand DNA breaks in primordial follicles, oocytes and granulosa cells (Soleimani, Heytens et al. 2011). Doxorubicin also caused apoptosis in a dose-dependent manner in cultured ovarian cortical tissues with a significant increase in the percentage of apoptotic follicles after exposure to 100µg/mL of doxorubicin. These findings were confirmed in an *in vivo* model in which SCID mice xenografted with human ovarian tissue were exposed to 10mg/kg of doxorubicin (Soleimani, Heytens et al. 2011). It has been proposed that doxorubicin may interfere with the electron transport chain leading to a release of cytochrome c, which in turn activates the caspase cascade and causes apoptosis (Smith and Kumar 2010). In the nucleus, Doxorubicin up-regulated p53 protein expression, which initiates apoptosis when there is significant damage to the DNA (Smith and Kumar 2010). Furthermore, the double-strand breaks caused by doxorubicin can lead to the activation of ataxia telangiectasia mutated protein kinase (AT), which also initiates apoptosis when there is extensive DNA damage (Soleimani, Heytens et al. 2011).

1.7.3 Cyclophosphamide in animal models

The administration of cyclophosphamide to rodents caused a dose-dependent loss of primordial and primary follicles (Meirow, Lewis et al. 1999, Petrillo, Desmeules et al. 2011). In a different study, ovaries from 4 day old mice exposed *in vitro* to 0 – 30µM 4-hydroxycyclophosphamide, had significantly fewer healthy primordial and small primary follicles (Desmeules and Devine 2006) with DNA double strand breaks in the oocytes (Petrillo, Desmeules et al. 2011). Granulosa

cells from rat ovaries had a reduction in mitochondrial transmembrane potential after exposure to cyclophosphamide which led to activation of the caspase family and apoptosis (Zhao, Huang et al. 2010). It has also been suggested that cyclophosphamide up-regulates the proapoptotic Bax protein as well as expression of H2AX, a marker of double-strand DNA breaks (Petrillo, Desmeules et al. 2011).

An immunodeficient mouse model in which human foetal ovary pieces were xenografted into mice which were then administered a single dose of 200mg/kg of cyclophosphamide, gave rise to a 93% reduction in primordial follicle density with cell death by apoptosis after 48h (Oktem and Oktay 2007). The oocytes showed evidence of apoptotic cell death earlier than the follicular granulosa cells (Oktem and Oktay 2007). In a subsequent study, ovarian cortical pieces from cancer patients were used to demonstrate that patients who had previously received any kind of chemotherapy had significantly fewer primordial follicles than the control group of patients who hadn't received chemotherapy (Oktem and Oktay 2007). Those who were treated with cyclophosphamide as part of their treatment regimens had fewer primordial follicles than patients who were treated with non-alkylating agents (Oktem and Oktay 2007).

1.7.4 Combination of Doxorubicin and cyclophosphamide in animal models

The therapeutic synergistic effect of AC has been studied in animal models. Corbett et al., (1975) found that the combination of doxorubicin and cyclophosphamide was more effective at killing 4 different mammary tumours implanted in mice than the optimum effective dose of either agent alone and that the therapeutic potentiation was not schedule dependent (Corbett, Griswold et al. 1975).

Similar results were reported when AC was administered to female mice inoculated with leukaemia L1210 ascites tumour cells (Avery and Roberts 1977).

1.8 Generalised overview of effects of chemotherapeutics on female fertility

A major problem associated with chemotherapy in women with breast cancer is premature ovarian failure, early menopause and reduced fertility (Letourneau, Ebbel et al. 2012). Chemotherapy is associated with reproductive aging of 10 years or more (Kim 2013, Haddidi 2015), hence the age at which breast cancer is diagnosed is one of the most important factors affecting incidence of ovarian failure (Morgan, Anderson et al. 2012). In cohorts of breast cancer patients ≤35 years old with a variety of cancers and who were treated with many different types of chemotherapeutic agents, the incidence of amenorrhea and ovarian failure 3 years after diagnosis was like women without breast cancer who did not receive chemotherapy, only 10%. However, the incidence of amenorrhea increased to 50% in women who were between 35 and 40 years old when they were diagnosed, and reached 85% in women older than 40 years old (Goodwin, Ennis et al. 1999, Sukumvanich, Case et al. 2010). While chemotherapy can damage the ovary at any age, the differences in the onset of permanent amenorrhea and premature ovarian failure can be attributed to older women having a smaller follicle reserve than younger women when they began chemotherapy (Meirow and Nugent 2001). Chemotherapy-induced cytotoxicity affects active granulosa cells, but it also causes ovarian cortical fibrosis and blood vessel damage(Morgan, Anderson et al. 2012). Hyalinization of cortical blood vessels, neovascularization, and cortical fibrosis

observed after exposure to multidrug chemotherapy result in local ischemia, which affects the growth and viabiltiy of primordial follicles (Meirow, Biederman et al. 2010). Similarly, formation of new vessels, which are critical for normal follicle growth, may also be impaired by damaged vasculature and fibrosis (Meirow, Dor et al. 2007).

Amenorrhea has a negative impact on the patient's quality of life (Fioroni, Fava et al. 1994, Bernhard, Zahrieh et al. 2007). Most women with cessation of menses present symptoms including hot flushes, genitourinary syndrome and sexual dysfunction(Bernhard, Zahrieh et al. 2007) and early onset of the menopause increases the risk of osteoporosis (Bruning, Pit et al. 1990), cardiovascular disease (Jeanes, Newby et al. 2007), and psychosocial problems, such as depression (Carter, Rowland et al. 2005). Partridge et al. (2007) found that chemotherapy-related amenorrhea can be an indicator of early menopause.

1.9 Patient age and resumption of menstruation

Clinically, chemotherapy can cause immediate, temporary amenorrhea, but the resumption of menses 12m after diagnosis is not necessarily indicative of ongoing 'long-term' menstruation (Petrek, Naughton et al. 2006), and chemotherapy can also have long–term deleterious effects on ovarian function, that result in permanent amenorrhea (Morgan, Anderson et al. 2012). The risk of chemotherapy-related ovarian failure depends on the patient's age and on the type and dose of the chemotherapeutic agents used (Goodwin, Ennis et al. 1999, Petrek, Naughton et al. 2006, Abusief, Missmer et al. 2010). Older patients have a higher risk to go through early menopause and complete infertility than younger patients. This higher prevalence can be explained by older women having less primordial follicles

(Maltaris, Weigel et al. 2008). Retrospective reports of breast cancer survivals entering premature menopause in their late 30s and early 40s (Rogers and Kristjanson 2002, Lee, Schover et al. 2006, Ajala, Rafi et al. 2010) are available in the literature; however, these studies have many limitations including the sample size or limited follow-up. To this date, the likelihood of chemotherapy-related premature menopause has not been thoroughly explored in breast cancer survivors (Partridge, Gelber et al. 2007).

1.10 Pregnancy in breast cancer survivors

Pregnancy after breast cancer is another area of investigation. Population based studies showed there is a 30-50% reduction in the probability of having a live birth in breast cancer patients (Green, Whitton et al. 2002, Magelssen, Melve et al. 2007, Cvancarova, Samuelsen et al. 2009). In a systematic review of cohort and case-control studies pregnancy rates after breast cancer treatment were on average 40% lower than those for the general population (Gerstl, Sullivan et al. 2018). Some studies reported that less than 5% of all breast cancer survivors will be able to conceive after treatment (Gerstl, Sullivan et al. 2018). The data on the use of chemotherapeutic agents to treat breast cancer women, is not uniformly reported in many studies; however it has been shown that the administration of adjuvant chemotherapy after surgery (mastectomy or breast-conserving surgery) reduced pregnancy rates to 14-47% compared to women who did not receive any chemotherapy (Maltaris, Weigel et al. 2008). The incidence of live births after breast cancer also varies with age. Among women < 45 years of age at diagnosis, only 3%

had full-term pregnancy (Mueller, Simon et al. 2003); and among women < 35 years at diagnosis, 8% were able to conceive (Blakely, Buzdar et al. 2004)

There has been concern that pregnancy after breast cancer may worsen the prognosis or cause genetic mutations to the embryo, hence women are usually advised to wait between 6 and 12 months before attempting natural conception (Petrek 1994, Gwyn and Theriault 2001). The waiting period depends on the type of malignancy (high levels of estradiol may increase rates of recurrence) and the type of chemotherapy. Approximately 50% of breast cancers are estrogen sensitive (Petrek, Naughton et al. 2006, Davies 2013) and it is standard to treat these patients with tamoxifen for 5y to prevent estrogen stimulating recurrence and growth of the tumours (Davies 2013). Petrek et al., (2006) reported that 90% of patients with ER+ breast cancer who were treated with tamoxifen and were <35y at diagnosis resumed menstruation 1y later, whereas only 8% of women with a median age of 40y at diagnosis were menstruating after 5y treatment with tamoxifen (Davies 2013).

Pregnancy rates after 2yr of diagnosis have been reported (Ives, Saunders et al. 2007). Out of 2539 breast cancer patients in Western Australia, 123 (5%) had at least one pregnancy after confirmed diagnosis, 49.5% of these were after the 2years mark (Ives, Saunders et al. 2007). 64% of all pregnancy were full-term, 15% ended in miscarriage and 34% resulted in voluntary termination. 41% received some form of chemotherapy; data of pregnancies after chemotherapy was not disclosed (Ives, Saunders et al. 2007).

In a retrospective study of 198 women diagnosed with non-metastatic breast cancer, >50% had hormone-sensitive tumours. In this cohort 173 of the 198 women had a mean age of 31y at diagnosis and conceived spontaneously when 35y old 42 months later. The remainder (n=25) were 33.7y at diagnosis and used ART to

become pregnant 48 months later when they were 38.5 years. Nine of these women had hormone-sensitive cancers. The percentage of successful pregnancies and live births was 77% in both the 35y and 38.5 year old groups of women. In the larger spontaneous pregnancy group, 10% of conceived embryos were lost through induced abortion and 12% through miscarriage, similar to the rates in unexposed women. When spontaneous pregnancy occurred 3 – 7 years after diagnosis, there was no increased recurrence of cancer which was 16%, nor increase in death rate, which was 6% 9 years after initial diagnosis (Goldrat, Kroman et al. 2015). Of the 25 ART patients, the malignancy recurred in 2 who had COH cycles, but these numbers were not high enough for statistical analysis (Goldrat, Kroman et al. 2015).

Druckenmiller et al. (2016) studied oocyte cryopreservation as a reproductive option for premenopausal cancer survivors. 176 patients with cancer (media age 31years), completed 182 oocyte cryopreservation cycles. Ten patients (11 cycles) returned to attempt pregnancy with their cryopreserved oocytes after 2 years. Out of the 10 cancer survivors that return to thaw their oocytes, 4 had successful pregnancies, delivering 5 neonates in total. Three of these pregnancies were through a gestational carrier, due to either hysterectomy or a breast cancer malignancy for which avoidance of posttreatment pregnancy was recommended (Druckenmiller, Goldman et al. 2016).

In a cohort of 497 cancer patients; out of which 262 (52.7%) had a confirmed diagnosed of breast cancer (median age between 31-33years), Moravek et al. (2018) compared the outcomes of cancer patients who pursued fertility preservation (FP) with those who did not. Of 204 (43.5%) cancer patients that initiated FP, 6.4% had a spontaneous and successful pregnancy, those who did not opt for FP (n=293; 56.5%), the spontaneous and successful pregnancy rate was 5.5%, no significantly

different. Of those who opted for FP and returned to use cryopreserved oocytes or embryos, 57% had live births, although of these 38% used gestational carriers (Moravek, Confino et al. 2018). It's important to note that no analyses were made in regards of the duration and type of chemotherapy used during disease management.

Del Mastro et al. (2011) determined the effect of the temporary ovarian suppression during chemotherapy in premenopausal women. Out 281 women (media age 39years) with stage I through III breast cancer who were candidates for adjuvant or neoadjuvant chemotherapy, 133 received only chemotherapy (anthracycline-based, anthracycline + Taxane- based, or Cyclophosphamide-Methotrexate-Fluorouracil-based treatment) and 148 received chemotherapy and Triptorelin, a Gonadotropin-releasing hormone (GnRH) analogue causes temporary ovarian suppression (Del Mastro, Boni et al. 2011). After a 12month follow up the rate of early menopause in the chemotherapy-alone group was 25.9%, significantly higher than the chemotherapy + Triptorelin group (8.9%) (95% confidence interval [CI], −26% to −7.9%; $P \leq 0.001$). However, the rate of early menopause for each specific chemotherapy regimen was not analysed.

Endometrial recovery and receptivity after chemotherapy treatment has also been evaluated in premenopausal breast cancer patients. Munoz et al. (2015) evaluated the implantation and successful pregnancy outcome of cancer survivors who required oocyte donation (OD). Out of 30 patients diagnosed with breast cancer 37.6% had successful oocyte implantation (Muñoz, Fernandez et al. 2015). Furthermore there were no significant differences in terms of pregnancy, implantation, miscarriages or delivery rates between the cancer group and patients who had not been exposed to chemo or radiotherapy (Muñoz, Fernandez et al. 2015). Endometrial receptivity in women treated and in remission was therefore

equivalent to that of general patients. It was concluded that chemotherapeutics, in general, appeared to have no effect on endometrial receptivity.

1.11 Reactive oxygen species

Reactive oxygen species are highly reactive molecules that possess one or more unpaired electrons. Under normal conditions, ROS are constantly produced as a by-product of the metabolism of molecular oxygen (Gomes, Fernandes et al. 2005, Valko, Leibfritz et al. 2007, Fan and Li 2014). These reactive molecules can interact with other biological products and impact the cell function in response to intracellular and extracellular stimuli (Fan and Li 2014). ROS are produced by the cell from different sources such as mitochondrial electron transport chain and cytochrome P450 reductase (Droge 2002, Li and Shah 2004, Valko, Leibfritz et al. 2007, Gutteridge and Halliwell 2010, Finkel 2011). Common ROS include superoxide radical (O2-), hydroperoxyl radical (HO2-), hydroxyl radical (HO-), peroxyl radical (ROO-), alkoxyl radical (RO-), hydrogen peroxide (H2O2), singlet oxygen (1O2), nitric oxide (NO) and hypochlorous acid (HOCl) (Murrant and Reid 2001, Fink 2002, Gomes, Fernandes et al. 2005).

ROS play a dual role as harmful and beneficial molecules; normally they are produce within the cells in small quantities, and are necessary for many vital biological functions including signal transduction, neurotransmission, platelet aggregation, blood pressure modulation, immune system control, etc. (Moncada 1991, Suzuki, Forman et al. 1997, Babior 2000, Azzi, Davies et al. 2004). However, ROS can also be produced as a consequence of exposure to different factors such as UV light, cigarette smoke, radiation and chemotherapy (Gomes, Fernandes et al.

2005). When overproduced, these molecules can affect cellular function by either regulating important signalling process that lead to altered gene transcription, or by causing oxidative cellular damage and resulting in apoptosis and cell death (Droge 2002, Whiteman, Hong et al. 2002, Biswas, Chida et al. 2006, Circu and Aw 2010, Finkel 2011).

Under physiological conditions, cells have an antioxidant defence mechanism, which scavenge ROS and prevent their excessive accumulation; among common antioxidant molecules there are non-enzymatic molecules such as uric acid, ascorbic acid and α-tocopherol; and antioxidant enzymes including catalase, glutathione peroxidase and superoxide dismutase (Verbon, Post et al. 2012, Vurusaner, Poli et al. 2012, Fan and Li 2014). The balance between ROS generation and ROS scavenging, is known as the cellular 'reDox state'; when the reDox balance is disturbed; either by overproduction of ROS or by a depletion of antioxidant molecules, the condition of oxidative stress occurs (Görlach, kietzmann et al. 2002, Vokurkova, Xu et al. 2007, Vurusaner, Poli et al. 2012).

1.11.1 Chemotherapy-induced ROS in the ovary

Oxidative stress in the ovary can affect significantly ovarian function(Agarwal, Gupta et al. 2005). ROS affects numerous reproductive processes including oocyte maturation, fertilization, embryo development, and pregnancy (Ishikawa 1993, Vega, Johnson et al. 1998, Jozwik, Wolczynski et al. 1999, Sugino, Takiguchi et al. 2000, Agarwal, Gupta et al. 2005) . ROS has also been implicated with decline fertility (De Bruin, Dorland et al. 2002, Agarwal, Gupta et al. 2005) and apoptosis in antral follicles (Luderer 2014). Devine et al. (2012) reported that depletion of antioxidant

molecules, such as glutathione, can lead to atresia of antral follicles *in vivo*, and apoptosis of granulosa cells in cultured antral follicles *in vitro* (Devine, Perreault et al. 2012).

Doxorubicin is known to cause ROS-induced cell death as part of its mechanism of action; we have discussed previously that one of the most severe toxic effect of this drug is the acute and/or chronic cardiotoxicity in cancer patients; the primary molecular mechanism underlying doxorubicin-induced cardiomyocyte death is the excess of reactive oxygen species (ROS) (Zhang, Liu et al. 2012). In regards of the ROS effect on the ovary, Zhang et al. (2017) found that the ROS was significantly increased in primary granulosa cells from mice, following 24h exposure to doxorubicin; they also found the increase in ROS was in a dose-dependent manner (Zhang, He et al. 2017). Some antioxidant molecules have been shown to reduce doxorubicin-induced oxidative stress in rat testes (Yeh, Liu et al. 2009, Attia, Bakheet et al. 2010). In mice ovarian cells and non-human primate ovaries, dexrazoxane and bortezomib, were found to attenuate doxorubicin-induced DNA damage (Roti Roti and Salih 2012, Roti, Ringelstetter et al. 2014, Kropp, Roti et al. 2015, Salih, Ringelstetter et al. 2015)

Similarly, cyclophosphamide has also been evaluated for ROS-related toxicity (Berrigan, Struck et al. 1987, Venkatesan and Chandrakasan 1995, Murata, Suzuki et al. 2004). Tsai-turton et al., (2007) found that exposure to 4-hydroperoxycylophoshhamide (4-Cyc), caused oxidative stress in COV434 granulosa cells *in vitro,* as indicated by an increase of ROS generation measured by DCF fluorescence, oxidative DNA damage, a decreased ratio of antioxidant molecules (Tsai-Turton, Luong et al. 2007). They also found that co-treatment with

antioxidants (glutathione ethyl ester, dithiothreitol or ascorbic acid) inhibited 4-Cyc-induced apoptosis.

1.12 Vitamin E

Vitamin E is an essential, fat-soluble vitamin that comprises eight lipophilic, structurally related molecules: four tocopherols (α, β, γ, δ) characterised by a saturated phytyl tail and four tocotrienols (α, β, γ, and δ) characterised by an unsaturated isoprenoid side chain (McKeown-Eyssen, Holloway et al. 1988, Cooney, Franke et al. 1993, Christen, Woodall et al. 1997, Jiang, Wong et al. 2004).

Among these, α-Tocopherol (αT) is the most predominant form in human plasma and tissues and for that reason is considered the “classic vitamin E”; on the other hand, γ-Tocopherol (γT) is the most abundant in the human diet and is found in vegetable oils such as soybean, corn and cottonseed (Brigelius-Flohe, Kelly et al. 2002). Tocotrienols are consumed more readily in some Asian countries and can be found primarily in palm and annatto oils (Ong and Tee 1992)

1.12.1 Metabolism of vitamin E

Dietary vitamin E is absorbed in the intestine and secreted via chylomicrons by enterocytes into the intestinal lymph system (Rigotti 2007). Further systemic circulation occurs via chylomicron remnants after hydrolysis of triglycerides. Vitamin E is rapidly taken up by the liver via LDL receptor (LDLR)-related proteins and LDLR-mediated uptake of chylomicron remnants (Fig 1.7) (Herrera and Barbas 2001). The transport of Vitamin E from the liver to blood circulation and its further distribution to

different organs and tissues is done through lipoproteins, which serve as carriers for lipophilic molecules (Herrera and Barbas 2001). Under normal physiological conditions vitamin E is packed into very low-density lipoproteins (VLDL) that are secreted into the circulation (Kayden and Traber 1993). αT but not γT, is transferred to VLDL via a hepatic α-tocopherol transfer protein (α-TTP). The main function of α-TTP is to regulate αT concentrations in the plasma and extrahepatic tissues (Drevon 1991); however the underlying mechanism of αT incorporation is still not well understood (Drevon 1991, Herrera and Barbas 2001). Whereas αT is preferentially released into circulation and transported to different tissues; γT is metabolized and excreted. In the liver, vitamin E is metabolized via cytochrome p450 4F2 into chromanol metabolites (Herrera and Barbas 2001). CYP4F2 catalyses the vitamin E-ω-hydroxylase reaction; this initial step is then followed by β-oxidation, which removes two or three carbon moieties, which results in the formation of a short chain metabolite, carboxylethyl hydroxychromans (CEHC) (Sontag and Parker 2002). Tocopherol omega-hydroxylase can bind to both αT and γT, but it has more affinity and higher catalytic activity toward γT; therefore γT is more rapidly metabolised and excreted than αT (Hensley, Benaksas et al. 2004). Swanson et al. (2003), showed that γT is excreted via the urinary system after metabolism as CEHC; additionally, excess γT is believed to be eliminated via the biliary system (Swenson, Bolcsak et al. 2003).

Figure 1.7 Schematic representation of vitamin E transport via lipoproteins.

LPL= lipoprotein lipase; s.c.= surface components transferred after the action of LPL on chylomicrons and VLDL; (X- and/or y-= (X- and/or y-tocopherol; FFA= free fatty acids; a-TTP= n-tocopherol transfer protein (Herrera and Barbas 2001)

1.12.2 Anticancer activity of tocopherols

The potential anticancer activity of tocopherols has been investigated for many years; however, the epidemiological evidence is limited and inconsistent, and conclusions on the role of vitamin E in breast cancer remain elusive (Brigelius-Flohe, Kelly et al. 2002, Smolarek and Suh 2011). Furthermore, in many studies the term

“vitamin E” is used loosely and the variant utilized is often not clarified (Smolarek and Suh 2011).

Schwenke (2002) investigated the relationship between dietary intake of αT with or without the presence of other tocopherols and breast cancer. Results suggested that vitamin E may modestly protect women from breast cancer; however, there was no evidence that vitamin E supplements offered any further protection against breast cancer (Schwenke 2002). Conversely, in the Shanghai Breast Cancer Study, it was suggested that vitamin E supplementation may reduce the risk of breast cancer in women with low dietary intake (Shrubsole, Jin et al. 2001). In a cohort study, the European Prospective Investigation into Cancer and Nutrition (EPIC) trial found that vitamin E did not decrease the risk of breast cancer but it had a small protection effect in post-menopausal women (Riboli, Hunt et al. 2002); on the other hand, Nechuta et al. (2011) found that supplementation with vitamin E in the first 6 months after diagnosis, may help reduce recurrence of breast cancer and overall risk of mortality (Nechuta, Lu et al. 2011).

The mechanisms for anticancer activity of tocopherols have also been studied in the past years and it has been suggested that chemopreventive effect of vitamin E can be via inhibition of ER (Chamras, Barsky et al. 2005, Barve, Khor et al. 2009), increase of peroxisome proliferator activated receptor γ (PPARγ) (Campbell, Stone et al. 2003), induction of Nrf2 (Feng, Liu et al. 2010, Yu, Khor et al. 2010), antioxidant and anti-inflammatory activities, or through induction of apoptosis (Gysin, Azzi et al. 2002, Jiang, Wong et al. 2004, Lee, Ju et al. 2009)

1.12.3 Antioxidant properties

Tocopherols are recognized for the antioxidant role and for the inhibition of lipid peroxidation caused by ROS (Traber 2007, Traber and Atkinson 2007). These antioxidant properties are mainly due to the phenolics hydrogens in the chromanol ring that are donated to lipid free radicals (Burton and Ingold 1989). It has been suggested that the structural differences in the *ortho*-positions for the methyl groups on the chromanol ring may be responsible for the different reductive power of the each tocopherol (Kamal-Eldin and Appelqvist 1996). αT has two *ortho*-methyl groups, which is believed to increase the solubility of αT in lipid substrates and therefore, make it a more potent hydrogen donor than either γT (with one *ortho*-methyl group) and δT (zero *ortho*-methyl groups) (Cillard and Cillard 1980, Kamal-Eldin and Appelqvist 1996)

In vitro studies showed that αT in membranes and lipoproteins, reduce fatty acid peroxyl radicals by in turn becoming α-tocopheroxyl radical (Niki 2014). α-tocopheroxyl radical may undergo several reactions, including scavenging another product to yield a more stable product, reacting with another α-tocopheroxyl radical to give a dimer, or it may be reduced by different reducing agents such as ascorbate and ubiquinol, to regenerate αT (Niki 2014).

1.12.4 Antioxidant protective role in ovarian follicles

Knockout of antioxidant genes in mice has been used to explore the role of reDox control y reproductive function (Devine, Perreault et al. 2012). Mice with mutations in glutathione reductase have been reported to have normal ovarian function (Ho, Magnenat et al. 1997); however, complete knockout of Gpx4 gene resulted in embryonic lethality (Yant, Ran et al. 2003). Sod1 null mice have been

reported to have reduced fertility, although the exact mechanism remains unclear(Matzuk, Dionne et al. 1998). Deletion of c-glutamyl transpeptidase 1 (Ggt1) caused complete infertility and lack of antral follicles and ovulatory response in mice (Kumar, Wiseman et al. 2000, Will, Fischer et al. 2000). The antioxidant GSH present in moderate concentrations in mice ovaries has also been associated with follicle depletion (Lopez and Luderer 2004). Reduced GSH concentration to 50% resulted in significant decrease of atretic antral follicles (Lopez and Luderer 2004); similarly, in antral follicles from rats, increasing levels of apoptosis were observed in absence of GSH (Tsai-Turton, Luong et al. 2007)

1.13 Hypotheses

- Combination of Dox and cyclophosphamide would be more cytotoxic *in vitro* to the human MCF-7 breast cancer cell line and the human ovarian granulosa tumour-derived KGN cell line, than each chemotherapeutic agent alone.

- The antioxidant properties of γToc will support and estrogen hormone production by the KGN cells.

- Both αToc, and γToc would reduce chemotherapy-related ROS generation.

- γToc but not αToc, would augment the cytotoxic activity of the combination of Dox and cyclophosphamide against breast cancer cells *in vitro.*

- γToc but not αToc would induce apoptosis in both breast cancer cell lines but would reduce chemotherapeutic-induced apoptosis in ovarian granulosa cells.

1.14 Aims

The aims of this PhD project are:

- To examine the effect of the combination of Dox and cyclophosphamide against MCF-7 breast cancer cell and KGN granulosa cells.

- To examine the effect of tocopherols (αToc, and γToc) co-administered with Dox and 4-Cyc on MCF-7 and KGN granulosa cell viability

- To determine the MCF-7 derived EC25 values of chemotherapeutics (Dox and 4-Cyc) and tocopherols (αToc, and γToc).

- To evaluate the effect of MCF-7 derived EC25 values of combined chemotherapeutics (Dox and 4-Cyc) and tocopherols on T47D, OVCAR-3 and COV434 cells.

- To develop a simple technique to monitor real time production of ROS in the same cells using a DCF assay and to assess the number of viable cells on the same microplates.

- To evaluate the antioxidant potential of tocopherols to reduce chemotherapeutic-stimulated ROS in ovarian granulosa cells whilst maintaining the anti-cancer efficacy.

- To evaluate apoptosis and caspase activity in breast cancer cells and ovarian granulosa cells after exposure to chemotherapeutics and/or tocopherols

2. CHAPTER II: MATERIALS AND METHODS

2.1 Reagents

All chemicals and reagents used in this research were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia) unless otherwise specified. Chemicals and reagents are grouped according to the experiments they were used for.

2.1.1 Cell line maintenance reagents

2.1.1.1 Cell line media

The MCF-7 human epithelial breast adenocarcinoma cell line was obtained from the America Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI medium supplemented with 10% Foetal Calf Serum (FCS) and 1% v/v of 10,000 units/mL penicillin + 10 mg/mL streptomycin. The KGN human granulosa carcinoma cell line (Nishi, Yanase et al. 2001) was donated by Dr. Theresa Hickey, Research Centre for Reproductive Health, Department of Obstetrics and Gynaecology, University of Adelaide, and maintained in DMEM/F12 supplemented with insulin (5 μg/mL), transferrin (5 μg/mL) and selenium (5 ng/mL, ITS), 10% FCS and 1% v/v of 10,000 units/mL penicillin + 10 mg/mL streptomycin. The OVCAR-3 human ovarian epithelial adenocarcinoma cell line (ATCC, Manassas, VA, USA) was maintained in RPMI medium, supplemented with 20% FCS and 5μg/mL insulin. The COV434 (ECACC 07071909) cell line was derived from a solid human ovary granulosa cell carcinoma and maintained in supplemented DMEM/F12 medium with 10% FCS and 1% v/v of 10,000 units/mL penicillin + 10 mg/mL streptomycin.

2.1.1.2 *Freezing medium for cell storage*

Complete medium was supplemented with 20% of FCS and 5% sterile dimethyl sulfoxide (DMSO). The freezing media was prepared fresh and was put on ice 30 minutes prior to use.

2.1.1.3 *PBS*

NaCl (AJAX Chemicals; 98 g), KCl (0.2 g), Na2H2PO4 (AJAX Chemicals; 1.44 g) and KH2PO4 (AJAX chemicals; 0.24 g) were added to 950 mL of MilliQ water. The pH was adjusted to 7.4 and then the volume increased to 1 L with MilliQ water.

2.1.2 Cell viability reagents

2.1.2.1 *Preparation of chemotherapeutics*

Doxorubicin and 4-hydroperoxycyclophosphamide (ThermoFisher Scientific, Victoria, Australia) were diluted complete medium to make stock solutions of 100 µM and 1000 µM respectively. These were stored at 4°C and -20°C respectively for a maximum of 3 months. Immediately before use were further diluted to the desired concentrations in complete medium.

2.1.2.2 *Preparation of tocopherols*

Alpha and gamma tocopherol were dissolved in DMSO to prepare stock solutions of 1000 µM. These were stored at 4°C for a maximum of 3 months. Immediately before use they were further diluted to the desired concentrations in complete medium.

2.1.2.3 *Crystal violet stain*

0.5 g Crystal violet was dissolved in 100 mL of a methanol solution (50%) and stored at room temperature until required.

2.1.2.4 *Trypan blue*

0.2 g of Trypan blue was dissolved in 0.9% NaCl solution, aliquoted and stored at 4°C until required.

2.1.3 ROS reagents

10 mL buffer Hank's balance salt solution (HBSS) (Abcam, Victoria, Australia) was added to 90 mL ddH_2O. DCFDA was diluted in 1X HBSS to generate a solution of 10 µM. Positive control, Ter-butyl hydrogen peroxide (TBHP) (Abcam, Victoria, Australia), was diluted in complete medium (supplemented RPMI or DMEM/F12) without phenol red, to give final concentrations of 12.5 and 50 µM.

2.1.4 Apoptosis reagents

2.1.4.1 *Caspase-Glo 3/7*

The Caspase-Glo® 3/7 Assay Kit (Promega, Victoria, Australia) was stored at -20°C in the dark prior to use. Buffer (10 mL) and lyophilized Caspase-Glo® 3/7 Substrate were pre-equilibrated to room temperature and mixed by inverting the contents until the substrate was thoroughly dissolved. Spare reconstituted reagent was stored at 4°C for up to 3 days protected from light. The Caspase positive control staurosporine was prepared in complete medium (RPMI or DMEM/F12) to give a final concentration of 1 μM.

2.1.4.2 *4',6- Diamidino-2-phenylindole dihydrochloride for fluorescence (DAPI)*

Stock solution of DAPI (1 mg/mL) was prepared in sterile water. Aliquots were stored at -20°C further diluted in sterile PBS to a concentration of 1 μM/mL.

2.1.5 DeadEnd™ Fluorometric TUNEL reagents

2.1.5.1 *rTDT incubation buffer*

Equilibrium buffer, nucleotide mix and rTDT enzyme (Promega, Victoria, Australia) were mixed at a ratio of 90:10:2 respectively to produce the rTDT incubation buffer (50 μL/ slide)

2.1.5.2 *Paraformaldehyde (4%)*

4 g of paraformaldehyde were mixed with 80 mL of PBS. Volume was then increase to 100 mL and solution was stirred and heated to 60°C until clear. 4% Paraformaldehyde was stored at 4°C for 2 weeks.

2.1.5.3 *Propidium Iodide (PI)*

Stock solution of PI (1 mg/mL) was prepared in MilliQ water, aliquoted and stored at 4°C. Further dilutions to 1 µg/mL in PBS were used for cell assays

2.1.5.4 *2X Sodium Chloride and Sodium Citrate (SSC) solution*

20X SSC supplied by the manufacturer (Promega, Victoria, Australia), was diluted in MilliQ water to a working concentration of 2X.

2.1.5.5 *Triton X-100 Solution (0.2%)*

0.2% Triton X-100 was prepared by mixing 0.2 mL of Triton X-100 with 90 mL of 1X PBS. Solution was mixed and stirred then increased to 100 mL with PBS.

2.2 Methods

2.2.1 Cell lines

2.2.1.1 *MCF-7 breast cancer cell line*

MCF-7 breast adenocarcinoma cell line was derived from pleural effusion of a metastatic breast cancer patient (Soule, Vazquez et al. 1973). MCF-7 cells maintain several of the functional characteristics of differentiated mammary epithelium including the dome-forming capability and the expression of cytoplasmic estrogen receptors (Huguet, McMahon et al. 1994).

2.2.1.2 *T47D breast cancer cell line*

T47D or human ductal breast carcinoma cell line was originally isolated from a pleural effusion of a 54 year old female breast cancer patient (Keydar, Chen et al. 1979, Liburdy, Levine et al. 1999). T47D cells have differentiated mammary epithelium morphology and grow in monolayers when cultured. Similar to MCF-7 cells, T47D cells contain specific receptors for estrogen, progesterone, glucocorticoids and androgens (Keydar, Chen et al. 1979).

2.2.1.3 *KGN granulosa cell line*

The human KGN granulosa cell line was derived from a patient with granulosa cell tumour. KGN granulosa cells possess similar to normal granulosa cells. Physiological characteristics include normal steroidogenic mechanisms, expression of the follicle stimulating hormone receptor (FSH), and responsiveness to FSH or its transduction molecule cAMP by increasing progesterone production (Nishi 2000).

2.2.1.4 *COV434 granulosa cell*

COV434 granulosa cell line was established from a human ovarian tumour (Zhang, Vollmer et al. 2000). A number of experiments have shown that COV434 cells contain several essential properties present in normal granulosa cell function; including production of estrogen in the presence of FSH and the ability to form intracellular connections with cumulus oophorus (Zhang, Vollmer et al. 2000).

2.2.1.5 *OVCAR-3 cancer cell line*

OVCAR-3 cell line was established from a patient with progressive adenocarcinoma of the ovary (Hamilton, Young et al. 1983). The tumour cells were injected into nude mice, and the resulting tumours disaggregated and used to generate the cell line. OVCAR-3 cells grow in monolayers and have an abnormal karyotype (Hamilton, Young et al. 1983)

2.2.2 Cell line maintenance

Cells were subcultured twice a week. Media in each 75cm^2 flask were removed and cells were washed twice with 1X PBS. Cells were detached by adding 1.5 mL of Trypsin-Ethylene diamine tetra acetic acid (EDTA) to the flask and incubated for 5-10 min at 37°C with 5% CO_2. Media were added and cells were collected and centrifuged for 5min at 612 x g. The supernatant was aspirated, and the cells were gently resuspended in 1mL of medium with was adjusted to a final volume of 5 mL. To determine the cell concentration, a trypan blue assay was performed. Cells were subcultured at a ratio of 1:4 in 75 cm^2 culture flask. Excess

cells were cryopreserved with freezing media and stored in liquid nitrogen until needed.

2.2.3 Mycoplasma test

Mycoplasma is a common contaminant in the cell culture laboratory but their presence may go undetected for months (Drexler and Uphoff 2002). To ensure the cells were contamination-free during this project, they were routinely tested for mycoplasma. MycoAlert assay (Lonza, Victoria, Australia) measures the presence of mycoplasma enzymes selectively. MCF-7, T47D, OVCAR-3, KGN and COV434 cells were passage as described in 2.2.2. Supernatant prior to trypsination was collected and stored at 4°C for ≤ 5 days and brought to room temperature immediately before the assay. Supernatant was centrifuged at 612 x g for 5 min and 100 µL of cleared supernatant was transferred into clear flat bottom, white polystyrene plate. 100 µL of Mycoalert reagent was added for 5min; luminescence (1 second integrated reading) was recorded as reading A. 100 µL of MycoAlert substrate was added for 10 min and second reading (reading B) was recorded. To calculate the ratio, results from reading B were divided by data from reading A. a result < 0.9 can be interpreted as negative for mycoplasma; a ratio between 0.9-1.2 is considered borderline and cells should be place in quarantine a retest in 24 h; results > 1.2 can be interpreted as positive for mycoplasma contamination. Results of mycoplasma testing can be found in appendix 7.1.

2.2.4 Short tandem repeat (STR) profiling

Short tandem repeat profiling is a genotype-based method that allows for human cell line authentication. STR profiling is based on the amplification of a

number of polymorphic STR loci by commercially available primers and the comparison of the PCR products with sample donors or established databases (Masters, Thomson et al. 2001). Samples of MCF-7, T47D, OVCAR-3 and COV434 cells were prepared and dispatched to CellBank, NSW, and Australia for identification testing. Cells were passage as described in 2.2.2 and counted by trypan blue assay (2.2.6). Cells were centrifuged 612 x g for 5 min and supernatant was removed. Pellets were stored at -80°C until ready for testing and were shipped frozen on dry ice. Results of STR can be found in appendix 7.2.

2.2.5 Cell viability assay

2.2.5.1 *Trypan blue exclusion assay*

Cell viability was assessed by trypan blue exclusion assay. In this assay dead cells are permeable to the dye and are stained blue(Strober 1997). 10 µL of cell suspension was mixed with 50 µL of Trypan Blue dye (TB), and loaded onto each chamber of the haemocytometer. Viable and dead cells were counted in the 4 large squares (1 mm^2) with a 10X objective light microscope. The average number of cells per square was then multiplied by the dilution factor (x2) and by 1,000 to determine the number of cells per mL. The concentration of each cell suspension was then adjusted for each experiment performed.

2.2.5.2 *Crystal violet assay*

The crystal violet assay simultaneously stains and fixes the nuclei of live cells only. After each experiment, cell culture supernatants were replaced with 50 µL of

Crystal Violet stain (0.5%). The cells were stained and fixed for 10 min at room temperature. Excess stain was rinsed away with demineralised water, and cells were left to air dry overnight. 50 µL of destain solution was added for 10 min. The optical density was read at 570 nm with correction at 630 nm (Reid, Lang et al. 2015). A crystal violet standard plot was produced in each replicate experiment f MCF-7, T47D, COV434 and OVCAR-3 cell densities ranged from 0 – 80,000 and KGN cell densities from 0 – 100,000 cells per well in replicates of 6 for each cell density. Absorbance readings were plotted against cell densities with an average linear correlation of R^2 = 0.99 (n=3). Numbers of viable cells after each experiment were determined by comparison with the CV standard curve.

2.2.6 Reactive Oxygen species detection

2.2.6.1 *DCFDA cell-free fluorescence*

Aliquots of 50 µM DCFDA or aliquots of 100 µM TBHP were added to either 1X kit dilution buffer or to phenol red-free RPMI with or without 10%FCS to obtain a concentration of 10 µM DCFDA or 20 µM TBHP. 100µL of each solution was added to wells in dark, clear bottom 96-well microplates in triplicate (Greiner CELLSTAR®, Sigma-Aldrich, Victoria, Australia). Fluorescence readings were taken using a plate spectrofluorometer (VICTOR X multilabel, Perkin Elmer, Australia) after 1 h incubation at 37°C in a humidified 5% CO_2. Fluorescence was measured using the following settings: excitation at 495 nm, emission at 530 nm; temperature 37°C; reading mode: bottom; number of reads: 3. Wells with only 1X buffer or RPMI with or without 10% FCS were included as background negative controls. Each experiment was repeated on three separate occasions (n=3). Relative fluorescence units (RFU)

were calculated by subtracting blank readings from all measurements. Plates were kept at 37°C in a humidified 5% CO_2 incubator in the dark between readings.

2.2.6.2 *Cellular ROS production*

Cells were exposed to 100 µL 10 µM DCFDA for 45 min at 37°C in a humidified 5% CO2 incubator in the dark. The DCFDA solution was removed, and cells were exposed to 100 µL of chemotherapeutics or tocopherols for 3 or 24 h. The ROS production was detected by recording fluorescence: immediately after addition of test agents (time 0), every hour for a 3 h incubation period, and after 24 h of continuous incubation (24h+). Fluorescence was measured according to protocol described

2.2.7 Hormone assays

2.2.7.1 *Estradiol Enzyme-linked Immunosorbent Assay (ELISA)*

Supernatants from each KGN culture experiment (n=3) were examined in a competitive Estradiol ELISA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse anti-rabbit IgG, and an acetylcholinesterase estradiol tracer. Detection ranges from 6.6 to 4000 pg/mL, and the intra-assay coefficient of variation (CoV) ranges from 7.8 to 18.8%. For this study, the estradiol standard was diluted in the DMEM/F12 cell culture medium to give concentrations that ranged from 6.6 to 4000 pg/mL. A separate standard plot was constructed for each experimental replicate (n=3) and the lowest R^2 value was 0.99. Concentration of estrogen was determined by comparison with the standard curve. Estrogen/cell concentration was calculated by dividing pg/mL of estrogen for each culture well by the numbers of viable cells in the same well.

2.2.8 Cell death assays

2.2.8.1 *DAPI staining of cells for detection of apoptotic nuclei*

MCF-7, T47D, OVCAR-3 or COV434 cells (30,000 cells per well) were added to Nunc Lab-Tek II – CC2 chamber slides (Promega, Australia). After an initial 24 h adherence period, cells were exposed to 300 µL of chemotherapeutics, tocopherols or staurosporine (positive control) and incubated for further 24h. Cells were then fixed by adding 4% paraformaldehyde in 1X PBS for 25 min at 4°C. After rinsing cells with 1X PBS, slides were incubated with DAPI stain prepared in sterile 1X PBS at a working concentration of 1µg/mL for 30min in the dark at room temperature. After rinsing with 1X PBS, Slides were mounted with buffered glycerol and examined using a Fluorescence microscope with filter Chroma 31000 at excitation 340-380 nm, Dichroic 400 and emission 435-485 nm. Four digital images of each well were taken at 20X magnification. Experiment was repeated in 3 separate occasions (n=3) for each of the four cell types.

2.2.8.2 *TUNEL assay for detection of apoptosis*

MCF-7, T47D, OVCAR-3 or COV434 cells (30,000 cells per well) were added to Nunc Lab-Tek II – CC2 chamber slides (Promega, Victoria, Australia). After an initial 24 h adherence period, cells were exposed to 300 µL of chemotherapeutics or tocopherols and incubated for further 24 h. Test agents were removed and cells were fixed by adding 4% paraformaldehyde in 1X PBS for 25 min at 4°C. After rinsing cells with 1X PBS, cells were permeabilized with 0.2% Triton X-100 solution

in 1X PBS. The positive control slide was prepared by adding 100 µL of DNase I. Slides were treated with 50 µL of recombinant terminal deoxynucleotidyl transferase (rTdT) incubation buffer containing: equilibrium buffer, Nucleotide mix and rTdT enzyme. Slides were incubated in a humidified chamber at 37°C for 1 h, protected from light. Slides were then rinsed in 2X sodium chloride (0.3 mM) and sodium citrate (0.03 mM) (SSC) solution for 15min at room temperature. 1 µg/mL Propidium Iodide (PI) solution was used as a counter stain for 15 min in the dark. After rinsing with 1X PBS slides were mounted with buffered glycerol and examined using a Fluorescence microscope (Olympus AX70) with filter Chroma 31001 at excitation 450-495 nm, Dichroic 505 and emission 515-555 nm for the green fluorescein of TUNEL, and Chroma 31002 at excitation 515-550 nm, Dichroic 565 and emission 575-615 nm for the red fluorescence of the PI staining. Four digital images of each well were taken at 20X magnification, TUNEL positive and negative cells were counted and the percentage of apoptotic cells was calculated. The experiment was repeated in 3 separate occasions (n=3) for each of the four cell types.

2.2.8.3 *Caspase -3, -7 assay*

After exposure to chemotherapeutics or tocopherols 50µL of test reagents were replaced with 50µL of luminescent Caspase-Glo® 3/7 reagent. Luminescence was recorded after 1h incubation at room temperature. A blank reaction (Caspase-Glo® 3/7 Reagent, vehicle and cell culture medium without cells) was used to measure background luminescence. Blank values were subtracted from experimental values. Negative controls (Caspase-Glo® 3/7 Reagent and vehicle-treated cells in medium) were included for determining the basal caspase activity of

the cell culture system. Staurosporine was included as a positive control for caspase activation (Belmokhtar, Hillion et al. 2001). Each concentration of chemotherapeutics was examined in three replicate wells and the experiment was repeated on 3 separate occasions (n=3) for each of the four cell types.

3. CHAPTER III: EFFECT OF TEST REAGENTS ON CELL VIABILITY AND HORMONE PRODUCTION

Some replication of content follows because thesis section 3.1 has been published as peer reviewed article

3.1 Effect of chemotherapeutics and tocopherols on MCF-7 breast adenocarcinoma and KGN ovarian carcinoma cell lines *in vitro*

3.1.1 Introduction

In Asia, approximately 25% of all breast cancer patients are premenopausal and younger than 35 years old (Tiong, Rozita et al. 2014). Worldwide, up to 90% of breast cancer patients can survive for 5-years following diagnosis (Ferlay, Soerjomataram et al. 2015, Yardley, Arrowsmith et al. 2017) but find that chemotherapy-induced premature ovarian failure and infertility reduce the survivors quality of life (Mor, Malin et al. 1994, Ganz, Rowland et al. 1998, Ganz, Greendale et al. 2003, Ganz 2005, Baucom, Porter et al. 2006, Mariotto, Rowland et al. 2007, Morgan, Anderson et al. 2012).

Many types of breast cancer are treated with a combination of chemotherapeutic agents such as doxorubicin (Adriamycin) and cyclophosphamide (Nabholtz, Falkson et al. 2003, Bray, Sludden et al. 2010, Yardley, Arrowsmith et al. 2017). Clinical administration (Dees, O'reilly et al. 2000, Jones, Savin et al. 2006) resulted in plasma concentrations of 1.8±0.4μM doxorubicin within 24h of infusion (Swenson, Bolcsak et al. 2003) and serum concentrations of 4-hydroxycyclophosphamide to be approximately 0.02μM 2-4h after administration (Struck, Alberts et al. 1987).

Cyclophosphamide, an alkylating agent, requires hepatic activation to form 4-hydroxycyclophosphamide and aldophosphamide, which coexist in equilibrium and diffuse freely into cells. Aldophosphamide is metabolized into phosphoramide mustard (Boddy and Yule 2000, Emadi, Jones et al. 2009) which causes intra and inter-strand crosslinking in DNA. This interferes with DNA replication (Dong, Barsky et al. 1995) and stimulates apoptosis (Boddy and Yule 2000). A synthetic compound, 4-hydroperoxycyclophosphamide (4-Cyc) is metabolised to 4-hydroxycyclophosphamide *in vitro* (Ozer, Cowens et al. 1982, Dees, O'reilly et al. 2000) and *in vivo* (Teicher, Holden et al. 1996, Yuksel, Bildik et al. 2015). Aldehyde dehydrogenase oxidises aldophosphamide to an inactive metabolite instead of into the active phosphoramide mustard, and hence cells with different levels of aldehyde dehydrogenase respond differently to 4-Cyc (Emadi, Jones et al. 2009).

Doxorubicin (Dox), an anthracycline agent, intercalates in DNA in a topoisomerase-II dependent manner, and inhibits DNA replication, synthesis and mitosis (Tewey, Rowe et al. 1984, Thorn, Oshiro et al. 2011). Dox also induces the production of reactive oxygen species (ROS) which causes lipid peroxidation and apoptosis (Gewirtz 1999). The combined administration of both drugs caused therapeutic synergism in a mouse model (Corbett, Griswold et al. 1975) that was attributed to these different mechanisms of action; cyclophosphamide cross-linking of DNA strands and Dox inhibition of DNA repair (Tobias, Parker et al. 1975).

The chemotherapeutic combination of Dox and cyclophosphamide causes premature ovarian failure in premenopausal breast cancer patients (Emadi, Jones et al. 2009, Meirow, Biederman et al. 2010, Morgan, Anderson et al. 2012). Ovaries contain follicles; a spherical structure consisting of a single oocyte (egg) surrounded by layers of dividing granulosa cells. Granulosa cells produce anti-Müllerian hormone

(AMH) which inhibits activation of small, quiescent primordial follicles (Durlinger, Kramer et al. 1999). It is thought that chemotherapeutics cause granulosa cell death (Downs and Utecht 1999, Zhao, Huang et al. 2010), which reduces AMH, and results in the activation of primordial follicles (Morgan, Anderson et al. 2012). The granulosa cells in the activated follicles proliferate and the follicles grow, but subsequent cycles of Dox and cyclophosphamide therapy cause granulosa cell death and loss of these follicles (Oktem and Oktay 2007, Soleimani, Heytens et al. 2011). Hence, chemotherapy to treat breast cancer reduces serum concentrations of AMH, depletes the ovary of its reservoir of quiescent primordial follicles, and advances infertility through premature ovarian failure (Yucebilgin, Terek et al. 2004, Morgan, Anderson et al. 2012). The administration of cyclophosphamide to rodents caused a dose-dependent loss of small follicles (Meirow, Lewis et al. 1999, Desmeules and Devine 2006, Oktem and Oktay 2007) with DNA double strand breaks in the oocytes (Petrillo, Desmeules et al. 2011). Dox caused apoptosis in mature murine oocytes (Perez, Knudson et al. 1997, Jurisicova, Lee et al. 2006) and the *in vivo* administration of Dox to mice significantly reduced the numbers of follicles, whilst increasing ovarian apoptosis (Ben-Aharon, Bar-Joseph et al. 2010) (Roti, Leisman et al. 2012). It is clear that cyclophosphamide alone, or Dox alone, have adverse effects on the follicular granulosa cells of the ovary, but there are no reports describing the cytotoxic effects of the combined regime (that is used to treat breast cancer patients), on ovarian granulosa cells.

Dox-induced ROS damage was significantly lower in mice administered vitamin E (Nagata, Takata et al. 1999, Thabrew, Samarawickrema et al. 1999), and vitamin E decreased the toxicity of Dox without reducing its effectiveness as a chemotherapeutic agent (Myers, McGuire et al. 1976, Krivit 1979, Lubawy, Whaley

et al. 1979, Herman and Ferrans 1983, Milei, Boveris et al. 1986, Geetha, Sankar et al. 1990). Vitamin E consists of eight structurally distinct compounds classified as tocopherols (alpha, beta, gamma and delta) and tocotrienols (alpha, beta, gamma and delta) (Brigelius-Flohe, Kelly et al. 2002, Lu, Xiao et al. 2010, Smolarek and Suh 2011, Bak, Das Gupta et al. 2017). Tocopherols have antioxidant activity against ROS-induced lipid peroxidation (Traber 2007, Traber and Atkinson 2007) and gamma tocopherol (γToc) is the prominent form in the human diet (Brigelius-Flohe and Traber 1999).

The administration of α-tocopherol (αToc) to 21 breast cancer patients prior to chemotherapy significantly elevated serum concentrations of αToc but did not augment efficacy of the chemotherapeutics, and did not decrease toxic side-effects, although ovarian function was not assessed in this study (Legha, Benjamin et al. 1982). It seems that long-term dietary supplementation with antioxidant vitamins reduces the incidence, but not the severity of cancer (Day and Bingham 1994, Heinonen, Koss et al. 1998). Klein et al (2011) reported that αToc did not have anti-cancer properties *in vivo*, but when the human breast cancer MCF-7 cell line was used to generate tumours in mice, the dietary administration of either αToc or γToc reduced tumour growth (Bak, Das Gupta et al. 2017). Delta and γToc increased the levels of pro-apoptotic proteins and inhibited expression of anti-apoptotic proteins *in vivo,* and also had anti-tumour activity in animal models of colon and prostate cancer (Smolarek and Suh 2011). γToc inhibited the proliferation of human breast cancer cells *in vitro* (Lee, Ju et al. 2009, Smolarek and Suh 2011), delayed the formation of breast cancer tumours in rodent models (Smolarek and Suh 2011) and induced apoptosis in breast cancer cells via up-regulation of DR5 expression (Klein, Thompson et al. 2011). Estrogen metabolism can generate ROS and this may

contribute to the pathogenesis of breast cancer (Bak, Das Gupta et al. 2017). This also suggests that antioxidant tocopherols may have more anti-cancer activity *in vivo* than in estrogen-free *in vitro* systems.

It was hypothesised that the combination of Dox and cyclophosphamide would be more cytotoxic *in vitro* to the human MCF-7 breast cancer cell line, and the human ovarian granulosa tumour-derived KGN cell line, than each chemotherapeutic agent alone (Corbett, Griswold et al. 1975). Both alpha and gamma tocopherol are antioxidants with the potential to reduce chemotherapeutic-induced ROS damage and consequently reduce cytotoxicity, but γToc additionally has anticancer activity. Therefore the hypothesis for this study was that γToc, but not αToc, would augment the cytotoxic activity of the combined Dox and cyclophosphamide regime *in vitro.*

3.1.2 Materials and methods

3.1.2.1 *Chemicals and Reagents*

All chemicals and reagents used in this study were obtained from Sigma-Aldrich (Victoria, Australia), unless specified otherwise.

3.1.2.2 *Preparation of solutions*

Stock solutions of 100 μM doxorubicin (Dox) and 1000 μM 4-hydroperoxycyclophosphamide (4-Cyc, ThermoFisher Scientific, Victoria, Australia) were prepared in RPMI media and 10% foetal calf serum (FCS, DKSH, Victoria, Australia) for MCF-7 cells or in DMEM/F12 media and 10% FCS for KGN cells.

These solutions were kept at 4°C and -20°C respectively for a maximum of 3 months and were diluted immediately before use, because these conditions maintain activity and stability (Hoffman, Grossano et al. 1979, Ulukaya, Ozdikicioglu et al. 2008). Stock solutions of alpha and gamma tocopherol (αToc and γToc) were prepared by diluting the compounds in dimethyl sulfoxide (DMSO) to yield solutions of 1000 µM. These were stored for a maximum of 3 months at 4°C. Further dilutions in the appropriate cell culture medium were prepared immediately before use, and cells were exposed to 0.8% DMSO. The 0.5% crystal violet stain was prepared in a 50% methanol (99.9% pure). 100% acetic acid was diluted to 33% with demineralised water, to be used as a destain solution in the crystal violet assay.

3.1.2.3 *Cell culture*

Although the KGN cell line was derived from an ovarian granulosa cell carcinoma, it can be used as a model for human ovarian granulosa cell growth, apoptosis and steroid hormone production (Nishi, Yanase et al. 2001). Media were replaced every 2-3 days and both cell lines were subcultured twice a week. Cell culture flasks containing 80% confluent cells in exponential growth phase were used for all experiments.

3.1.2.4 *Effect of Doxorubicin, 4-hydroperoxycyclophosphamide, α and γ tocopherol on MCF-7 and KGN cell viability*

MCF-7 cells (20,000 cells per well) and KGN cells (25,000 cells per well) were added to 96-well microplates. After a 24h adherence period, supernatants were

removed and cells were exposed to 100 µL of chemotherapeutics or tocopherols (Table 3-1). The concentrations selected for this *in vitro* study bracket the clinical, *in vivo* serum concentrations of Dox (Swenson, Bolcsak et al. 2003) and 4-hydrocyclophosphamide (Struck, Alberts et al. 1987) (Table 3-1). Cells were exposed to chemotherapeutics and tocopherols according to four different schedules: 24h exposure, 24h exposure + 24h culture in media, 24h exposure + 48h culture in media, or 72h continuous exposure where reagents in medium + 10% FCS were replenished every 24h. After exposure to chemotherapeutics and tocopherols, media containing reagents were collected and frozen, and the cell viability was assessed by the crystal violet (CV) assay. Each test condition was examined in three replicate wells and each experiment was repeated on 3 separate occasions (n=3) for the two cell types.

Single agents	Concentrations (µM)
Dox	0.5, 10, 25
4-Cyc	0, 0.5, 1, 2.5
αToc	0, 50, 75, 100
γToc	0, 50, 75, 100

Combined agents		Concentrations (µM)
Dox + 4-Cyc	Low	10 (Dox) + 1 (4-Cyc)
Dox + 4-Cyc	High	25 (Dox) + 2.5 (4-Cyc)
Dox + 4-Cyc + αToc	Low	10 (Dox) + 1 (4-Cyc) + 75 (αToc)
Dox + 4-Cyc + αToc	High	25 (Dox) + 2.5 (4-Cyc) 75 (αToc)
Dox + 4-Cyc + γToc	Low	10 (Dox) + 1 (4-Cyc) + 75 (γToc)
Dox + 4-Cyc + γToc	High	25 (Dox) + 2.5 (4-Cyc)+ 75 (γToc)

Table 3-1 Clinically relevant concentrations of chemotherapeutics and tocopherols.

Dox: Doxorubicin, 4-Cyc: 4-hydroperoxycyclophosphamide, αToc: α-Tocopherol, γToc: γ-Tocopherol.

3.1.2.5 *Crystal violet (CV) cell viability assay*

Cell culture supernatants were replaced with 50 µL of Crystal Violet stain (0.5%). The cells were stained and fixed for 10min at room temperature. Excess stain was rinsed away with demineralised water, and cells were left to air dry overnight. 50 µL of destain solution was added for 10 min. The optical density was read at 570 nm with correction at 630 nm (Reid, Lang et al. 2015). A crystal violet standard plot was produced in each replicate experiment in which MCF-7 cell densities ranged from 0 – 80,000 and KGN cell densities from 0 – 100,000 cells per well in replicates of 6 for each cell density. Absorbance readings were plotted against cell densities with an average linear correlation of R^2 = 0.99 (n=3) replicate experiments for MCF-7 cells and R^2 = 0.97 (n=3) replicate experiments for KGN cells. Numbers of viable cells after exposure to chemotherapeutics and/or tocopherols were determined by comparison with the CV standard curve for the same experimental replicate.

3.1.2.6 *Estradiol Enzyme-linked Immunosorbent Assay (ELISA)*

Supernatants from each KGN culture experiment (n=3) were examined in a competitive Estradiol ELISA (Cayman Chemical ELISA, Ann Arbor, MI, USA) that uses a mouse anti-rabbit IgG, and an acetylcholinesterase estradiol tracer. Detection ranges from 6.6 to 4000 pg/mL, and the intra-assay coefficient of variation (CoV) ranges from 7.8 to 18.8%. For this study, the estradiol standard was diluted in the DMEM/F12 cell culture medium to give concentrations that ranged from 6.6 to 4000

pg/mL. A separate standard plot was constructed for each experimental replicate (n=3) and the lowest R^2 value was 0.99. Concentration of estrogen was determined by comparison with the standard curve. Estrogen/cell concentration was calculated by dividing pg/mL of estrogen for each culture well by the numbers of viable cells in the same well.

3.1.2.7 *Statistical analysis*

To examine the dose-dependent effect of chemotherapeutics and/or tocopherols a one-way ANOVA with Tukey HSD and Bonferroni post-hoc was conducted. To examine the effect of the four different exposure schedules on cell viability, an ANOVA was conducted that used the periods of culture as independent factors. Statistical significance was assessed by Tukey HSD and Bonferroni post-hoc tests. A one-way ANOVA with Tukey HSD post-hoc was conducted to examine estrogen production. These statistical analyses were performed using SPSS statistics software (V22.0 IBM, Australia). Statistical significance was set at $p \leq 0.05$. All experiments were performed as three independent replicates, and all data expressed as mean ± standard deviation.

3.1.3 Results

KGN (25,000) and MCF-7 (20,000) cells were added to each well, and after 24h adherence and 24h culture in control conditions, there were 113,600±15,600 KGN cells/well and 38,100±4400 MCF-7 cells/well. After 24h adherence and 72h in

culture there were 119072±8750 KGN and 83383±13546 MCF-7 cells per well in control medium.

Doxorubicin killed both MCF-7 and KGN cells (Fig 3.1). A 24h exposure to 5μM Dox significantly decreased MCF-7 to 46±22% ($p<0.0001$) and KGN to 65±3% ($p<0.01$) percent of control (n=3, Fig 3.1A). Cells were exposed to Dox for 24h, then the cells were washed and cultured for an additional 24 or 48h in medium alone (Fig 3.1B and 3.1C) with media replenished at 24h intervals. There was a time-dependent decrease in the numbers of viable cells during the subsequent 48h culture (Fig 3.1B and 3.1C). There were similar numbers of viable cells after 72h continuous exposure to Dox (with media replenishment every 24h, Fig 3.1D) as there were after 24h exposure and a further 48h culture (Fig 3.1C).

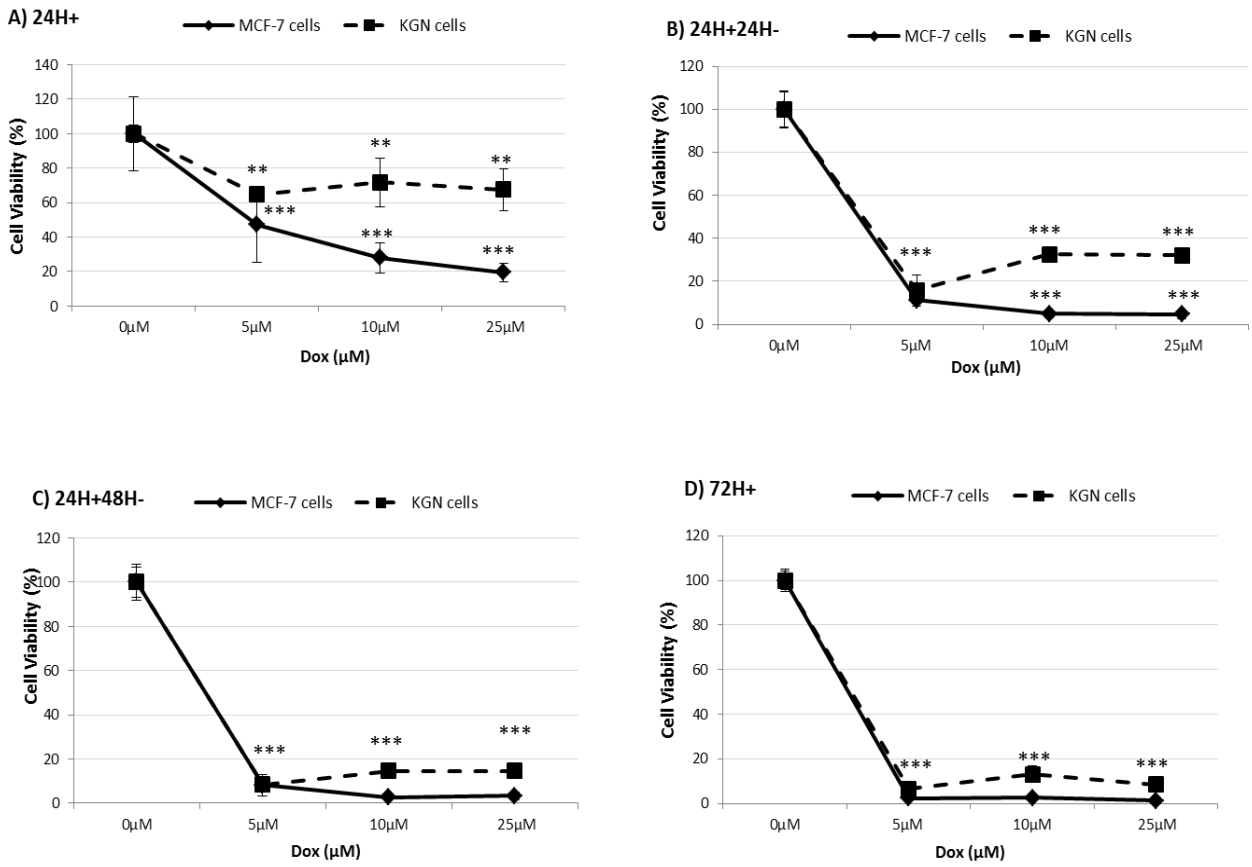


Figure 3.1. Doxorubicin-induced cytotoxicity.

MCF-7 and KGN cells were exposed to Dox 0, 5, 10, 25µM for **A)** 24h (24H+), **B)** 24h exposure and 24h culture with medium (24H+24H-), **C)** 24h exposure and 48h culture with medium (24H+48H-), or **D)** 72h continuous exposure (72H+). Complete RPMI (MCF-7) or DMEM/F12 (KGN) without Dox (0µM) was used as a control. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve and % cell viability was calculated from medium control. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. ** $p \leq 0.01$, *** $p \leq 0.0001$ compared to control.

4-Cyc had no effect on KGN cell viability (Fig 3.2A) and only the longest 72h exposure to the highest concentration (2.5µM) of 4-Cyc significantly reduced the numbers of viable MCF-7 cells to 56354±1657 cells per well ($p<0.05$).

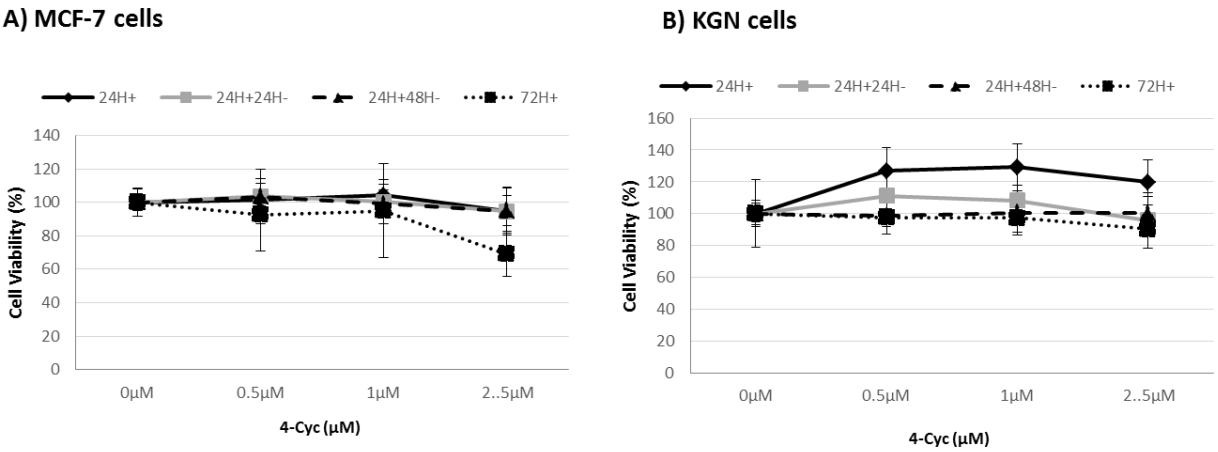


Figure 3.2 Effect of 4-Cyc on cell viability.

a) MCF-7 and **b)** KGN cells were exposed to 4-Cyc 0, 0.5, 1, 2.5µM for 24h exposure (24H+), 24h exposure and 24h culture with media (24H+24H-), 24h exposure and 48h culture with media (24H+48H-), or 72h continuous exposure (72H+). Complete RPMI or DMEM/F12 without 4-Cyc (0µM) was used as a control. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve and % cell viability was calculated from medium control. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. No statistical significance.

Exposure to αToc had no significant effect on MCF-7 or KGN cell viability (Fig 3.3) but γ-Toc was significantly more cytotoxic to MCF-7 cells than to KGN cells (Fig 3.4). A dose- and time-dependent decrease in MCF-7 cell viability was observed after a 24h or a 72h continuous exposure to γToc. (Fig 3.4), but increasing concentrations of γToc had no significant effects on KGN cell viability compared to the vehicle control (Fig 3.4). The percentage of viable KGN cells after 24h exposure to 100µM γToc was 113±16% per cells/well, similar to the percentage of viable cells after exposure to the same concentration of αToc (109±13% cells/well, Fig 3.3).

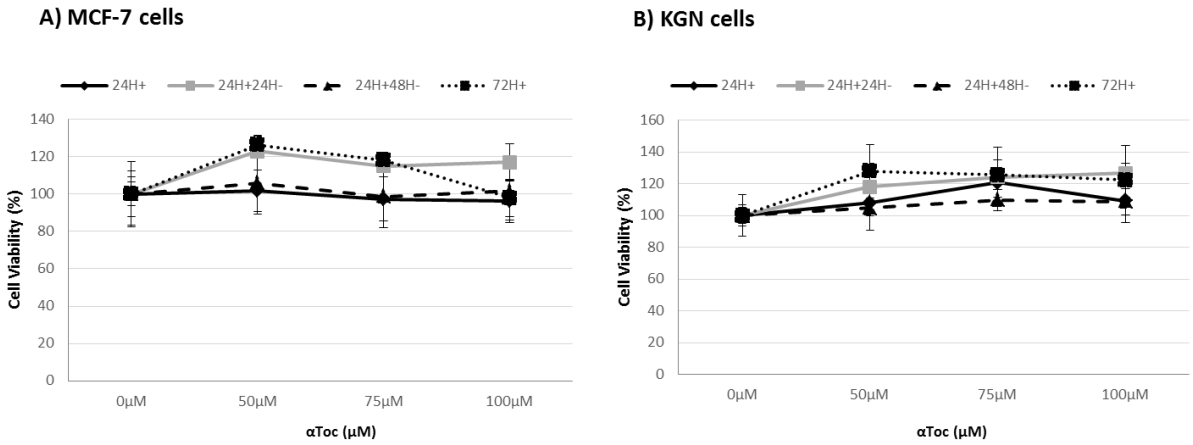


Figure 3.3 Effect of αToc on cell viability.

MCF-7 and KGN cells were exposed to αToc 0, 50, 75, 100μM for 24h exposure (24H+), 24h exposure and 24h culture with media (24H+24H-), 24h exposure and 48h culture with media (24H+48H-), or 72h continuous exposure (72H+). Culture media containing 0.8% DMSO was used as a control. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve and % cell viability was calculated from vehicle control. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. No statistical significance.

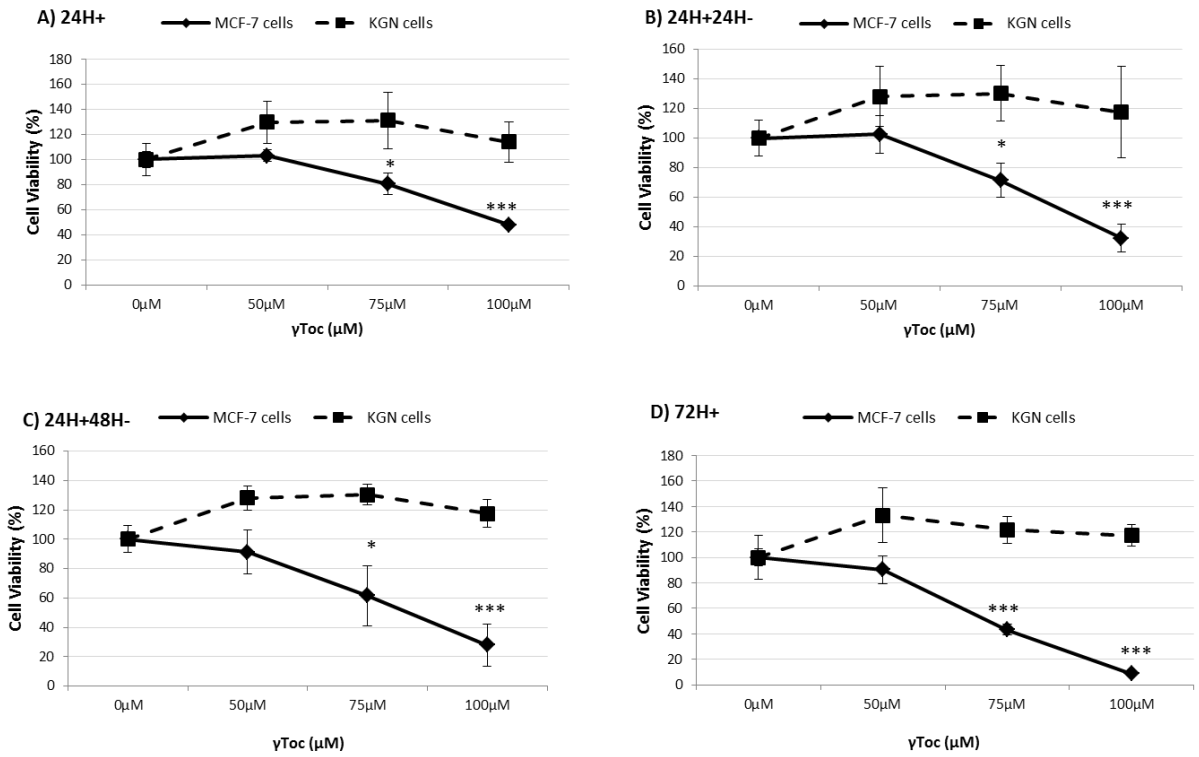


Figure 3.4 Effect of γToc on cell viability.

MCF-7 and KGN cells were exposed to γToc 0, 50, 75, 100μM for 24h exposure (24H+), 24h exposure and 24h culture with media (24H+24H-), 24h exposure and 48h culture with media (24H+48H-), or 72h continuous exposure (72H+). 0.8% DMSO in RPMI or DMEM/F12 was used as a control. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve and % cell viability was calculated from vehicle control. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *$p \leq 0.05$; *** $p \leq 0.0001$ compared to control.

The viability of MCF-7 cells was reduced to 31±7% percent of control by a 24h exposure to the low concentration combination of Dox (10μM) and 4-Cyc (1μM), similar to that observed with the same (10μM) concentration of Dox alone (data not shown). When the MCF-7 cells were exposed to the combination of higher concentrations of Dox (25μM) and 4-Cyc (2.5μM) for 24h, the combination also had the same effect as Dox (25μM) alone; viable MCF-7 cells were reduced to 16±6% of control (Fig 3.5A). Adding αToc to this combination had no effect on cell viability

(23±7% of control), but the addition of γToc (75μM) to the combination decreased MCF-7 cell viability to 9±3% cells per well after 24h exposure, significantly lower than Dox alone ($p<0.05$, Fig 3.5A) or 4-Cyc alone (2.5μM, Fig 3.2A, 95±13% of control), or compared to the combination of Dox and 4-Cyc (Fig 3.5A).

The combination of Dox (25μM) and 4-Cyc (2.5μM) caused significantly more KGN cell death than Dox alone (Fig 3.5B). After 72h exposure to this combination there were 1763±1494 KGN cells per well (1.4±1 % of control, Fig 3.5B), significantly lower than after a 72h exposure to Dox alone (10555±4797, $p<0.01$), equivalent to 8.7±3.4 percent of control (Fig 3.5B). The addition of αToc to this combination reduced KGN cell death so that it was the similar to Dox alone, 7305±1823 cells per well, equivalent to 7.9±1 percent of control (Fig 3.5B). The addition of γToc to the combination did not augment the cytotoxicity of Dox and 4-Cyc in KGN cells (Fig 3.5A). Overall, γToc combined with Dox and 4-Cyc was more cytotoxic towards MCF-7 than KGN cells in the first 24h of culture (Fig 3.5).

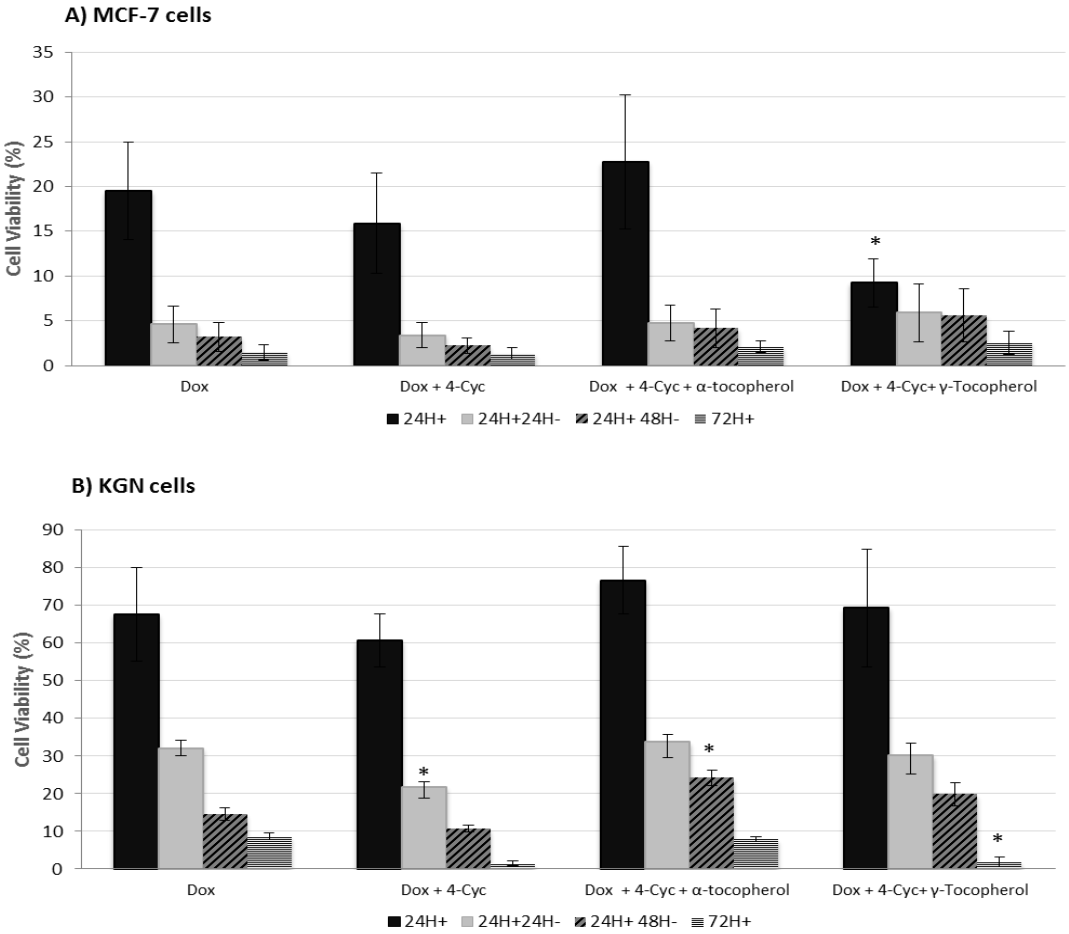


Figure 3.5 Cytotoxicity of combined chemotherapeutic regime.

A) MCF-7 and **B)** KGN cells were exposed to a combination of chemotherapeutics (25μM Dox + 2.5μM 4-Cyc), or a combination of chemotherapeutics + 75μM αToc, or a combination of chemotherapeutics + 75μM γToc for 24h (24H+); 24h exposure and 24h culture with media (24H+24H-); 24h exposure and 48h culture with media (24H+48H-), or 72h continuous exposure (72H+). Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard cue and % cell viability was calculated from vehicle control. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *$p \leq 0.05$ compared to control same concentration of doxorubicin alone (25μM).

After 24h culture KGN cells produced 1.2±0.1 pg/cell of estrogen, and 0.8±0.08 pg/cell in the last 24h of a 72h culture under control conditions (Fig 3.6A and 3.6B). A 24h exposure to 5μM Dox significantly reduced KGN cell viability (Fig 3.1A) but had no effect on estrogen per cell production, which was 1.2±0.03 pg/cell (Fig 3.6A). However, a continuous 72h exposure to Dox, during which media were replenished every 24h and the number of viable cells decreased (Fig 3.1D), caused a significant increase to 13±3 pg/cell ($p<0.01$, Fig 3.6A) in the last 24h culture period.

The same 72h continuous exposure to 2.5μM 4-Cyc had no effect on cell viability (Fig 3.2B) and3. no effect on estrogen production, which was 0.81±0.08 pg/cell in the last 24h culture period (Fig 3.6B).

When KGN cells were exposed to tocopherols, the 24h+48h$^-$ control KGN cells were exposed to almost the same conditions as the 72h+ control cells; 72h *in vitro* with media replenished every 24h. The only difference was that the 72h+ continuously exposed cells were cultured with 0.8% DMSO throughout, whereas the 24h+48h$^-$ control KGN cells were only cultured in the presence of 0.8% DMSO for the first 24h. The 72h+ exposure to 0.8% DMSO did not significantly affect KGN cell viability (Fig 3.3B), but it stimulated significantly more estrogen production (1.32±0.07 pg/cell) in the last 24h period of culture than the 24h+48h- exposure which supported production of 0.76±0.14 pg/cell ($p< 0.05$, Fig 3.6C and 3.6D).

KGN cells in the 0.8% DMSO control produced 1.1±0.4 pg/cell after 24h *in vitro*. The same 24h exposure to αToc had no effect on estrogen per cell production (Fig 3.6C) whereas 100μM γToc stimulated the production of 1.6±0.5 pg/cell (Fig 3.6D). A 72h continuous exposure to either αToc or γToc significantly reduced estrogen per cell production compared to control medium containing 0.8% DMSO (Fig 3.6C and 3.6D). The highest (100μM) concentration of αToc and γToc supported higher levels of estrogen synthesis than the lowest (50μM) concentrations of the tocopherols.

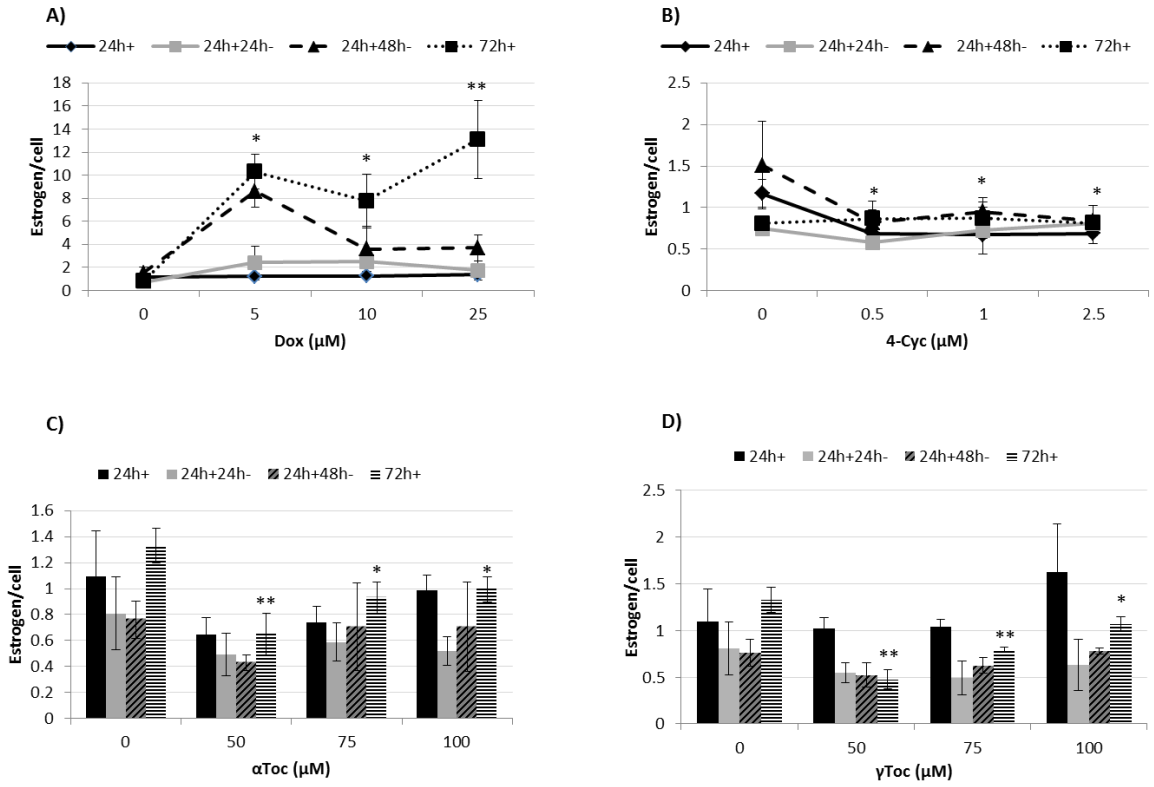


Figure 3.6 Effect of chemotherapeutics and tocopherols on estrogen production.

KGN cells were exposed to Dox (0,5,10,25μM), 4-Cyc (0,0.5,1,2.5μM), αToc (0,50,75,100μM) or γToc (0,50,75,100μM) for 24h exposure (24h+), 24h exposure and 24h culture with fresh DMEM/F-12 complete medium (24h+24h-), 24h exposure and 48h culture with DMEM/F-12 complete medium (24h+48h-), or 72h continuous exposure where reagents in medium + 10% FCS were replenished every 24h (72h+). Estrogen production was assessed in supernatant at the end of each exposure using an estradiol Enzyme Linked Immunoassay, in which concentration of estrogen (pg/mL) was obtained by comparison with a standard curve, and estrogen/cell concentration was calculated by dividing pg/mL of estrogen by the number of viable cells in the same well. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. *$p \leq 0.05$; ** $p \leq 0.01$ compared to the same exposure control.

A continuous 72h exposure to the combination of Dox and 4-Cyc reduced cell viability (Fig 3.5B) but stimulated the highest recorded estrogen per cell production; 39±22 pg/cell in the last 24h culture period (Fig 3.7). This was also higher than the estrogen per cell concentration caused by 72h exposure to Dox alone (Fig 3.6A). The addition of αToc or γToc to the combination of Dox and 4-Cyc had no statistically significant effect on estrogen per cell production (Fig 3.7), although it was

noted that 72h exposure to the combination of Dox and 4-Cyc with 75μM αToc resulted in 13±2 pg/cell.

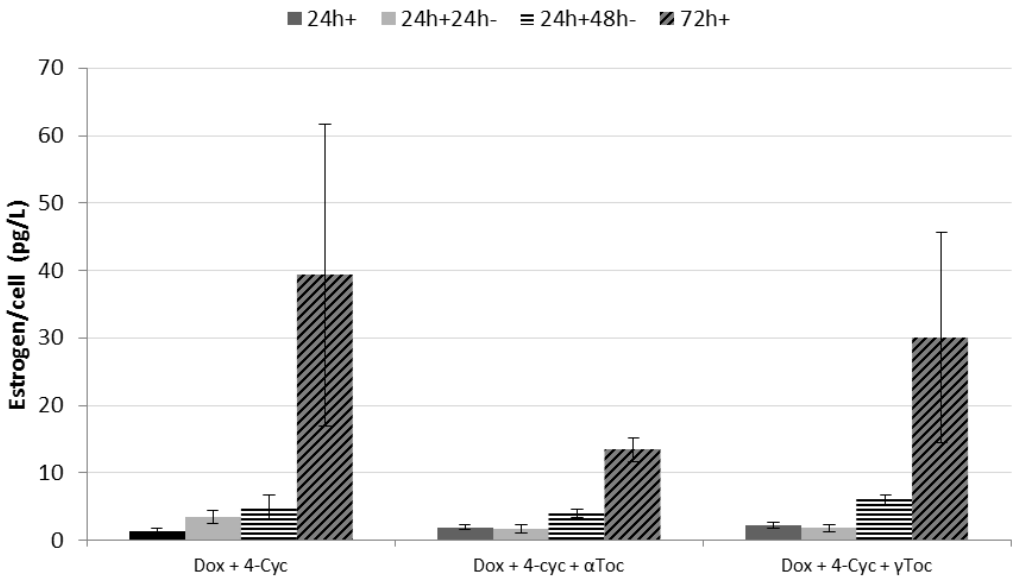


Figure 3.7 Effect of chemotherapeutics and tocopherols on estrogen production.

Combined chemotherapeutic regime (25μM Dox with 2.5μM 4-Cyc), combined regime + 75μM αToc, or combined regime + 75μM γToc for 24h exposure (24h+), 24h exposure and 24h culture with fresh DMEM/F-12 complete medium (24h+24h-), 24h exposure and 48h culture with DMEM/F-12 complete medium (24h+48h-), or 72h continuous exposure where reagents in medium + 10% FCS were replenished every 24h (72h+). Estrogen production was assessed in supernatant at the end of each exposure by using an estradiol ELISA, in which concentration of estrogen (pg/mL) was obtained by comparison with a standard curve, and estrogen/cell concentration was calculated by dividing pg/mL of estrogen by the number of viable cells in the same well. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. No statistical significance compared to control same concentration of doxorubicin alone (10μM).

3.1.4 Discussion

The combination of Dox and cyclophosphamide has been used as a standard chemotherapy option for breast cancer patients since 1975 (Younis, Rayson et al. 2011, Yardley, Arrowsmith et al. 2017). Although it is a successful treatment for

breast cancer (Ferlay, Soerjomataram et al. 2015) it causes premature ovarian failure and infertility (Morgan, Anderson et al. 2012). This study showed for the first time that the combination of Dox and 4-Cyc caused the same cytotoxicity to MCF-7 breast cancer cells *in vitro* as Dox alone, but there were different cytotoxic effects towards the KGN ovarian granulosa cell line; the Dox and 4-Cyc combination was significantly more cytotoxic than Dox alone. Similarly, γToc affected the two cell lines differently; it augmented the cytotoxicity of the Dox and 4-Cyc combination towards MCF-7 cells but did not affect cytotoxicity of the combination towards the KGN cells.

Breast cancer patients are administered multiple cycles of Dox and cyclophosphamide (Yardley, Arrowsmith et al. 2017) and although this can result in 90% survival for 5y (Ferlay, Soerjomataram et al. 2015), chemotherapeutic-resistant cells are known to cause recurrence of the cancer. The exposure and culture schedules used in this *in vitro* study resulted in only 54% of MCF-7 and 35% of KGN cells being killed in the first 24h of exposure. In our *in vitro* model 'viable' meant cells that were adherent to the floor of the culture vessel, whereas non-adherent dead cells were washed away. Cells with damaged DNA may still function and adhere to the culture vessel, and it is likely that DNA damage only manifests as cell death or loss in the crystal violet assay when the cell attempts to go through mitosis. Since the doubling time for MCF-7 is 29h (Sutherland, Hall et al. 1983) and was originally reported as being 46h for the KGN cell line (Nishi et al 2001) we expected to see further cell loss in the 48 – 72h following removal of the chemotherapeutics, and this proved to be the case; fewer than 10% of the cells were viable after 72h *in vitro*. We conclude that additional time in culture, sufficient for the MCF-7 to undergo mitosis, would be needed to be able to determine if this surviving ≤10% would give rise to

Dox-resistant cells or if these would also die. Further development is required to determine if this *in vitro* system can be used to derive chemoresistant cells.

Resistance or sensitivity to chemotherapeutics *in vivo* is affected by a number of interacting factors including the hepatic clearance of the chemotherapeutics and intracellular levels of metabolising enzymes such as glutathione S-transferase (McGown and Fox 1986) or aldehyde dehydrogenase, which *in vitro* metabolises 4-Cyc to its inactive form (Emadi, Jones et al. 2009). KGN cells were more sensitive to Dox but less sensitive to 4-Cyc than MCF-7 cells. We concluded this because a 72h continuous exposure to 4-Cyc reduced the number of viable MCF-7 cells but had no effect on KGN cells. It is possible that KGN cells express higher levels of aldehyde dehydrogenase than MCF-7 cells and hence metabolised 4-Cyc to its inactive form (Hoffman, Grossano et al. 1979).

A relatively short 24h *in vitro* exposure to 2.5µM 4-Cyc had no effect on MCF-7 cells, although this concentration is two orders of magnitude higher than the plasma concentration (0.02µM) of the pharmacologically equivalent 4-hydrocyclophosphamide 2-24h after administration of cyclophosphamide *in vivo*. The pharmacokinetics of cyclophosphamide has been well characterised (Grochow and Colvin 1979, Moore 1991, de Jonge, Huitema et al. 2005), but much less is known about the kinetics of the metabolites of cyclophosphamide. The hepatic metabolite 4-hydrocyclophosphamide has a plasma half-life of only a few minutes *in vivo* (de Jonge, Huitema et al. 2005) because it undergoes spontaneous alteration into phosphoramide mustard (Boddy and Yule 2000, Emadi, Jones et al. 2009). However, phosphoramide mustard may be ionised at physiological pH with a consequent reduction in cytotoxicity, and the oxidation of 4-hydrocyclophosphamide can produce inactive metabolites (de Jonge, Huitema et al. 2005). Therefore, the

clinically relevant dose of cyclophosphamide necessary to treat breast cancer patients might differ from the *in vitro* effective concentration.

Dox was more cytotoxic to MCF-7 cells than 4-Cyc. Although 2.5μM 4-Cyc did kill MCF-7 cells after 72h continuous exposure, when the same 2.5μM concentration of 4-Cyc was combined with Dox for 72h the numbers of surviving cells were comparable to those recorded after exposure to Dox alone, suggesting that in this *in vitro* model 4-Cyc did not potentiate the *in vitro* effect of Dox in the MCF-7 cells. Corbett et al. (1975) found that the growth of murine mammary adenocarcinomas *in vivo* was slower after administration of Dox as a single agent than after cyclophosphamide alone, meaning that the Dox was more cytotoxic than cyclophosphamide *in vivo*. However, the combination of Dox and cyclophosphamide delayed the *in vivo* development of mammary adenocarcinoma's for longer than after the administration of each single agent (Corbett, Griswold et al. 1975) which suggested therapeutic synergism between the two chemotherapeutics *in vivo*.

The combination of Dox and 4-Cyc reduced MCF-7 viability by 85% whereas exposure to 75μM γToc for 24h caused a 20% reduction in viable cell numbers. The addition of 75μM γToc to Dox and 4-Cyc for 24h reduced cell viability by 91%, less than the amount of cytotoxicity predicted by adding the activity of γToc to Dox and 4-Cyc. More studies using lower concentrations of reagents are needed to determine if there are synergistic interactions between γToc, Dox and 4-Cyc.

A long 72h continuous exposure to 2.5μM 4-Cyc had no effect on KGN cell viability or estrogen per cell production, a 72h exposure to Dox was cytotoxic, and exposure to the combination of Dox and 2.5μM 4-Cyc was more cytotoxic than exposure to Dox alone. This result suggested synergism between Dox and 4-Cyc, but a mechanism for that synergism cannot be deduced from this study. It is possible

that 4-Cyc caused DNA cross-linking (Emadi, Jones et al. 2009), but this damage was repaired in KGN cells exposed to 4-Cyc alone, whereas the addition of Dox to 4-Cyc prevented the damage from being repaired (Tobias, Parker et al. 1975), and hence caused KGN cell death.

In a previous study, KGN cells incubated with androstenedione for 72h synthesised and secreted significant amounts of estrogen into the culture medium (Nishi, Yanase et al. 2001). In the present study, a 24h culture in DMEM/F-12 medium containing 10% FCS and ITS resulted in the production of 1.2±0.1 pg/cell, and that rate of production was maintained for 72h when the culture medium was replenished every 24h. Foetal calf serum is rich in fatty acids and cholesterol, the substrate for the whole steroidogenic pathway (Miller and Bose 2011). Fatty acids, like arachidonic acid, play an essential role in StAR protein expression (Wang, Walsh et al. 2000) and the *in vitro* synthesis of steroid hormones such as progesterone and estrogen. In this study, the use of DMEM/F12 with 10% FCS and ITS, was enough to support steroidogenesis; androstenedione was not required to support estrogen synthesis and secretion.

Bak et al. (2017) reported that estrogen induced the expression of cyclin D1 and c-myc, and hence increased mitosis in MCF-7 cells *in vitro*, and that γToc, but not αToc, inhibited expression of these cell-cycle genes and reduced estrogen-stimulated MCF-7 cell proliferation. The MCF-7 cells in our study were not exposed to estrogen; therefore this was not the cause of the significant cell death caused by γToc in our study, suggesting that γToc is cytotoxic through another estrogen-independent mechanism of action. Lee et al. (2009) showed that γToc was cytotoxic to breast cancer cells because it enhanced the transactivation of PPARγ which caused apoptosis and inhibited cell cycle progression. γToc has also shown

anticancer activity in numerous cancer models, including colon (Campbell, Stone et al. 2006), prostate (Jiang, Wong et al. 2004), and lung cancer (Li, Lee et al. 2011) in the absence of estrogen. KGN cells synthesised estrogen, which raises the possibility that there may have been interactions between estrogen and γToc, but γToc alone did not cause cytotoxicity towards KGN cells in the presence of 75 to 183 pg/mL estrogen, and neither did γToc increase the cytotoxicity of the combination of Dox and 4-Cyc, which suggests that the pro-apoptotic effect that Bak et al. (2017) reported in estrogen-stimulated MCF-7 exposed to γToc does not apply to KGN cells.

Exposure to Dox for 72h caused significant KGN cell death, and counter-intuitively, also caused a significant increase in estrogen production per KGN cell. This effect has been reported in other steroid hormone-synthesising reproductive cell lines *in vitro*. An extract from a marine snail was significantly cytotoxic to a human Jar choriocarcinoma placental cell line. As the number of viable cells decreased, secreted progesterone increased (Edwards, Markovic et al. 2008). Gross et al. (2001) also described dying primary-derived granulosa cells increasing progesterone production. It is possible that the cytotoxic mechanisms of action in these cases disrupted membranes and dysregulated steroidogenesis, resulting in massive overproduction of steroid hormones. This confounding effect might be avoided in future by measuring production of another non-steroid hormone, AMH, which is important for fertility.

Four test reagents (γToc, αToc, Dox and 4-Cyc), were each tested at several different concentrations in four exposure schedules. This generated a relatively high number of test conditions which justified the use of human cell lines. Further studies examining ROS generation and cell death will support the selection of a reduced

number of test conditions. At this point MCF-7 cells could be replaced with heterogeneous populations of primary-derived breast cancer cells from different tumour types, and the KGNS could be replaced with 3D primary-derived ovarian follicle culture (Asaduzzaman, Figueroa et al. 2018) to better model the effects of chemotherapeutics with or without tocopherols on breast cancer and the ovary.

In summary, 4-Cyc was active because a 72h continuous exposure killed MCF-7 cells and reduced KGN estrogen per cell production. Both γToc and Dox (applied as single agents) significantly reduced the numbers of viable MCF-7 and KGN cells within 24h of exposure, while αToc reduced the cytotoxic effects of the Dox and 4-Cyc combination in KGN cells. The 4-Cyc concentration, although two orders of magnitude higher than effective clinical plasma concentrations may have been too low for this *in vitro* model, of the Dox and 4-Cyc combination in MCF-7 cells too. The hypotheses were partially supported: although the Dox and 4-Cyc combination was not more cytotoxic than Dox alone towards MCF-7 cells, the combination displayed therapeutic synergism towards the ovarian KGN granulosa cells. γToc, but not αToc, augmented the cytotoxic activity of Dox and 4-Cyc in the MCF-7 cells, but not the KGN cells. This study supports further work to explore the potential of γToc to increase the chemotherapeutic efficacy of Dox and 4-Cyc against breast cancer cells *in vitro*.

3.2 MCF-7 derived EC25 values

3.2.1 Background

Breast cancer treatment depends on the histological subtypes, analyses of gene-expression and individual characteristics of the patient (Wendy and Graham 2007) and often involves the use of one or more chemotherapeutic agents, either before surgery (neoadjuvant) or after surgery (adjuvant)

The combination of chemotherapeutics is a foundation of cancer therapy (Blagosklonny 2004, Yap, Omlin et al. 2013). Agents are chosen based on their potential synergistic effect (Pritchard, Lauffenburger et al. 2012, Bayat Mokhtari, Homayouni et al. 2017), and on their mechanisms of action (Pritchard, Lauffenburger et al. 2012). To target tumour cell heterogeneity, minimize the likelihood of therapeutic resistance, and reduce the occurrence of side effects, the drugs selected should not have similar mechanisms of action (Pritchard, Lauffenburger et al. 2012). For breast cancer patients, combined chemotherapy provided higher rates of response and longer progression to recurrence in comparison to single agent chemotherapy (O'Shaughnessy, Miles et al. 2002, Sledge, Neuberg et al. 2003). Although there is ongoing debate about the balance between increased toxicity and survival benefit, adjuvant therapy with two or more chemotherapeutics is widely used in the treatment of ER- and/or PR-positive breast cancer (Park, Lee et al. 2017).

AC is commonly used as a therapy for breast cancer (Younis, Rayson et al. 2011, Yardley, Arrowsmith et al. 2017) and is often administered in two infusions; intravenous administration of Dox (60mg/m^2) followed by cyclophosphamide (600mg/m^2) (Dees et al 2000, Jones et al 2006) which result in plasma concentrations of 1.8±0.4μM Dox (Swenson et al 2003) and whilst serum

concentrations of 4-hydroxycyclophosphamide (cyclophosphamide metabolite) of approximately 0.5ug/mL (0.02uM) after 2-4h (Struck et al. 1987). We previously demonstrated that the *in vitro* use of concentrations of 4-Cyc that bracketed the clinical i*n vivo* therapeutic dosage did not affect MCF-7 or KGN cell viability; furthermore the cytotoxic effect of the combination of Dox and 4-Cyc was not additive or synergistic towards MCF-7 breast cancer cells (Figueroa et al., 2019). Therefore the aim of this study was to determine the *in vitro* effective concentrations that kill 25% of MCF-7 cells for chemotherapeutics and tocopherols (αToc and γToc).

3.2.2 Materials and methods

3.2.2.1 *Preparation of chemotherapeutics*

Stock solutions of Dox (100 µM) and 4-Cyc (1000 µM) were prepared in RPMI medium and stored at 4°C and -20°C respectively (Hoffman et al. 1979; Ulukaya et al. 2008). Immediately before use they were diluted further to the desired concentrations in RPMI + 10% FCS.

3.2.2.2 *Preparation of Tocopherols*

α-Tocopherol (αToc) and γ-tocopherol (αToc) were diluted in DMSO to obtain stock solutions of 1000 µM, and stored at 4°C. Immediately before use they were diluted further to the desired concentrations in RPMI + 10% FCS.

3.2.2.3 *Cell Culture*

The human epithelial breast adenocarcinoma cell line (MCF-7) was obtained from the America Type Culture Collection (ATCC) and maintained in RPMI media, supplemented with 10% FCS and 1% v/v of 10,000 units/mL penicillin and 10mg/mL streptomycin. Medium was replaced every 2-3 days and cells were sub-cultured when 80% confluent.

3.2.2.4 *Chemotherapeutics and tocopherols dose response*

20,0000 MCF-7 cells per well were added to 96-well microplates. After a 24h culture, supernatants were replaced with 100µL of chemotherapeutics or tocopherols for 24h (Table 3-2) and then cell viability was assessed by the crystal violet assay. Each test condition was examined in three replicate wells and each experiment was repeated on 3 separate occasions (n=3).

Reagents	Test reagents concentration (µM)							
αToc	50	75	100	200				
γToc	50	75	100	200				
Dox	2.5	5	10	20	25			
4-Cyc	0.5	1	2.5	5	10	15	20	40

Table 3-2 Concentrations of chemotherapeutics and tocopherols.

Dox: Doxorubicin, 4-Cyc: 4-hydroperoxycyclophosphamide, αToc: α-Tocopherol, γToc: γ-Tocopherol.

3.2.2.5 *The Crystal Violet Assay*

Media containing test reagents were removed and 50µL of Crystal Violet stain (0.5%) was added to the cells for 10min.Excess stain was rinsed away with demineralised water, and 50µL of destain solution was added for 10min. The optical density was read at 570nm with correction at 630nm (Reid, Lang et al. 2015) using an automatic spectrophotometer and KC Junior software. A crystal violet standard curve was plotted in each replicate experiment. MCF-7 cell densities ranging from 0 – 80,000 cells per well were seeded with 6 replicates each. Absorbance readings were plotted against cell densities with an average linear correlation of R^2 = 0.99 (n=3) replicate experiments for MCF-7 cells. Numbers of viable cells after exposure to chemotherapeutics and/or tocopherols were determined by comparison with the CV standard curve for the same experimental replicate.

3.2.2.6 *Determination of Effective Concentrations (EC values)*

The effective concentration that killed 50% (EC50) of MCF-7 cells was calculated by a non-linear regression analysis performed using GraphPad Prism (Version 5.00, San Diego, California, USA). EC25 and EC75 were determined using calculation template software http://www.graphpad.com/quickcalcs/Ecanything1.cfm.

3.2.3 Results and Discussion

EC25 and EC50 were determined for Dox, 4-Cyc and γToc. 200 µM of αToc was cytotoxic, but the equivalent concentration of DMSO used to dilute αToc (1.8

μM) was also cytotoxic. Therefore, the concentration of αToc that killed cells was confounded by interference by the vehicle. Dox caused a dose dependent decrease of MCF-7 cell viability (Fig 3.8). The estimated EC50 value for Dox was 3.63μM, which is higher than the plasma concentrations (1.8±0.4 μM) reported after administration of Dox to breast cancer patients (Swenson et al 2003). 4-Cyc concentrations used for this experiment ranged from 0.5 to 40μM and a dose dependent decrease in MCF-7 cell viability was observed after exposure to 4-Cyc (Fig 3.8). The EC50 value calculated from the data was 63.69μM (Table 3-3) which was much higher than that of its plasma concentration *in vivo* (0.2 μM) (Struck et al. 1987).

The EC50 values for γToc were similar (105.3 μM) (Table 3-3) to the highest one used in our previous experiments (Figueroa et al. 2019).

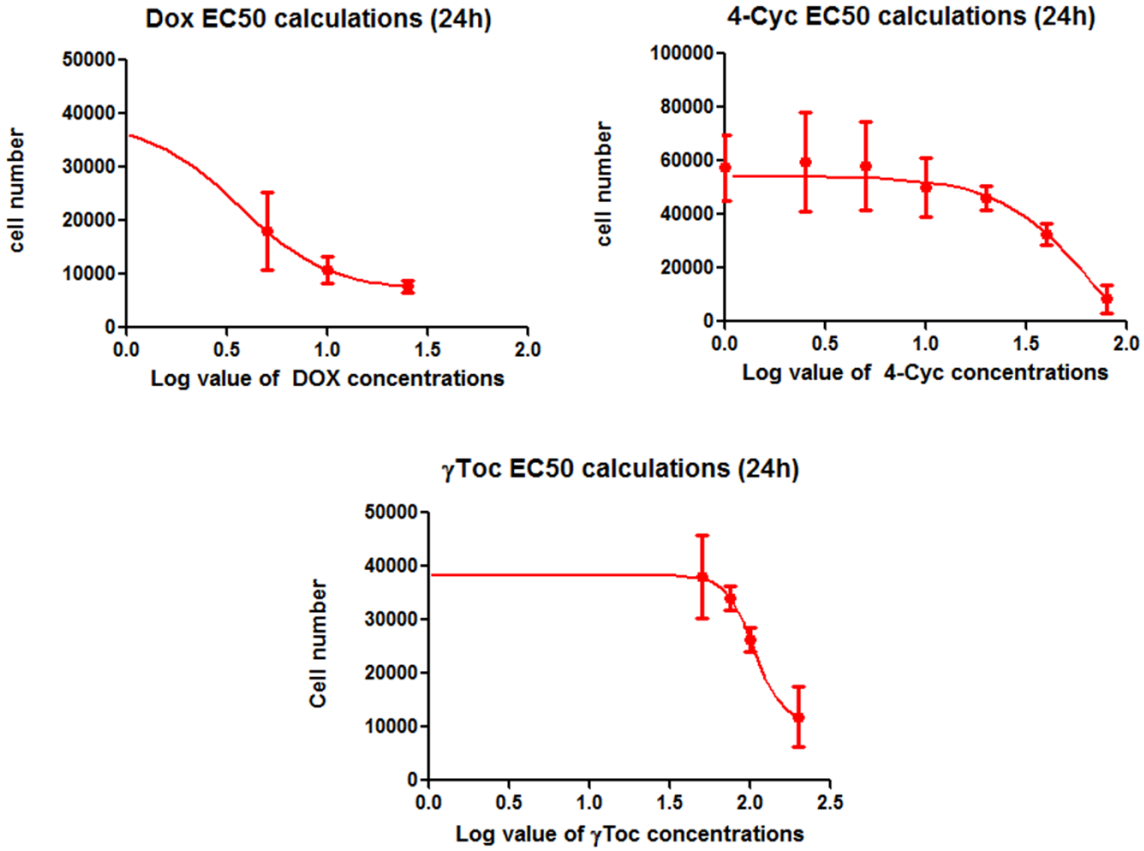


Figure 3.8 MCF-7 derived EC50 values.

20,000 MCF-7 cells per well were exposed to incremental concentrations of test reagents for 24h. Cell viability was examined in a crystal violet assay. EC25 and EC50 values were calculated using a non-linear regression analysis generated by GraphPad Prism (Version 5.00, San Diego, California, USA). Data is presented as the log (log 10) of the concentration. Mean±Std deviation of 3 experimental replicates is presented.

	24H+ Exposure		
	EC25 (µM)	EC50 (µM)	EC75 (µM)
Dox	1.21	3.63	10.89
4-Cyc	21.23	63.69	191.07
γToc	35.1	105.3	315.9
αToc	EC50 not found.		

Table 3-3: EC50 values of chemotherapeutics and tocopherols

The results from this study indicate that the clinically relevant doses of both Dox and 4-Cyc differ from the *in vitro* effective concentration. Based on this data, it was decided that for future experiments the *in vitro* MCF-7 derived EC25 values would be used instead of the *in vivo* concentrations that bracket clinically relevant doses.

4. CHAPTER IV: EFFECT OF TEST REAGENTS ON ROS PRODUCTION

Some replication of content follows because thesis sections 4.1 and 4.2 have been published or submitted as manuscripts for publication.

4.1 Real time monitoring and quantification of reactive oxygen species in breast cancer cell line MCF-7 by 2',7'–dichlorofluorescin diacetate (DCFDA) assay.

4.1.1 Introduction

Reactive oxygen species (ROS) are highly reactive molecules with one or more unpaired electrons produced by the reduction of oxygen (Gomes, Fernandes et al. 2005, Valko, Leibfritz et al. 2007, Fan and Li 2014). Common ROS include superoxide radical ($O_2^{\cdot-}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydroxyl radical ($HO^{\cdot}$), peroxyl radical ($ROO^{\cdot}$), alkoxyl radical ($RO^{\cdot}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), nitric oxide (NO) and hypochlorous acid (HOCl) (Murrant and Reid 2001, Fink 2002, Gomes, Fernandes et al. 2005).

Exposure to UV light, radiation, chemotherapeutics or infectious agents may induce the production of ROS (Halliwell 1991, Cooke, Evans et al. 2003) and ROS are also produced within the body as a by-product of respiratory metabolic processes facilitated by mitochondrial electron transport chains, NADPH oxidase, cytochrome P450 reductase, xanthine oxidase and nitric oxide synthase (Halliwell 1991, Droge 2002, Li and Shah 2004, Valko, Leibfritz et al. 2007, Gutteridge and Halliwell 2010, Finkel 2011). ROS also play an essential role as second messengers

in different intracellular signalling processes (Li and Shah 2004). ROS production and elimination is mediated by a wide range of enzymatic and non-enzymatic molecules with strong reductive power (Li and Shah 2004, Verbon, Post et al. 2012). The balance between these two processes (ROS generation and ROS scavenging) is known as cellular “reDox state” (Fan and Li 2014). When there is an alteration of this balance, either by overproduction of ROS or depletion of the antioxidant defence molecules, oxidative stress occurs, which causes the oxidation of lipids, tissue proteins, DNA and other biomolecules, and can lead to aberrant cell signalling, dysfunctional reDox control, apoptosis and cell death (Gomes, Fernandes et al. 2005).

Detection of ROS is commonly used to assess the mechanism of action of different drugs and compounds, and is also used as a marker for biological functions and cell cycle progression (Lautraite, Bigot-Lasserre et al. 2003). Some reactive oxygen species have a very short half-life; for instance OH radicals have a $T_{1/2}$ of approximately 10^{-9} seconds; therefore they exert effects close to their site of generation (Valko, Leibfritz et al. 2007, Forkink, Smeitink et al. 2010). Other molecules like O_2^- and H_2O_2 have longer half-lives, with approximate values of 10^{-6} and 10^{-5} seconds respectively; these molecules have wider diffusion distances, moving within and between cells to interact with reDox-sensitive molecules that in turn regulate cell cycle progression (Giorgio, Trinei et al. 2007). Although the precise mechanism of ROS signalling is poorly understood, it is currently thought that local ROS generation induces reversible post-translational changes at cysteine (Miki and Funato 2012), selenocysteine (Hawkes and Alkan 2010), methionine (Hoshi and Heinemann 2001) and histidine (Lee and Helmann 2006) protein residues with subsequent modifications to protein conformation, binding sites and surface

properties (Woolley, Stanicka et al. 2013). It is also well known that ROS can modulate the response of many important cell-signalling molecules including mitogen-activated protein kinases, nuclear factor-kB, tumour suppressor protein p53, and other cell cycle check point proteins (Li and Shah 2004, Menon and Goswami 2007, Vurusaner, Poli et al. 2012). Intracellular changes in ROS levels can therefore influence whether cells progress through or withdraw from the cell cycle, or undergo apoptosis (Fan and Li 2014). The rate at which the cell cycle is promoted or inhibited will ultimately depend on ROS levels, and varies according to the type of cell, extracellular stimuli and the duration of exposure (Fan and Li 2014).

Different techniques have been used to quantify ROS, including electrochemical quantification, electro spin resonance and fluorescent signalling (Fan and Li 2014). The fluorescence methodology is based on the use of suitable probes to measure ROS generation. Brandt and Keston (Brandt and Keston 1965) used 2',7'– dichlorofluorescin diacetate (DCFDA) as a fluorometric probe for the detection of H_2O_2. It was later demonstrated that DCFDA is oxidized by other reactive oxygen species including peroxyl and hydroxyl radicals (Gomes, Fernandes et al. 2005). DCFDA is a non-fluorescent, lipophilic and non-ionic compound capable of diffusing and crossing the cell membrane into the cytoplasm. Once inside the cells, DCFDA is deacetylated by intracellular esterases, producing 2',7'–dichlorofluorescin (DCFH), a non-fluorescent and membrane-impermeable compound that reacts with intracellular ROS (Brandt and Keston 1965, Rosenkranz, Schmaldienst et al. 1992, Rota, Chignell et al. 1999, Rota, Fann et al. 1999). Intracellular levels of non-fluorescent DCFH might vary widely with cell type, initial incubation time, DCFDA concentration, esterase activity and possible subsequent leakage (Chen, Zhong et al. 2010).

Experimental conditions in the DCFDA assay often vary in different studies due to the requirements of the experimental design and/or the preference of individual researchers. The lack of uniformity of experimental conditions makes it difficult to compare results from different studies, as it has been shown that the slope of the ROS dose-response curves differ greatly depending on the DCFDA concentration in the cell culture media (Wan, Zhou et al. 2003, Wan, Zhou et al. 2005). DCFDA concentrations range from 5-50μM (Valko, Leibfritz et al. 2007, Chen, Zhong et al. 2010) and cells can be incubated with DCFDA from 30min to 1h (Chen, Zhong et al. 2010) before or after challenging the cells with a ROS analogue or inducer. In the presence of ROS, DCFH is oxidised to fluorescent 2', 7'–Dichlorofluorescin (DCF). The emitted fluorescence is measurable by spectrofluorometry (Kim, Lee et al. 2005) or flow cytometry (Epling, Stites et al. 1992), with the magnitude of DCF fluorescence proportional to ROS activity (Halliwell and Whiteman 2004). In most cases the emitted fluorescence is measured once at a single designated time-point and the result used as a qualitative marker of cellular oxidant stress (Chen, Zhong et al. 2010). Gong et al. (Gong, Yu et al. 2016) examined real-time ROS production in mouse macrophages (RAW264) by incubating the cells with phosphorus and nitrogen doped carbon dots (PN-CDs) for 24h at 37°C. Real time confocal imaging was used to continuously monitor the cells for 50min after lipopolysaccharide (LPS) exposure to induce ROS. There was a significant fluorescence emission which decreased gradually after exposure to LPS. Oparka et al. (Oparka, Walczak et al. 2016) investigated ROS-dependent M-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate) oxidation in fibroblasts by measuring fluorescence every 1.5 minutes for 30 minutes, but to our knowledge there are no reports of studies that monitor ROS generation

over extended periods of time in the same cells using a microplate fluorescence spectrophotometer. The development of a real-time measurement method for the determination of intracellular ROS concentration would be valuable for the elucidation of cell cycle progression and the mechanism of action of different drugs; furthermore, using a microplate reader will give the advantage of *in situ* measurement, without the need for trypsinization; provide a large amount of data in a short period of time and allow parallel cytotoxicity screening of drugs and other compounds.

Ter-butyl hydrogen peroxide (TBHP) is a short-chain analogue of lipid hydroperoxides and is widely used as pro-oxidant agent *in vitro* because it mimics the toxic effect of peroxidized fatty acids (Martín, Martínez et al. 2001, Kučera, Endlicher et al. 2014).TBHP is well known for its damaging effects towards DNA, lipids and other macromolecules, and for causing oxidative stress to cells (Alia, Ramos et al. 2005). Some studies have also suggested that TBHP promotes peroxidation of membrane lipids more efficiently than H_2O_2 (Guidarelli, Cattabeni et al. 1997), which makes this hydroperoxide an excellent compound to use when establishing and characterising optimal conditions for an assay to measure ROS. TBHP diffuses through cell membrane easily and generates intracellular alkoxyl and peroxyl radicals, as well as H_2O_2, in a reaction assisted by metal ions (Guidarelli, Cattabeni et al. 1997, Alia, Ramos et al. 2005). These RO species induce several physiological alterations such as lipid peroxidation, oxidative DNA damage, the depletion of intracellular glutathione, and cell death by apoptosis or necrosis (Sandström 1991, Guidarelli, Cattabeni et al. 1997, Kim, Kang et al. 1998). Kweon et al., (Kweon, Jung et al. 2004) studied the cytotoxic effect of TBHP in Bovine aortic endothelial cells (BAECs). BAECs were exposed to 1mM TBHP for 1-24h, and then

assessed for cell viability using the MTT assay, and ROS generation with a DCF fluorometric assay. TBHP-initiated ROS generation preceded cell death and the oxidative stress induced by 1mM TBHP caused significant cell death (>50%) 6h after TBHP exposure, and >90% cell death 18h later (Kweon, Jung et al. 2004).

For over 40 years DCFDA has been used extensively as a fluorescent probe, and it has been assumed that DCFDA does not affect cell metabolism or viability even though few studies have confirmed the absence of toxicity. Bass et al., (Bass, Parce et al. 1983) pre-incubated human leukocytes with 5-12.5μM DCFDA for 15min, then examined chemotactic, degranulation and oxidative metabolic responses. Directional migration of leukocytes was not significantly affected by DCFDA, and neither was lysozyme release. Similarly, Mehanna et al., (Mehanna, Baudouin et al. 2011) found that a 20min exposure to 20μM DCFDA did not affect the viability of HTM-3 cells. In contrast, Andoh et al., (Andoh, Mizutani et al. 2006) found that DCFDA seemed to contribute significantly to cellular anti-toxicity defence. In this study, HeLa cells were incubated with 10μM sodium arsenite, 1μM cadmium chloride and 10μM hemin in the absence or presence of 20-40μM DCFDA for 24h. Cell viability was monitored by measuring the activity of lactate dehydrogenase, and in a trypan blue exclusion assay. DCFDA protected the cells against the cytotoxic effects of hemin, arsenite and cadmium (Andoh, Mizutani et al. 2006).

In the crystal violet staining assay (CV), dye dissolved in methanol stains the nuclei of live cells and fixes cells to the culture plates (Saotome, Morita et al. 1989). After solubilisation with acetic acid, the optical density can be measured with a spectrophotometer. The crystal violet assay is an excellent alternative to enzymatic assays (Saotome, Morita et al. 1989, Bechtel and Bonavida 2001, Siddiqui, Shabbir et al. 2006, Reid, Lang et al. 2015), as it does not rely on the metabolic function of

specific enzymes (Saotome, Morita et al. 1989). Measuring cell viability after ROS assay would not only give more information about the effects of DCFDA, but also would aid in the correlation of cell response after being challenged with a ROS inducer.

Therefore the aim of this study is to monitor real time production of ROS in the same cells using a DCF assay and to assess the number of viable cells on the same microplates after exposure to DCFDA and/or TBHP.

4.1.2 Materials and methods

4.1.2.1 *Chemicals and Reagents*

All chemicals and reagents used in this study were obtained from Sigma-Aldrich (Australia), unless specified otherwise. The DCFDA cellular ROS detection assay kit was purchased from Abcam (Melbourne, Australia).

4.1.2.2 *Preparation of solutions*

100 mL 1X DCFDA kit dilution Buffer (Abcam, Australia) was prepared by mixing 10 mL 10X dilution buffer with 90 mL ddH_2O. DCFDA was diluted in 1X kit dilution buffer to generate a stock solution of 50µM, which was further diluted in 1X kit dilution buffer to give 5, 10 and 25µM on the day of use. Ter-butyl hydrogen peroxide (TBHP) dilutions were prepared in complete RPMI with 10% FCS without phenol red, to give final concentrations of 12.5, 25 and 50µM. The 0.5% crystal violet stain was prepared in a 50% diluted methanol solution. 100% Acetic acid was diluted

to 33% with demineralised water and used as a destain solution in the crystal violet assay.

4.1.2.3 *4.1.2.3 Cell culture*

The MCF-7 human epithelial breast adenocarcinoma cell line was obtained from the America Type Culture Collection (ATCC) and maintained in RPMI media, supplemented with 10% FCS and 1% v/v of 10,000 units/mL penicillin + 10mg/mL streptomycin. Media were replaced every 2-3 days and cells were harvested with 0.1% trypsin/EDTA solution and subcultured twice a week. Cell culture flasks containing 80% confluent cells in exponential growth phase were used for all experiments.

4.1.2.4 *4.1.2.4 Cell-free DCFDA fluorescence*

Aliquots of 50µM DCFDA or aliquots of 100µM TBHP were added to either 1X kit dilution buffer or to phenol red-free RPMI with or without 10%FCS to obtain a concentration of 10µM DCFDA or 20µM TBHP. 100µL of each solution was added to wells in dark, clear bottom 96-well microplates in triplicate (Greiner CELLSTAR®, Sigma-Aldrich Australia). Fluorescence readings were taken using a plate spectrofluorometer (VICTOR X multilabel, Perkin Elmer, Australia) after 1h incubation at 37°C in a humidified 5% CO_2. Fluorescence was measured using the following settings: excitation at 495nm, emission at 530nm; temperature 37°C; reading mode: bottom; number of reads: 3. Wells with only 1X buffer or RPMI with or without 10%FCS were included as background negative controls. Each experiment

was repeated on three separate occasions (n=3). Relative fluorescence units (RFU) were calculated by subtracting blank readings from all measurements. Plates were kept at 37°C in a humidified 5% CO_2 incubator in the dark between readings.

4.1.2.5 *4.1.2.5 DCFDA concentration range study*

MCF-7 cells (20,000 cells per well) were added to dark, clear bottom 96-well microplates according to DCFDA kit manufacturer's instructions and incubated at 37°C in 5% CO_2 for 24h to allow adherence. Cells were washed with 1X warm phosphate buffered saline (PBS) and exposed to 100 µL of 0, 5, 10, 25 or 50µM DCFDA in 1X kit dilution buffer for 45min at 37°C in a humidified 5% CO_2 incubator in the dark. The DCFDA solution was replaced with RPMI and 10% FCS and the basal ROS production detected by recording fluorescence according to three different schedules: every hour for a 6h incubation period, only once after a single 6h continuous incubation (6h+), and after 24h continuous TBHP exposure (24h+).

4.1.2.6 *4.1.2.6 TBHP- stimulated ROS generation; Dose-response curve*

Additional MCF-7 cells (20,000 cells per well) were added to dark, clear bottom 96-well microplates according to DCFDA kit manufacturer's instructions and exposed to a range of concentrations of DCFDA 0-50µM (described above). DCFDA solutions were removed, and cells washed with 1X PBS. MCF-7 cells were then exposed to 100 µL 0, 12.5, 25 or 50µM TBHP in complete RPMI with 10% FCS without phenol red. Fluorescence was detected according to the 6h, 6h+, 24h+ schedule described above.

Controls were cells in media only (background negative control), cells loaded with DCFDA but not exposed to TBHP (basal ROS production), wells containing DCFDA and TBHP but no cells, and cells exposed to ranging concentrations of TBHP (0-50 µM) without DCFDA (autofluorescence control). RFU were calculated by subtracting background readings (cells in media only), from all measurements and determining the fold change. Each concentration of DCFDA and TBHP was examined in two replicate wells. Each experiment was repeated on three separate occasions (n=3).

4.1.2.7 *4.1.2.7 Image acquisition*

Cell morphology was examined using an inverted light microscope (Olympus CKX41) and camera (Olympus DP22). Four digital images of each well were taken with a 20X (0.40php) objective using an image acquisition system (DP2- SAL, Olympus).

4.1.2.8 *4.1.2.8 Cell viability study after DCFDA- cellular reactive oxygen species detection assay*

The crystal violet (CV) assay was performed on the same MCF-7 cells that were examined in the DCFDA – ROS assay (described above). Media containing TBHP were removed and replaced with 50 µL of Crystal Violet stain (0.5%). The cells were stained and fixed for 10min at room temperature. Excess stain was rinsed away with demineralised water, and cells were left to air dry overnight. 50 µL of

destain solution was added for 10 min. The optical density was read at 570nm with correction at 630 nm (Reid, Lang et al. 2015). A separate crystal violet standard curve was produced in each replicate experiment in which cell densities ranged from 0 – 80,000 cells per well in replicates of 6 for each cell density. To generate standard curves, absorbance readings were plotted against cell densities with an average linear correlation of R^2 = 0.99 (n=3 replicate standard curves). Numbers of viable cells after exposure to DCFDA and/or TBHP were determined by comparison with the CV standard curve generated for the same replicate experiment.

4.1.2.9 *Statistical analysis*

A mixed analysis of variance (ANOVA) was performed to test the dose-dependence effect of DCFDA and TBHP and pair-wise comparisons with Bonferroni post-hoc adjustment for multiple comparisons were used to investigate statistical significance. To examine the effect of the three different time schedules on ROS production, an ANOVA was conducted that used time, DCFDA and TBHP concentrations as independent factors. Statistical significance was assessed by Tukey HSD and Bonferroni post-hoc tests. A one-way ANOVA with Tukey HSD post-hoc was conducted to examine the fluorescence signal in MCF-7 when loaded with varying concentrations of DCFDA, and to examine the fluorescence signal in the cell-free system. These statistical analyses were performed using SPSS statistics software (V22.0 IBM, Australia). Statistical significance was set at $p \leq 0.05$.

To examine the effects of the DCFDA and TBHP on MCF-7 cell viability a two-way ANOVA with Tukey HSD post-hoc was conducted. All experiments were

performed as three independent replicates, and all data expressed as mean ± standard deviation.

4.1.3 Results

In cell free controls, 1h incubation of 10µM DCFDA in 1X ROS assay kit buffer generated the same background fluorescence as DCFDA-free buffer (Fig 4.1). The same concentration of DCFDA in phenol-red free RPMI cell culture medium, either with or without 10% FCS, generated significantly higher levels of fluorescence than DCFDA in buffer (Fig 4.1), but the presence or absence of 10% FCS had no significant effect on fluorescence. In the absence of DCFDA, the ROS generator TBHP (25µM) did not cause fluorescence in cell free buffer or in RPMI medium (Fig 4.1).

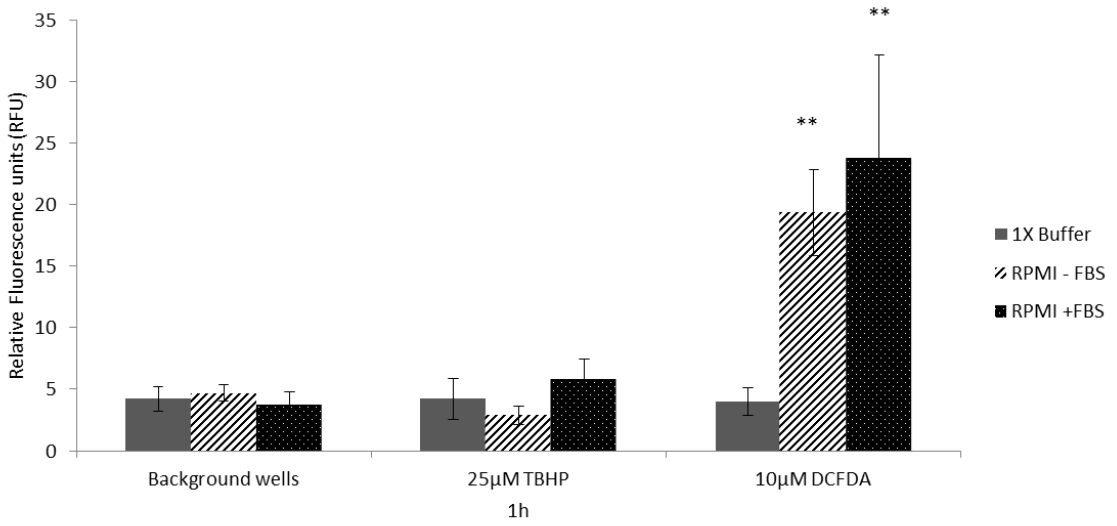


Figure 4.1 Cell-free 2',7'- dichlorofluorescin diacetate (DCFDA) and TBHP fluorescence.

The non-specific background fluorescence of 10µM DCFDA or 25µM TBHP was assessed in wells containing either 1X ROS assay kit buffer or Phenol red-free RPMI cell culture medium with or without 10% foetal calf serum (FCS) after 1h incubation at 37°C in a humidified 5% CO_2. Background wells contained only 1X buffer, or RPMI with or without 10%FCS. Means ± SD of 3 independent experiments shown. Data analysed by One-way ANOVA with Tukey's post-hoc test. ** $p \leq 0.01$ compared to background control.

MCF-7 cells generated ROS in the absence of TBHP (Fig 4.2, 4.3). Background (or basal) fluorescence values after 6h continuous incubation were significantly higher in cells loaded with 50µM DCFDA (55 ± 13.02 RFU per well) than in cells loaded with 5- 25µM DCFDA (Fig 4.3). The addition of TBHP caused a significant dose-dependent increase in ROS generation at each time point compared to the background values from the same time-point (Fig 4.2).

A time-dependent, significant increase in fluorescence was detected up to 3h compared to pre-incubation readings (0h, $p<0.05$, Fig 4.2). After 3h there was no significant increase in fluorescence compared to the 0h levels.

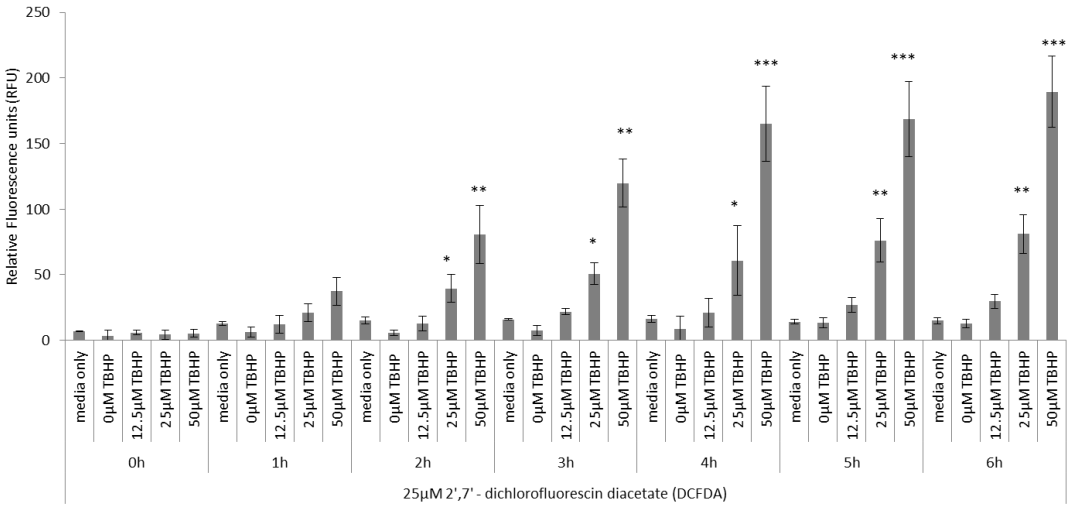


Figure 4.2TBHP-stimulated ROS generation by MCF-7 cells loaded with 25µM 2',7'- dichloroflurescin diacetate (DCFDA).

DCFDA fluorescence was assessed every hour for 6h in cells exposed to 0, 12.5, 25 and 50µM TBHP. Cells in phenol red-free RPMI without DCFDA or TBHP were used as background negative control. Means ± SD of 3 independent experiments shown. Data analysed by mixed ANOVA within factor of time with pair-wise post-hoc test. *$p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.0001$ compared to 0µM TBHP vehicle control within each time of detection.

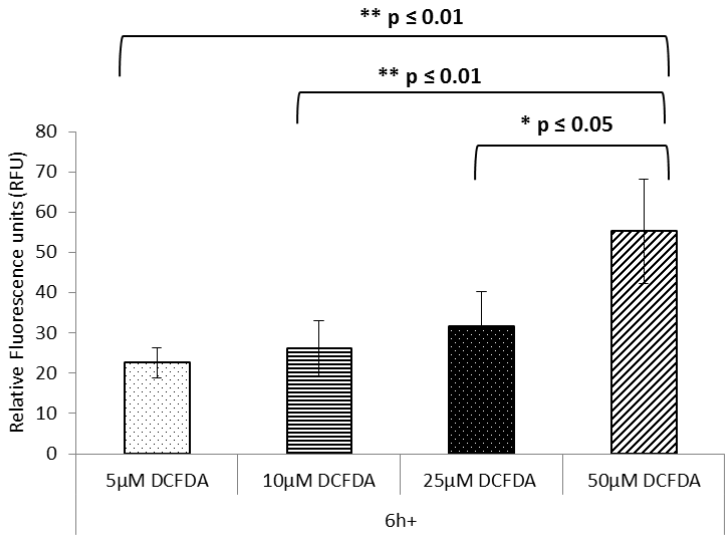


Figure 4.3 ROS generation by MCF-7 cells loaded with 5-50µM 2',7'- dichlorofluorescin diacetate (DCFDA). Background (or basal) DCFDA fluorescence in cells that were cultured for 6h culture in phenol red-free RPMI without TBHP. Means ± SD of 3 independent experiments shown. Data analysed by one-way ANOVA with Tukey HSD post-hoc test. *p ≤ 0.05; ** p ≤ 0.01.

Reading the fluorescence every hour for 6 hours did not affect the emission signal, because there was no significant difference between the final 6th hour OD result and those generated by cells that were read only once, after continuous 6h incubation (Fig 4.4).

Although exposure to TBHP for 6h generated the same amount of ROS as a shorter 3h exposure (Fig 4.2), an extended 24h exposure to TBHP generated significantly higher levels of ROS than the shorter 6h exposure (Fig 4.4), even after accounting for the increase in background ROS production.

DCFDA was required to be able to detect TBHP generated ROS, because cells exposed to 0-50µM TBHP in the absence of DCFDA showed no increase in fluorescence after 6 or 24h (data not shown).

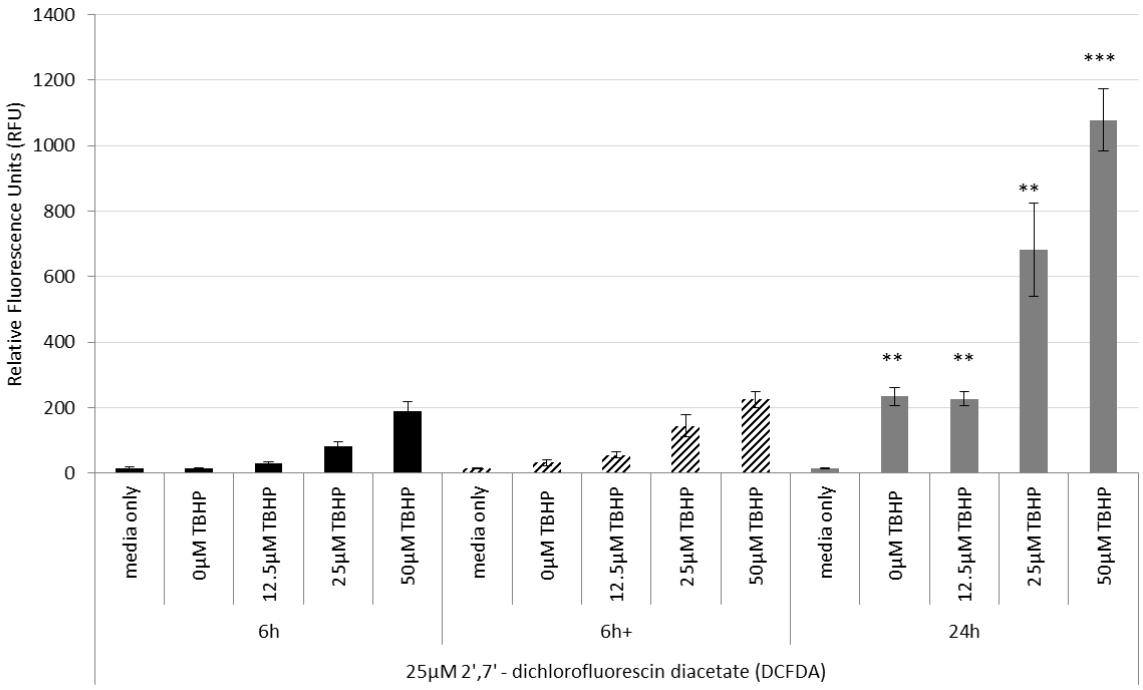


Figure 4.4 Effect of continuous real time monitoring on DCFDA assay.

MCF-7 cells were loaded with 25µM 2',7'- dichloroflurescin diacetate (DCFDA) for 45min, then exposed to 0, 12.5, 25 or 50µM TBHP for 6 or 24h. The DCF fluorescence was read every hour for 6h, or after 6h+ or 24h+ uninterrupted *in vitro* cultures. Means ± SD of 3 independent experiments shown. Data analysed by three-way between factors ANOVA with Pair-wise post-hoc test. ** $p \leq 0.01$, *** $p \leq 0.0001$ significant difference from +6h continuous exposure at same TBHP concentration.

When DCFDA concentrations were 0-25µM, the addition of 0 - 25µM TBHP had no significant effect on cell viability (Fig 4.5) but there was a TBHP concentration-dependent decrease in cell viability, compared to the medium control, when cells were loaded with 50µM DCFDA (Fig 4.5).

Exposing 20,000 MCF-7 cells to the combination of 50µM DCFDA and 50µM TBHP seemed to be more toxic after 24h (11,655 ± 6753 cells per well) than exposing the cells to 50µM TBHP alone (22,032 ± 1388 cells per well) (Fig 4.6); however the difference in viability was not statistically significant.

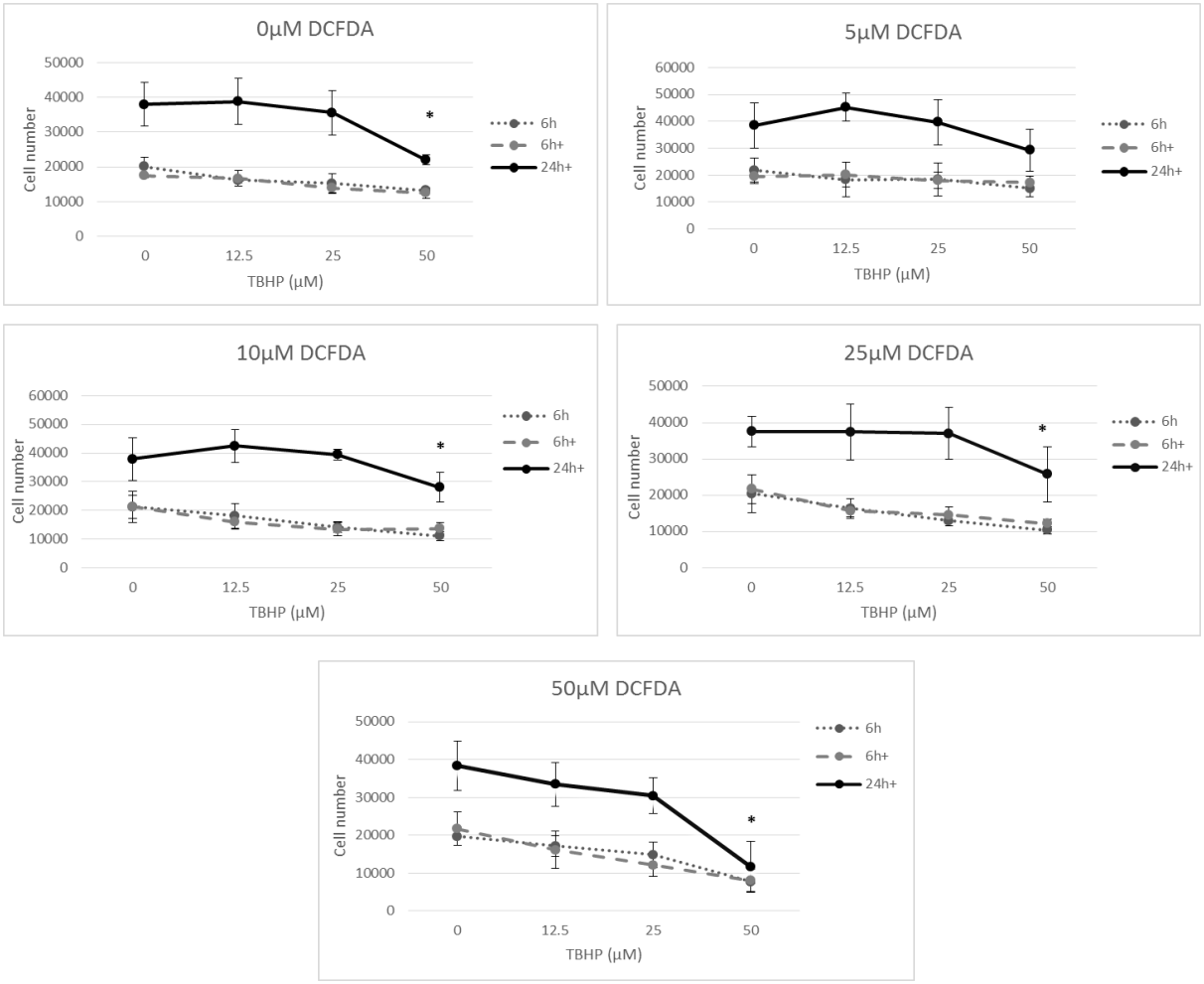


Figure 4.5 Effect of DCFDA and TBHP on MCF-7 Cell viability.

Cells were loaded with 0-50µM 2',7'- dichloroflurescin diacetate (DCFDA) for 45min then washed and exposed to 0, 12.5, 25 and 50µM TBHP for 6 or 24h. Fluorescence was read every hour for 6h or after 6h+ or 24h+ uninterrupted *in vitro* culture. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data were subjected to 2-way ANOVA with Tukey post-hoc test, and difference from media control from same incubation period shown. * $p \leq 0.05$.

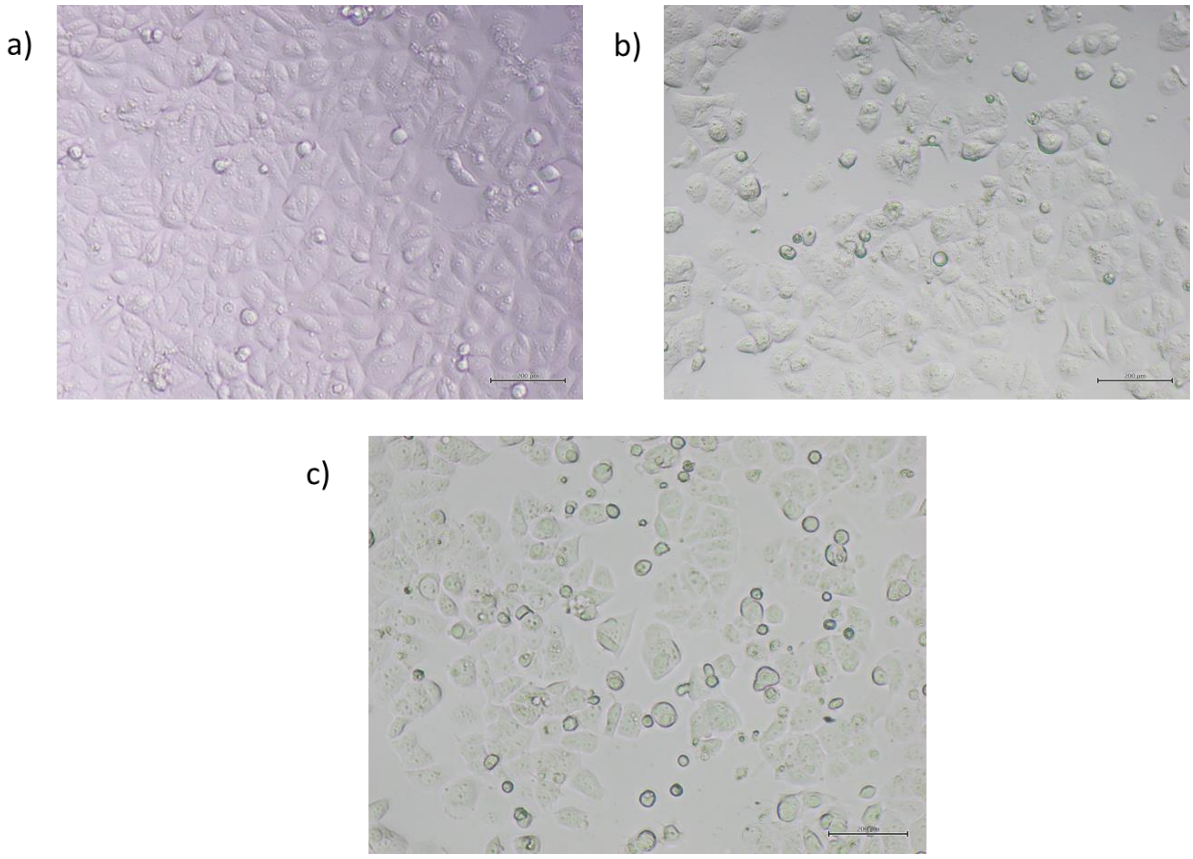


Figure 4.6 Effect of DCFDA and TBHP on MCF-7 Cell viability.

Cells were loaded with 0-50µM 2',7'- dichloroflurescin diacetate (DCFDA) for 45min then washed and exposed to 0, 12.5, 25 and 50µM TBHP for 24h. Cell morphology was examined using an inverted microscope; digital images were taken with DP2-SAL, Olympus acquisition system of **a)** Medium only control; **b)** DCFDA- free cells exposed to 50 µM TBHP; and **c)** cells loaded with 50µM DCFDA and exposed to 50µM TBHP for 24h.

4.1.4 Discussion:

Reactive oxygen species are difficult to measure because they are present in low concentrations intracellularly, and they have short lifetimes (Gomes, Fernandes et al. 2005). The majority of published studies report measurement of the ROS detection

signal at only one time-point (Chen, Zhong et al. 2010) but this single snapshot is unlikely to capture the peak or nadir of ROS levels in cells, and this approach is also sensitive to assay conditions such as the DCFDA loading concentration (Wan, Zhou et al. 2003, Wan, Zhou et al. 2005). This is the first study to characterise ROS generation in the same cells at hourly intervals for 6h, and which additionally describes the effects of a range of concentrations of DCFDA on ROS detection and cell viability. To our knowledge, this report is the first to explore a DCFDA-based assay's potential for real time ROS detection in breast cancer cells.

Under normal physiological conditions, cancer cells generate high levels of ROS to activate different signaling pathways that are crucial for cell proliferation and survival (Jackson and Loeb 2001, Benhar, Engelberg et al. 2002, Storz 2005); and also express high levels of antioxidant molecules to regulate ROS and prevent cell damage or death (Simon, Haj-Yehia et al. 2000, Cadenas 2004). In our study, increasing levels of ROS in MCF-7 cells were detected in the absence of TBHP, suggesting an imbalance of the intracellular ReDox state that could be a consequence of insufficient antioxidant molecules in medium supplemented with 10% FCS or insufficient ROS scavenging inside the cells (Chen, Zhong et al. 2010), spontaneous deacetylation of DCFDA to DCF (Royall and Ischiropoulos 1993, Zhou, Diwu et al. 1997), artefactual signal due to light-induced oxidation (Winterbourn 2014) or competitive inhibition of antioxidant molecules by DCFDA (Wrona, Patel et al. 2005). High fluorescence levels were also detected in a cell-free model with RPMI with or without FCS. Brubacher and Bols (2000) observed that tyrosine combined with ubiquitous metal contaminants of physiological buffers can result in high levels of oxidation, which may be wrongly interpreted as cellular ROS (Brubacher and Bols 2001). RPMI and other common use media contain different amino-acids, including

tyrosine, which could potentially influence fluorescence intensity; therefore we recommend testing the reaction of culture media with DCFDA, as part of ROS assay establishment. Basal ROS production by MCF-7 cells loaded with 5 – 50µM DCFDA (in the absence of TBHP) was detected after 6h culture, with the apparent ROS levels dependent on the loading concentration of DCFDA. DCF assay results are often expressed as the net increase of cellular fluorescence over that of the background control group (Wan, Zhou et al. 2005), but subtracting the basal ROS had no effect on the DCFDA dose-dependent increase in ROS levels. Adding TBHP increased ROS generation to levels that exceeded the capacity of 5µM DCFDA to metabolise the ROS (Bass, Parce et al. 1983, Royall and Ischiropoulos 1993, Wrona, Patel et al. 2005, Chen, Zhong et al. 2010) and ROS detection after loading cells with 25µM DCFDA and stimulating with TBHP was significantly higher than with 5µM DCFDA. The higher 25µM DCFDA concentration was not limiting after 6h culture because ROS detection values were higher after the extended 24h culture period, demonstrating the capacity of 25µM DCFDA for detecting higher ROS levels.

Two factors seemed to affect MCF-7 cell viability; the amount of ROS stimulated by TBHP and the concentration of DCFDA. 50µM DCDFA was not cytotoxic to MCF-7 cells grown for 24h, but the addition of 50µM TBHP caused an increase in ROS and a consequent decrease in cell viability. The same conditions of 24h exposure and 50µM TBHP however were less toxic to MCF-7 cells in the absence of DCFDA, suggesting that there was an interaction between DCFDA, TBHP (ROS), and the culture conditions; hence we recommend using the lower 25µM concentration of DCFDA in order to prevent non-specific ROS generation.

Real time monitoring every hour for 6h did not significantly reduce the DCFDA fluorescent signal; suggesting that for MCF-7 cells, the dose-dependent increase in

fluorescent signal was not compromised by exposing the cells every hour to the detecting light beam of the fluorescence plate reader; validating our proposal that the DCFDA assay can be used to measure ongoing ROS production in real time. This is important because reactive oxygen species are highly reactive and extremely short-lived molecules, which can be generated within seconds of stimulation. ROS have also the tendency toward chain reaction and generation of other RO molecules (Maneesh and Jayalekshmi 2006), triggering an amplified cascade of intracellular events and activation of different pathways (Liou and Storz 2010). Therefore, it is possible that shorter incubation periods could be applicable to the detection of intracellular ROS, with a fluorescent signal that extends over the hours as more detectable ROS are generated within the cells. It's important to highlight that because of the non-specific oxidation of DCFDA and the multiple pathways that can lead to DCF fluorescence, this assay must be utilised as a qualitative marker for total ROS production, rather than a precise indictor of any specific kind of ROS (Tarpey and Fridovich 2001).

ROS detection is dependent on the concentration of DCFDA that maximises assay sensitivity and ROS detection, whilst minimising dye-induced cytotoxicity. In this study that balance lay between 10- 25µM DCFDA combined with a loading exposure of 45min. Cells loaded with 10-25µM DCFDA and exposed to 0-50µM TBHP yielded a more precise estimation of ROS, with a significant and dose-dependent increase in fluorescence signal in all three independent experiments. We conclude that MCF-7 cells can be loaded with 10-25µM DCFDA for 45min, and that a 6h exposure to 0-50µM TBHP is sufficient to generate a sensitive and reproducible standard curve. The finding that the DCF fluorescent signal can be measured

repeatedly in the same cells could potentially increase the utility of the assay for future research applications.

4.2 γ- tocopherol reduced chemotherapeutic-induced ROS and cytotoxicity in an ovarian granulosa cell line, but not in breast cancer cell lines *in vitro*

4.2.1 Introduction

The generation and accumulation of intracellular reactive oxygen species (ROS, (Gomes, Fernandes et al. 2005, Valko, Leibfritz et al. 2007, Fan and Li 2014) is crucial for normal cell metabolism (Halliwell 1991, Droge 2002, Valko, Leibfritz et al. 2007, Guéraud, Atalay et al. 2010, Gutteridge and Halliwell 2010, Finkel 2011, Costa, Scholer-Dahirel et al. 2014). ROS generation is highly regulated by either enzymatic (catalases, peroxidases and dismutases) or non-enzymatic (vitamin A, C or E) reductive molecules. Disturbances in cellular reDox balance can lead to an over-accumulation of ROS (Costa, Scholer-Dahirel et al. 2014, Fan and Li 2014) which can cause irreparable cellular damage, apoptosis and death (Gomes, Fernandes et al. 2005, Costa, Scholer-Dahirel et al. 2014). Cells also produce ROS after exposure to radiation or chemotherapeutics (Halliwell 1991, Cooke, Evans et al. 2003). Many chemotherapeutic drugs induce ROS as part of their mechanism of action (Nogueira and Hay 2013) by increasing ROS accumulation or inhibiting ROS scavenging, both of which can result in an escalation of ROS to toxic levels (Nogueira and Hay 2013).

Breast cancer patients are commonly administered an infusion of adriamycin (‘A’, later named doxorubicin ‘Dox’) intravenously (60mg/m^2) then an infusion of

cyclophosphamide ('C' 600mg/m^2, (Dees, O'reilly et al. 2000, Jones, Savin et al. 2006) and different types of breast cancer have additional agents added to their treatment regimens (Nabholtz, Falkson et al. 2003, Joerger, Huitema et al. 2007, Bray, Sludden et al. 2010) including the addition of paclitaxel to four cycles of AC (Henderson, Berry et al. 2003, Yardley, Arrowsmith et al. 2017). Breast cancer is not treated with either chemotherapeutic in isolation.

Dox is an anthracycline agent that causes apoptosis by intercalating into double-stranded DNA and inhibiting topoisomerase-II (Tewey, Rowe et al. 1984). A second mechanism of action involving ROS has also been described (Doroshow 1986, Gewirtz 1999, Mizutani, Tada-Oikawa et al. 2005).

Cyclophosphamide is an alkylating agent that requires hepatic metabolic activation (Emadi, Jones et al. 2009). Hydroxylation of cyclophosphamide in the liver by cytochrome P450 generates 4-hydroxycyclophosphamide and aldophosphamide (Boddy and Yule 2000). Aldophosphamide is metabolized into phosphoramide mustard and acrolein which increases ROS production in a variety of cell lines (Luo and Shi 2004, Mythili, Sudharsan et al. 2004, Luo and Shi 2005, Liu, Li et al. 2012).

Dox and cyclophosphamide are associated with a variety of adverse effects *in vivo*, including T-cell suppression, chronic cardiotoxicity and premature ovarian failure (Legha, Benjamin et al. 1982, Ozer, Cowens et al. 1982, Meirow, Biederman et al. 2010, Morgan, Anderson et al. 2012). It has been proposed that chemotherapeutics cause premature ovarian failure by targeting proliferating granulosa cells in growing follicles (Meirow, Biederman et al. 2010, Morgan, Anderson et al. 2012). Since granulosa cells synthesise anti-Müllerian hormone (AMH), the loss of follicles due to chemotherapeutic-induced cytotoxicity causes a consequent depletion in circulating AMH, which results in the activation and

recruitment of dormant primordial follicles into the growing pool (Morgan, Anderson et al. 2012). It is thought that the *in vivo* administration of repeated cycles of Dox and cyclophosphamide diminishes the cohort of active growing follicles, reduces the reserve of primordial follicles, and results in premature ovarian failure (Perez, Knudson et al. 1997, Meirow, Lewis et al. 1999, Jurisicova, Lee et al. 2006, Oktem and Oktay 2007, Oktem and Oktay 2007, Petrillo, Desmeules et al. 2011, Soleimani, Heytens et al. 2011, Morgan, Anderson et al. 2012).

Although the toxicity of cyclophosphamide and Dox (as single agents) on the ovary is well established (Perez, Knudson et al. 1997, Meirow, Lewis et al. 1999, Jurisicova, Lee et al. 2006, Oktem and Oktay 2007, Oktem and Oktay 2007, Petrillo, Desmeules et al. 2011, Soleimani, Heytens et al. 2011, Morgan, Anderson et al. 2012), to our knowledge there are no reports that describe the effect of the combination of Dox and cyclophosphamide on proliferating granulosa cells.

The tocopherols (alpha, beta, gamma and delta) and tocotrienols (alpha, beta, gamma and delta) that together form Vitamin E (Iqubal, Khan et al. 2014) act as free radical scavengers in cell membranes (Brigelius-Flohe, Kelly et al. 2002). α-tocopherol (αToc) is the most abundant form in nature while γ-Tocopherol (γToc) is the most common form in the human diet (Brigelius-Flohe, Kelly et al. 2002).

It has been proposed that Dox-induced cardiotoxicity is the result of ROS-induced membrane lipid peroxidation (Myers, McGuire et al. 1977) and it has been observed that vitamin E deficiency results in histological features that are comparable to Dox-treated cardiac tissue (Myers, McGuire et al. 1977, Legha, Benjamin et al. 1982).

When αToc was administered to breast cancer patients before chemotherapy, although serum concentrations of αToc were elevated 8-fold there were no other

observable effects (Legha, Benjamin et al. 1982). However, the effect of αToc on post-chemotherapeutic ovarian function was not examined.

Although αToc may protect against the development of cancer (Day and Bingham 1994, Stolzenberg-Solomon, Sheffler-Collins et al. 2009) γToc delayed the formation of breast cancer tumours in rodent models (Smolarek and Suh 2011) and induced apoptosis in breast cancer cells *in vitro* (Klein, Thompson et al. 2011, Gopalan, Yu et al. 2012). It has therefore been proposed that γToc may prevent breast cancer *in vivo* (Constantinou, Papas et al. 2008, Lee, Ju et al. 2009). Additionally, a mixture of γ and delta tocopherol down-regulated the expression of estrogen receptor and inhibited estradiol-induced human MCF-7 breast cancer cell proliferation *in vitro* (Lee, Ju et al. 2009).

Both α and γ tocopherol are antioxidants with the potential to reduce chemotherapeutic-induced ROS damage and consequently reduce premature ovarian failure. Reduced ROS however, could also lead to decreased efficacy against breast cancer cells. γToc has both reductive power and anticancer activity (Smolarek and Suh 2011) and this led to our hypothesis that gamma tocopherol, but not alpha tocopherol, would augment the cytotoxic activity of the combination of Dox and cyclophosphamide against breast cancer cells *in vitro,* whilst simultaneously reducing ROS generation.

4.2.2 Materials and methods

4.2.2.1 Chemicals and Reagents

All chemicals and reagents used in the study were obtained from Sigma-Aldrich (Australia), unless specified otherwise. The 2',7'–dichlorofluorescin diacetate (DCFDA) cellular ROS detection assay kit was purchased from Abcam (Melbourne, Australia).

4.2.2.2 Preparation of solutions

Supplemented RPMI was prepared by mixing 500mL of phenol red-free RPMI with 1% v/v of 10,000 unit/mL penicillin + 10mg/mL streptomycin. Foetal calf serum (FCS) (DKSH, Victoria, Australia) was added to 10% (v/v) for MCF-7 and T47D and 20% for OVCAR-3 cells. Supplemented RPMI with 20% FCS additionally contained 5µg/mL of recombinant human insulin. Supplemented DMEM/F-12 was prepared by mixing phenol red-free DMEM/F-12 with 1% (v/v) of 10,000 unit/mL penicillin + 10mg/mL streptomycin and adding FCS to 10% (v/v). 10 mL Hank's balanced salt solution (HBSS, provided by the DCFDA ROS assay kit manufacturer) was added to 90 mL ddH_2O. DCFDA was diluted in 1X HBSS to generate a solution of 10µM. The DCFDA ROS assay positive control, ter-butyl hydrogen peroxide (TBHP), was diluted in supplemented media (RPMI or DMEM/F12) without phenol red, to give final concentrations of 12.5 and 50µM. Stock solutions of 100µM Dox and 1000µM 4-hydroperoxycyclophosphamide (4-Cyc) (ThermoFisher Scientific, Victoria, Australia) were prepared in supplemented media (RPMI or DMEM/F-12) and kept at 4°C and -20°C respectively for a maximum of 3 months. α and γ tocopherol were diluted in

100% DMSO to a concentration of 1000µM. These stock solutions were kept at 4°C for a maximum of 3 months. Further dilutions were made using supplemented media, and the concentration of DMSO the cells were exposed to was lower than 0.8% DMSO. The crystal violet stain (0.5%) was prepared in a 50% methanol (99.9% pure). Destain solution for the crystal violet assay was prepared with 100% acetic acid diluted to 33% with demineralised water.

4.2.2.3 Cell culture

The MCF-7 human epithelial breast adenocarcinoma cell line and the T47D human epithelial breast ductal carcinoma cell line were obtained from the America Type Culture Collection (ATCC) and maintained in supplemented RPMI medium with 10% FCS. The OVCAR-3 human ovarian epithelial adenocarcinoma cell line (ATCC, USA) was maintained in RPMI medium, supplemented with 20% FCS and 5µg/mL insulin. The COV434 (ECACC 07071909) cell line was derived from a solid human ovary granulosa cell carcinoma and maintained in supplemented DMEM/F12 medium. Media in each 75cm^2 flask of cells were replaced every 2-3 days and each cell line was subcultured twice a week. Cells that had undergone fewer than 25 passages were used for all experiments when they were 80% confluent, and in the exponential growth phase.

4.2.2.4 Determination of MCF-7 Effective Concentration (EC) values

MCF-7 cells (20,000 cells per well) were exposed to increasing concentrations of chemotherapeutics or tocopherols for 24h and cell viability was examined in a crystal violet assay. The experiment was repeated on 3 separate occasions.

Single agents	Concentrations (μM)
Doxorubicin	1.21
4-Cyc	21.23
αToc	100
γToc	35.1

Combined agents	Concentrations (μM)
Dox + 4-Cyc	1.21 (Dox) + 21.23 (4-Cyc)
Dox + 4-Cyc + αToc	1.21 (Dox) + 21.23 (4-Cyc) + 100 (αToc)
Dox+ 4-Cyc + γToc	1.21 (Dox) + 21.23 (4-cyc)+ 35.1 (γToc)

Table 4-1 24h MCF-7 – derived EC25 chemotherapeutics and tocopherols values.

Dox: Dox, 4-Cyc: 4-hydroperoxycyclophosphamide, αToc: α-Tocopherol, γToc: γ-Tocopherol.

4.2.2.5 Effect of Dox, 4-hydroperoxycyclophosphamide (4-Cyc), α or γ tocopherol on ROS generation

MCF-7, T47D, OVCAR-3 or COV434 cells (20,000 cells per well) were added to dark, clear bottom 96-well microplates for 24h adherence before replacing media

with 100µL 10µM DCFDA for 45min at 37°C in a humidified 5% CO_2 incubator in the dark. The DCFDA solution was removed, and cells were exposed to 100µL of chemotherapeutics or tocopherols (Table 4-1) in triplicate wells for 3 or 24h using concentrations that killed 25% of the MCF-7 cells (EC25). Since a cytotoxic concentration of αToc was not determined, the highest concentration tested was selected for further examination.

Controls were cells in medium only (background negative control), and cells exposed to low (12.5 µM) or high (50 µM) concentrations of TBHP (positive controls) (Figueroa, Asaduzzaman et al. 2018). Each experiment was repeated on three separate occasions (n=3).

4.2.2.6 ROS measurement by DCFDA assay

The ROS production was detected by recording fluorescence immediately after addition of test agents (time 0), every hour for a 3h incubation period, and after 24h continuous incubation (24h+). Fluorescence was measured according to protocol described by Figueroa et al., (Figueroa, Asaduzzaman et al. 2018) Fluorescence readings were made using a plate spectrofluorometer (GloMax® Explorer, Promega, Australia). Relative fluorescence units (RFU) were calculated by subtracting background readings (cells in media only), from all fluorescence values obtained from DCFDA loaded cells in media + test reagents. Each concentration of DCFDA and TBHP was examined in two replicate wells. Plates were kept at 37°C in a humidified 5% CO_2 incubator in the dark between readings.

4.2.2.7 Crystal Violet assay

After measurement of ROS, cell viability was determined using the crystal violet (CV) assay. Media containing test agents and non-adherent dead cells were replaced with 50µL of crystal violet stain (0.5%). Cells were stained for 10min, and then rinsed with demineralised water to remove any excess stain. Cells were left to air dry overnight. 60µL destain solution of 33% acetic acid was added. After 10min, absorbance was read at 570nm with correction at 630nm (Reid, Lang et al. 2015). The numbers of viable cells remaining after exposure to test agents were determined by a comparison with a CV standard curve using densities of 0-80,000 cells per well ($R^2 = 0.99$) generated for the same replicate experiment.

4.2.2.8 Statistical analysis

One-way ANOVA tests were performed to examine the effect of exposure and reagent on the numbers of viable cells. ROS per cell values were subjected to one-way ANOVA with Newman-Keuls post-hoc test. The RFU values obtained from 0, 1, 2, 3 and 24h exposures to chemotherapeutics and tocopherols were subjected to Two-way ANOVA with Bonferroni post-hoc test. These statistical analyses were performed using GraphPad Prism. Statistical significance was set at $p \leq 0.05$. All experiments were performed as three independent replicates, and all data expressed as mean ± standard deviation.

4.2.3 Results

In each experiment 20000 cells per well underwent a 24h preculture and a 24h period of control culture. This gave rise to approximately 35000 viable cells per well in control media for the three cell lines OVCAR, T47D and MCF-7, but only 20000 viable COV434 cells per well (Fig 4.7). The cells in control media generated 700 to 1300 RFU indicative of normal metabolic ROS (Fig 4.7). A 24h exposure to the previously determined MCF-7 derived EC25 values of 4-Cyc or Dox (Table 4-1) decreased the MCF-7 cell viability by 25%, to 74±7.9% and 72±8.2% of the media controls, respectively (Fig 4.7D).

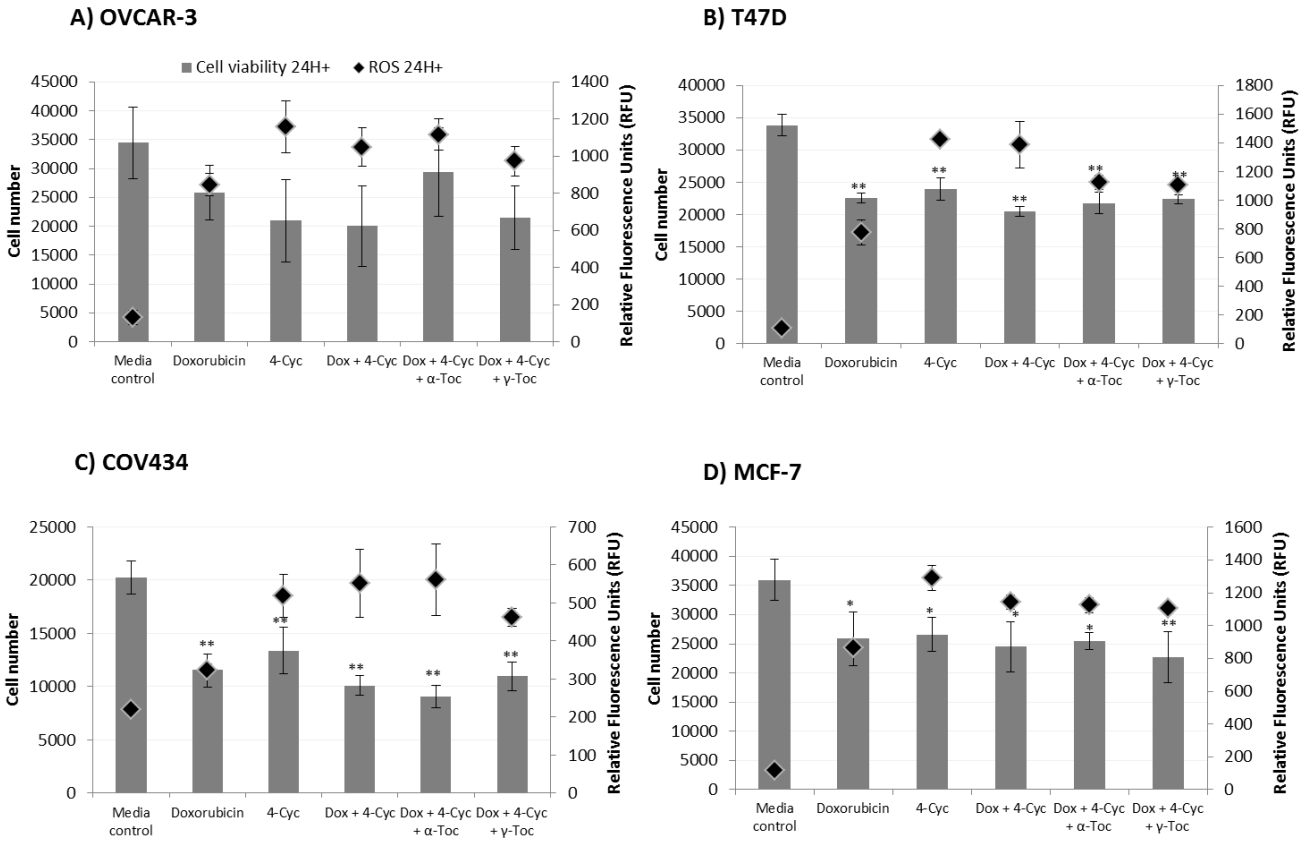


Figure 4.7 Effect of 24h exposure to chemotherapeutics and tocopherols on cell viability.

OVCAR-3, B) T47D, C) COV434, and D) MCF-7 cells were loaded with 10μM DCFDA for 45min then washed and exposed to reagents at concentrations that reduced MCF-7 viability by 25% (EC25). These were doxorubicin (Dox, 1.21μM), 4-hydroperoxycyclophosphamide (4-Cyc, 21.23μM), a combination of Dox (EC25) and 4-Cyc (EC25), Dox (EC25) and 4-Cyc (EC25) and α-Tocopherol (αToc,100μM), or Dox (EC25) and 4-Cyc (EC25) and γ-Tocopherol (γToc, 35.1 μM). Reactive oxygen species were assessed by reading fluorescence after 24h+ *in vitro* culture (relative fluorescent units, diamonds) and the numbers of viable cells were determined in a crystal violet assay after comparison with a standard curve (columns). The experiment (and crystal violet standard curve) was repeated on three separate occasions (n=3) and mean± stdev shown. Viable cell numbers subjected to One-way ANOVA with Tukey post-hoc test, significant difference from Media control * $p<0.05$, ** $p< 0.01$.

Exposure to the MCF-7 derived EC25 values of Dox significantly reduced T47D, OVCAR-3 and COV434 cell viability to 73±5.6%, 75±5% and 57±1.9%

respectively (Fig 4.7). Similarly, MCF-7 EC25 values of 4-Cyc significantly decreased the cell viability of T47D, OVCAR-3 and COV434 to 68±2.2%, 60±14% and 66±8.6% of the media controls, respectively (Fig 4.7). No significant differences in cytotoxicity were observed between cells exposed to the combination of Dox and 4-Cyc, and the cytotoxicity caused by each agent alone, in any of the cell lines tested (Fig 4.7). To illustrate, in MCF-7 cells, the combination of Dox (EC25) and 4-Cyc (EC25) only caused cell death in 37% of the cells after 24h exposure (Fig 4.7D). The percentage of cells killed by the combination of 4-Cyc and Dox did not support synergistic interactions between the two agents.

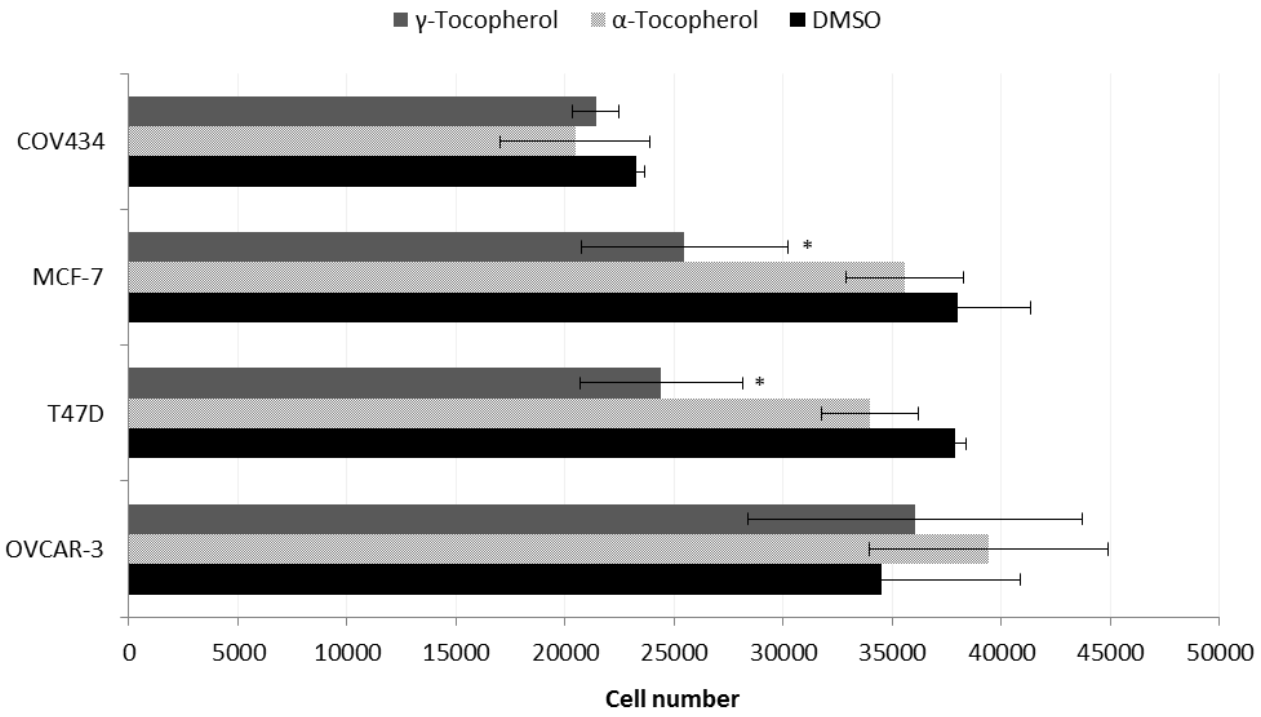


Figure 4.8 Effect of 24h exposure to tocopherols on cell viability.

COV434, MCF-7, T47D and OVCAR-3 cells were loaded with 10μM DCFDA for 45min then washed and exposed to αToc (100μM), or γToc (35.1μM). Fluorescence was read after 24h *in vitro* culture then cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data were

subjected to One-way ANOVA with Tukey post-hoc test. * $p \leq 0.05$ significant difference compared to DMSO control.

Exposure to 0.8% DMSO (the vehicle for tocopherols) did not affect cell viability, for example there were 33837±1642 T47D cells after 24h in control medium (Fig 4.7B) and 37897±495 after 24h culture in control medium containing 0.8% DMSO (Fig 4.8). Exposure to αToc had no effect on cell viability in any of the cell lines (Fig 4.8) but exposure to the MCF-7 derived EC25 value of γToc significantly decreased MCF-7 and T47D cell viability to 70±1.9% and 72±7.7% of DMSO controls. It is interesting that the concentration of γToc that killed breast cancer cells had no effect on OVCAR-3 or COV434 cell viability (Fig 4.8). Although γToc was cytotoxic to breast cancer cells as a single agent, it did not increase the cytotoxicity of the combined Dox + 4-Cyc regimen (Fig 4.7). The cytotoxic effects of 4-Cyc and Dox and γToc were therefore not additive.

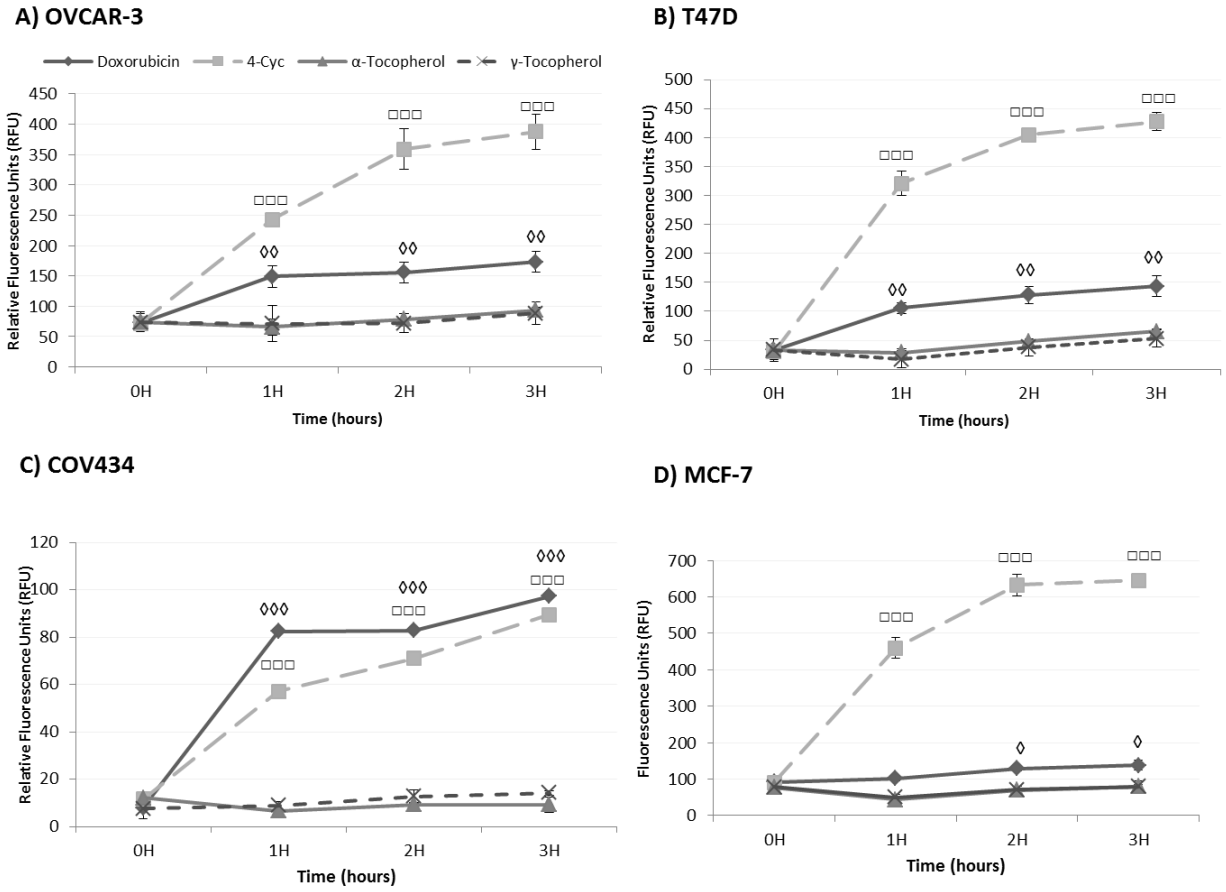


Figure 4.9 Effect of 3h exposure to chemotherapeutics and tocopherols on ROS production.

A) OVCAR-3, B) T47D, C) COV434, and D) MCF-7 cells were loaded with DCFDA for 45min then washed and exposed to reagents at concentrations that reduced MCF-7 viability by 25% (EC25). Fluorescence was read every hour for 3h *in vitro* culture. Means ± SD of 3 independent experiments shown. Relative fluorescent units (RFU) were subjected to Two-way ANOVA with Bonferroni post-hoc test. ◊ Doxorubicin, □ 4-Cyc, ◊ or □ $p \leq 0.05$, ◊◊ or □□ $p \leq 0.01$, ◊◊◊ or □□□ $p \leq 0.001$ significant differences from 0h control.

Higher levels of ROS were detected in all cell lines after 24h exposure to chemotherapeutics compared to control media (Figs 4.7 and 4.9). 4-Cyc caused the highest levels of ROS in all the cell lines and although a 24h exposure to Dox generated ROS as well, the amount of ROS caused by exposure to the combination of Dox and 4-Cyc was similar to that of 4-Cyc alone; ROS generation was not additive (Figs 4.7 and 4.9) except in COV434 cells. A 24h exposure to either αToc or

γToc caused the T47D cells to produce significantly more ROS than the DMSO controls (Fig 4.9B). The addition of γToc (but not αToc) to the combined regimen significantly decreased the amount of ROS produced by the COV434 ovarian granulosa cells (Fig 4.9C) and this reduction in Dox and 4-Cyc-stimulated ROS by γToc was significant after only 1h in COV434 cells (Fig 4.11C).

Acute time-dependent, significant increases in ROS levels were detected in all cell lines during the first 3h exposure to the MCF-7 EC25 values of Dox (Fig 4.10), whereas 4-Cyc caused the highest ROS levels in OVCAR, T47D and MCF-7 cells. In the COV434 cells in 4-Cyc and Dox stimulated the same amount of ROS during the first 3h and the amount of ROS generated by 4-Cyc was lower than in the other 3 cell lines (Fig 4.10C). Unlike the T47D 24h dataset, a 3h exposure to αToc or γToc had no effect on ROS generation (Fig 4.10B).

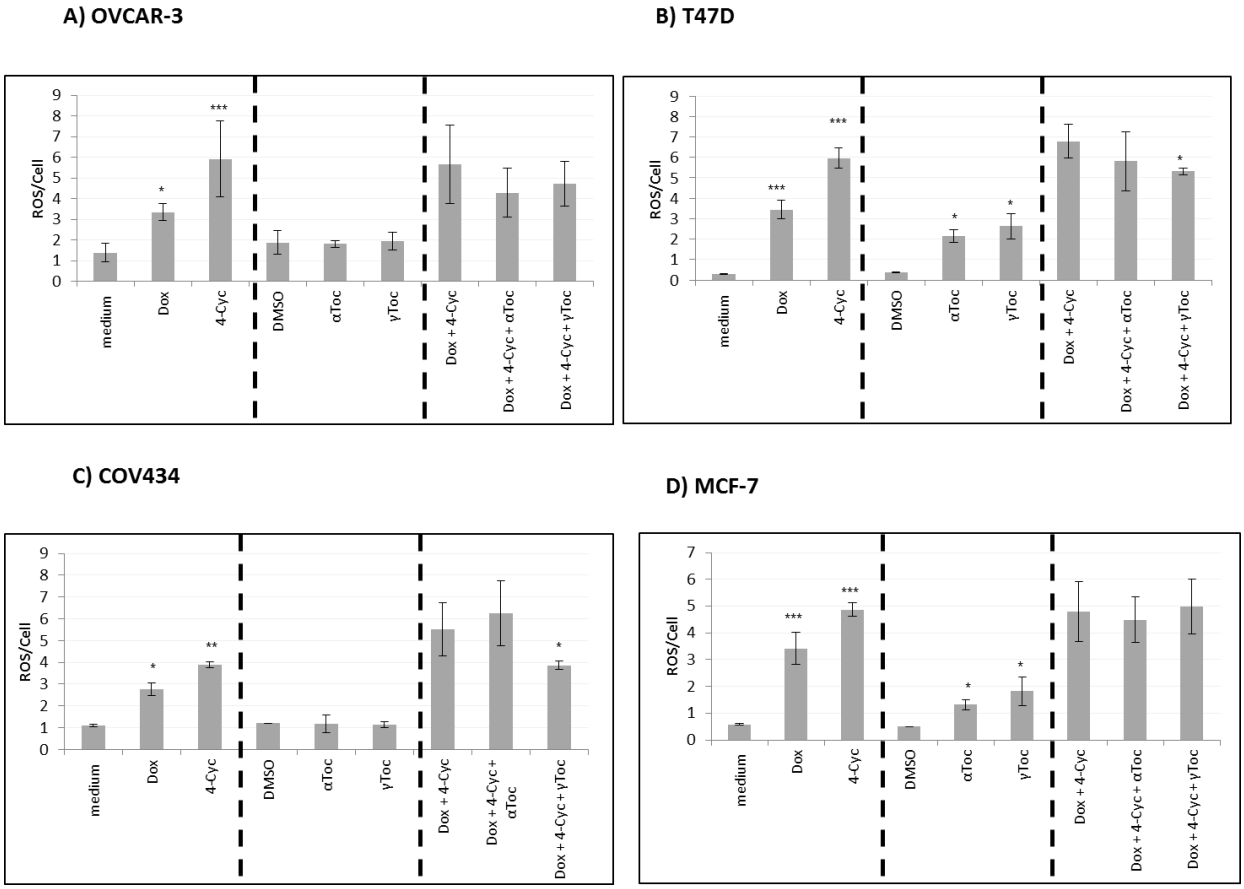


Figure 4.10 Effect of 24h exposure to chemotherapeutics and tocopherols on ROS production.

A) OVCAR-3, B) T47D, C) COV434, and D) MCF-7 cells were loaded with 10µM DCFDA for 45min then washed and exposed to reagents at concentrations that reduced MCF-7 viability by 25% (EC25). Fluorescence was read after 24h+ uninterrupted *in vitro* culture. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. ROS values were expressed per viable cell. Means ± SD of 3 independent experiments shown. Data were subjected to One-way ANOVA with Newman-Keuls post-hoc test. Significant differences compared to medium control (1st section) or DMSO medium control (2nd section) or combination of Dox + 4-Cyc (3rd section), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

In three of the cell lines a 3h exposure to the combined regimen of 4-Cyc and Dox caused ROS generation intermediate to the levels generated by each agent alone (Fig 4.11). To illustrate, 4-Cyc generated 387±29 and Dox generated 173±17 RFU in OVCAR cells (Fig 4.10A) whereas the combined regimen caused production

of 322±27 RFU (Fig 4.11A). In contrast, the COV434 cells responded to the combined regimen by generating more ROS (122±9 RFU, Fig 4.11C) than after a 3h exposure to 4-Cyc (89±7 RFU) or Dox (97±7 RFU) as single agents (Fig 4.11C). T47D cells were like OVCAR and MCF-7 cells in that the addition of either of the antioxidant tocopherols to the combined regimen did not decrease ROS levels during a 3h exposure (Fig 4.11) whereas the COV434 cells responded differently; the addition of α or γToc to the combination of 4-Cyc and Dox for only 1h caused significant decreases in ROS levels, and the protective effect of the tocopherols against the chemotherapeutic-induced ROS continued for the 3h exposure (Fig 4.11C).

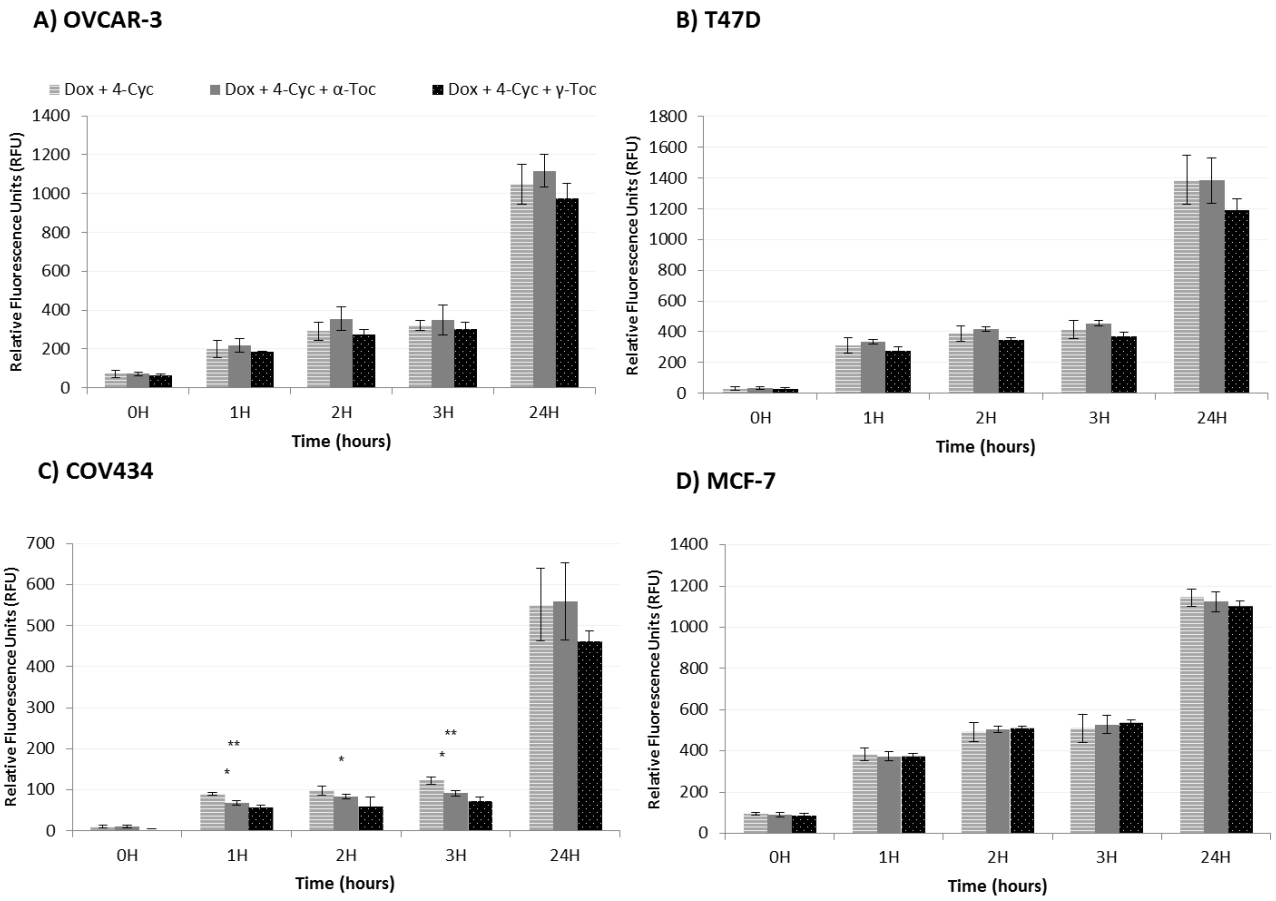


Figure 4.11 Effect of 3h exposure to chemotherapeutics and tocopherols on ROS production.

A) OVCAR-3, B) T47D, C) COV434, and D) MCF-7 cells were loaded with 10µM DCFDA for 45min then washed and exposed to the combination of Dox (EC25) and 4-Cyc (EC25), Dox (EC25) and 4-Cyc (EC25) and αToc (100µM), or Dox (EC25) and 4-Cyc (EC25) and γToc (EC25) for 3 or 24h. Fluorescence was read every hour for 3h or after 24h+ *in vitro* culture. Means ± SD of 3 independent experiments shown. Data were subjected to two-way ANOVA with Bonferroni post-hoc test. * $p \leq 0.05$, ** $p \leq 0.01$ significant difference compared to combination of Dox and 4-Cyc.

4.2.4 Discussion

This is the first study to examine the effects of a clinically relevant combination of the chemotherapeutics Dox and 4-Cyc on cytotoxicity and ROS production by human breast and ovarian cell lines *in vitro.* We found that the ovarian granulosa-derived COV434 cells were twice as sensitive to the chemotherapeutic

agents as the cell lines derived from epithelial cells (MCF-7, T47D or OVCAR-3 cells), they were resistant to the cytotoxic activity of γToc, and in COV434 cells γToc rapidly reduced chemotherapeutic-generated ROS production. Together these differential activities towards breast and ovarian granulosa-derived cells suggest potential for the development of a fertility preservation therapy for breast cancer patients.

The four cell lines displayed different sensitivities to the cytotoxic, ROS-inducing and antioxidant activities of the test agents used in this study (Dox, 4-Cyc, αToc, γToc, and the combination of Dox and 4-Cyc with αToc or γToc). Transformed cell lines commonly have different sensitivities to test agents (Edwards, Benkendorff et al. 2014). In the present study exposure to the MCF-7 derived EC25 values for 4-Cyc were expected to reduce the numbers of viable cells to 75%, but actually reduced the numbers of viable COV434 cells to 66%, OVCAR-3 to 61% and T47D to 68%. Cell line sensitivity to the cytotoxic effects of Dox was slightly different; COV434 cells were the most sensitive (57%), but the OVCAR-3 cells (75%) were more resistant to the cytotoxic effects of Dox than 4-Cyc. The combination of the EC25 value for 4-Cyc with the EC25 value for Dox did not cause death of 50% of MCF-7, T47D nor OVCAR-3 cells as expected; only 37%, and 50% of the more sensitive COV434 cells. For these four cell lines, cytotoxicity caused by the two chemotherapeutics was not synergistic, and the rates of cell death obtained with the combined regimen were only 1.3-fold higher than with a single agent. This is important because in early animal studies, the combination of Dox and cyclophosphamide was clearly much more effective against four murine mammary adenocarcinomas than Dox as a single agent, and the authors attributed this synergism to different mechanisms of action of cyclophosphamide and Dox (Corbett,

Griswold et al. 1975). In humans, adriamycin (Dox) was found to be effective against breast cancer (Carter 1972, Hoogstraten, George et al. 1976) but it was also cardiotoxic, and it was determined that a lower dose of adriamycin could be combined with cyclophosphamide to achieve the same anti-cancer efficacy whilst reducing off-target adverse effects. There were subsequently a number of clinical trials to optimise the doses of adriamycin ('A' or Dox) and cyclophosphamide ('C'), and these were followed by studies which compared the combination of AC to other combination therapies such as CMF or AC+F (Bonadonna, Brusamolino et al. 1976, Colleoni, Price et al. 1998, Evans, Yellowlees et al. 2005). In a phase III randomised trial, the combination of AC showed a response rate of 47% in metastatic breast cancer patients, and complete remission in 7% (Mansi, Yellowlees et al. 2010).

Several factors affect cell line toxic responses *in vitro*. One factor is the cell doubling time, because some chemotherapeutics are phase-specific agents and only kill cells that are passing through the relevant cell cycle phase (Orth, Tang et al. 2008, Shi, Orth et al. 2008, Gascoigne and Taylor 2009). This 'fractional kill' theory predicts a strong correlation between proliferation rate and drug sensitivity (Berenbaum 1972). Neither Dox or cyclophosphamide are considered cell cycle phase-specific drugs although they have been known to preferentially target the more metabolically active cells (Morgan, Anderson et al. 2012) and Fan et al., (2014) found that Dox inhibited the growth of HepG2 cells by induction of G2/M cell cycle arrest. It's possible therefore that the *in vitro* cytotoxicity of Dox and 4-Cyc is dependent on the mitotic rate and that cell lines with fast doubling times (more metabolically active) might be more sensitive to their cytotoxic effects. The COV434 cells are reported as having doubling rates from 24h (Cowley, Weir et al. 2014) to 36h (Verga Falzacappa, Mangialardo et al. 2009), and they had the slowest doubling

rate in the present study. Nevertheless, they were the most sensitive to the cytotoxic activity of Dox or 4-Cyc. The next most sensitive cell line was OVCAR-3, with the longest reported doubling time of 48h (Hamilton, Young et al. 1983). Doubling times are reported as being 30h for MCF-7 (Dougherty, Schumaker et al. 2004) and 39h for T47D (Dougherty, Schumaker et al. 2004). In the present study then, the differing proliferation rates of the four cell lines did not explain the data. Added to this, although MCF-7 and ovarian granulosa KGN cells have different doubling times, in a previous study in which they were exposed to 5μM Dox or 4-Cyc for 24h then cultured for a further 48h, or exposed for 72h continuously (Figueroa, Asaduzzaman et al. 2019) there was no difference in cytotoxicity between these two protocols; >80% of both cell lines died within the first 24h.

Another factor that affects *in vitro* responses to test agents is the origin and phenotype of the cell line. Both MCF-7 and T47D cell lines were isolated from a pleural effusion of patients with breast carcinoma (Soule, Vazquez et al. 1973, Keydar, Chen et al. 1979). MCF-7 cells maintain several of the functional characteristics of differentiated mammary epithelium including the expression of estrogen receptors (Huguet, McMahon et al. 1994). Similarly, the T47D line also expresses steroid hormone receptors (Keydar, Chen et al. 1979). The COV434 cell line was derived from a solid primary human ovarian granulosa cell carcinoma but is a good *in vitro* model for normal healthy granulosa cells because the cells are mitotic and maintain many of the functional characteristics required for follicle growth and development (Zhang, Vollmer et al. 2000). The present study confirms a previous report that a 24h exposure to 25μM 4-Cyc caused a 40% decrease in viable COV434 cells (Yuksel, Bildik et al. 2015). The OVCAR-3 cell line however was obtained from a patient who had an epithelial adenocarcinoma of the ovary and who had been

treated with the combination of Dox, cyclophosphamide and cisplatin 8 months before donation of ascites fluid containing ovarian adenocarcinoma cells. The tumour cells were injected into nude mice, and the resulting tumours disaggregated and used to generate the OVCAR-3 cell line. These cells have an abnormal karyotype and are resistant to doxorubicin, although the effects of 4-Cyc have not been reported (Hamilton, Young et al. 1983). This explains why OVCAR-3 cells were more resistant then T47D and COV434 cells to the cytotoxic activity of Dox. Here we report for the first time that in our hands OVCAR cells were less resistant to 4-Cyc than they were to Dox, and that they were more sensitive to 4-Cyc than T47D and MCF-7 cells in the same experiment.

Neither α nor γToc affected the viability of either of the ovarian cell lines, but γToc as a single agent (and not αToc) killed both breast cancer cell lines. This type of differential activity at comparable doses has been observed elsewhere. A 96h exposure to 25μM γToc induced apoptosis in prostate and lung cancer cells but had no effect on the viability of primary-derived prostate epithelial cells (Jiang, Wong et al. 2004). γToc also induced apoptosis in MCF-7 and MDA-MB-435 human breast cancer cells via upregulation of JNK/CHP/DR5 mediated apoptosis and de novo ceramide synthesis (Klein, Thompson et al. 2011, Gopalan, Yu et al. 2012), and DR5-mediated apoptosis is thought to be characteristic of cancer but not normal cells (Kruyt 2008). Although γToc as a single agent caused cytotoxicity in the two breast cancer cell lines, its addition to the combined regimen of Dox and 4-Cyc neither increased nor decreased breast cancer cell death. This led to the conclusion that the cytotoxic effects of 4-Cyc and Dox and γToc were not additive.

Exposure to 4-Cyc caused the production of more ROS than exposure to Dox, except in COV434 cells, in which Dox and 4-Cyc stimulated the same, low

levels of ROS. Dox-stimulated ROS production has been reported previously, including in H9c2 cardiac muscle cells which were exposed for 1h to a concentration of Dox that was 10 times higher than in our study, and caused a 4-fold increase in ROS (Tan, Wang et al. 2010). In COV434 cells, although Dox induced similar levels of ROS per cell as in other cell lines, 4-Cyc induced lower levels. Tsai-Turton et al., (2007) found that COV434 cells exposed to 50μM 4-Cyc for 2h, or to 1μM for 6h, increased production of ROS significantly (Tsai-Turton, Luong et al. 2007). In that study however, the concentration of DCFDA (100μM) was 100 times higher than in the present study, and higher DCFDA concentrations can be toxic and contribute to ROS generation (Figueroa, Asaduzzaman et al. 2018). Nevertheless, the amount of ROS generated by COV434 cells after 24h exposure to 21μM 4-Cyc in our study was in broad agreement with the study by Tsai-Turton et al (2007). Although the effects of 4-Cyc or Dox on ROS have been reported previously, this is the first study to examine the effects of the combined regimen. A 3h exposure to the individual chemotherapeutics generated ROS levels that bracketed the ROS levels measured after exposure to the combined regimen, meaning that the combination of 4-Cyc and Dox resulted in ROS levels that were the average of the individual agents, but that ROS levels were not additive. Again, COV434 cells were the exception; ROS levels after exposure to the combined regimen were slightly higher than after exposure to each separate chemotherapeutic. Neither of the tocopherols caused an increase in ROS in any of the cell lines within the first 3h of exposure, but between 3h and 24h both tocopherols increased ROS in the T47D cells. It is surprising that antioxidant molecules should cause an increase in ROS, and this observation led to the speculation that after 3h *in vitro* the tocopherols disrupted the breast cancer cell REDOX balance or regulation (Costa, Scholer-Dahirel et al. 2014, Fan and Li 2014).

The levels of ROS generated by the tocopherols in the breast cancer cells were approximately half that generated by the chemotherapeutics, and the addition of αToc or γToc to the combined regimen did not increase ROS.

ROS are associated with wide-spread adverse effects impacting many different cell types *in vivo* (Myers, McGuire et al. 1976, Krivit 1979, Lubawy, Whaley et al. 1979, Nagata, Takata et al. 1999, Thabrew, Samarawickrema et al. 1999), whereas cytotoxic activity tends to impact a smaller sub-set of proliferating cells. Each of 4-Cyc (Kalich-Philosoph, Roness et al. 2013) and Dox (Ben-Aharon, Bar-Joseph et al. 2010) caused ovarian granulosa cell death in rodents *in vivo.* Dox caused granulosa cell damage within 2h, and oocyte apoptosis 12h after administration. Roti et al., (2012) concluded that fertility preservation designed to protect against the damage caused by chemotherapy should act immediately and rapidly (Roti, Leisman et al. 2012) Although the role that ROS play in the chemotherapy-mediated depletion of ovarian follicles has not been examined, it is known that alkylating agents such as cyclophosphamide cause more ovarian damage than other chemotherapeutics (Yuksel, Bildik et al. 2015) and in the present study 4-Cyc generated more ROS than Dox. We speculate that the clinical regimen of cyclophosphamide and Dox is cytotoxic to the granulosa cells in ovarian follicles of breast cancer patients via a number of mechanisms including DNA intercalation (Tewey, Rowe et al. 1984) and ROS production (Mizutani, Tada-Oikawa et al. 2005, Liu, Li et al. 2012) and that it is possible that decreasing ovarian ROS levels might have fertility preserving effects in breast cancer patients treated with AC. One way to improve existing anti-cancer treatments would be to reduce off-target adverse effects without reducing efficacy against breast cancer. In the present study the addition of γToc to the combination of Dox and 4-Cyc had no effect on cytotoxicity in three

epithelial-derived cancer cell lines, but differentially and specifically reduced ROS levels after only 1h in the ovarian granulosa cell line COV434.

Primary-derived patient tumour samples are a heterogeneous mixture of different cell types, and primary-derived physiologically normal human ovarian granulosa cells are relatively difficult to obtain. It is sensible to use homogenous cell lines initially to establish protocols, as has been done in this study, but important to a) acknowledge that the cell lines have very limited extrapolation to the *in vivo* situation and b) that results must be assessed in the context of the characteristics of each cell line; COV434 granulosa cells differ from physiologically normal granulosa cells *in vivo*. With that in mind, conclusions from the present *in vitro* study are that the neither the cytotoxic nor the ROS-inducing activities of 4-Cyc and Dox were synergistic since the combination of Dox and 4-Cyc did not increase cell death to 50% as was expected by adding EC25 Dox to EC25 4-Cyc. It is likely that the two chemotherapeutics therefore have some cytotoxic mechanisms of action in common. 4-Cyc was responsible for an 8.6-fold increase in ROS production within 3h, whereas Dox only increased ROS 5-fold, and the combined regimen increased ROS production 8.5-fold. The ovarian granulosa-derived COV434 cells were less resistant to the cytotoxic activities of the chemotherapeutic agents than the breast cancer cell lines but were also protected against chemotherapeutic-stimulated ROS by both tocopherols within 1h, an important attribute for fertility preservation (Roti, Leisman et al. 2012) αToc was not cytotoxic whereas γToc killed the two breast cancer but not the two ovarian cell lines. Although γToc was cytotoxic as a single agent it did not increase the cytotoxicity of the combination of Dox and 4-Cyc. Despite this we recommend further studies to determine if the antioxidant γToc has the potential to

reduce chemotherapeutic-stimulated ROS in ovarian granulosa cells whilst maintaining the anti-cancer efficacy of the established therapeutic regimen of AC.

5. CHAPTER V: APOPTOSIS INDUCTION BY CHEMOTHERAPEUTICS AND TOCOPHEROLS

5.1 Introduction

Cyclophosphamide and doxorubicin are commonly used for treating breast cancer (Younis, Rayson et al. 2011, Yardley, Arrowsmith et al. 2017). The combination is believed to have synergistic effects (Corbett, Griswold et al. 1975, Blagosklonny 2004, Pritchard, Lauffenburger et al. 2012, Bayat Mokhtari, Homayouni et al. 2017) and reduced recurrence rates of stage II breast cancer, supported a response rate of 47% in metastatic breast cancer patients, and complete remission in 7% (Nabholtz, Falkson et al. 2003). Doxorubicin is an anthracycline that intercalates and interferes with DNA and RNA synthesis (Tewey, Rowe et al. 1984, Thorn, Oshiro et al. 2011). It also induces the production of reactive oxygen species (ROS) which cause lipid peroxidation and apoptosis (Doroshow 1986, Gewirtz 1999, Mizutani, Tada-Oikawa et al. 2005). Cyclophosphamide is an alkylating agent that requires hepatic metabolic activation to release the active forms: phosphoramide mustard and acrolein (Boddy and Yule 2000, Emadi, Jones et al. 2009). Phosphoramide mustard intercalates into DNA and causes intra and inter-strand cross-links (Dong, Barsky et al. 1995), whereas acrolein has shown to increase ROS production in a variety of cell lines (Luo and Shi 2004, Mythili, Sudharsan et al. 2004, Liu, Li et al. 2012).

Activation of apoptosis pathways is a key mechanism by which chemotherapeutics kill cancer cells (Debatin 2005). Characteristic apoptotic changes have been described in cancer cells from mice after treatment with various

chemotherapy agents, including 5-fluorouracil (5FU), doxorubicin, cyclophosphamide and cisplatin, among others (Kaufmann and Earnshaw 2000)

Apoptosis is a distinct, intrinsic programmed cell death that occurs in several physiological and pathological conditions and is characterized by typical morphological features such as cell shrinkage, DNA fragmentation and membrane blebbing (Hengartner 2000). The underlying mechanism for initiation of apoptosis upon therapy is likely to vary according to the chemotherapeutic drug, but most apoptosis signalling pathways result in the activation of caspases (Debatin 2004).

Caspases are a family of proteolytic enzymes that act as effector molecules during apoptosis (Thornberry and Lazebnik 1998, Degen, Pruijn et al. 2000, Hengartner 2000) and are classified as initiator (or “upstream”) molecules, which are responsible for initiating the proteolytic cascade; or as effector (or “downstream”) molecules, which cleave cytoplasmic or nuclear substrates (Earnshaw, Martins et al. 1999, Slee, Adrain et al. 1999, Utz and Anderson 2000). Initiator caspases include caspase -2, -8, -9 and -10 (Earnshaw, Martins et al. 1999, Slee, Adrain et al. 1999, Utz and Anderson 2000); which are responsible for activating the effector caspases -3,-6,-7 (Earnshaw, Martins et al. 1999, Hengartner 2000)

Activation of caspases can be triggered by two different mechanisms; at the plasma membrane by death receptor mediated signalling (extrinsic pathway) or at the mitochondria (intrinsic pathway) (Thornberry and Lazebnik 1998, Hengartner 2000). Extrinsic death signalling pathway involves stimulation of members of the tumour necrosis factor (TNF) receptor superfamily which include death receptors (DR) 4 and 5, CD95 (APO-1/Fas) or TRAIL receptors. These aggregate and recruit Fas-associated death domain (FADD) and capase-8 molecules with subsequent activation of effector caspases -3, -6 or -7 (Schulze-Osthoff, Ferrari et al. 1998,

Krammer 2000, Walczak and Krammer 2000). On the other hand, the intrinsic mitochondrial-mediated death signalling pathway involves the release of factors such as cytochrome c followed by activation of caspase -3, through the formation of a caspase -9 complex (Costantini, Jacotot et al. 2000, Kroemer and Reed 2000, Martinou and Green 2001).

Keane et al. (1999) found that TRAIL receptors DR4 and DR5 were expressed by T47D, malignant breast cell lines and that exposure of cells to doxorubicin or 5-fluorouracil significantly augmented TRAIL-induced apoptosis, indicative of the extrinsic apoptotic pathway (Keane, Ettenberg et al. 1999). On the other hand, Sharifi et al. (2015) found that treatment of MCF-7 cells with doxorubicin caused a time dependent increase in caspase-9 levels and in the Bax/Bcl-xL ratio (Sharifi, Barar et al. 2015). Events associated with the intrinsic pathway. Similarly, MCF-7 cells exposed to cyclophosphamide showed increased caspase -9 activity in a dose dependent manner (Sharifi, Barar et al. 2015)

Doxorubicin and cyclophosphamide, as single agents, are associated with a variety of adverse effects *in vivo*, including T-cell suppression and chronic cardiotoxicity (Legha, Benjamin et al. 1982, Ozer, Cowens et al. 1982). Chemotherapy treatment in premenopausal women is associated with a high risk of premature ovarian failure. Although the exact mechanism is still uncertain, it has been proposed that this is caused by the loss of primordial follicles, not necessarily as direct effect of the chemotherapeutic agents, but through the targeting of proliferating granulosa cells in growing follicles (Meirow et al., 2010; Morgan et al., 2012). Granulosa cells produce anti-Müllerian hormone (AMH) (Durlinger, Kramer et al. 1999) which regulates the rate of primordial follicle activation (Morgan, Anderson et al. 2012). Depletion of circulating AMH results in an increased recruitment of

quiescent primordial follicles to replace damaged developing follicles (Morgan, Anderson et al. 2012). Repeated cycles of doxorubicin and cyclophosphamide may therefore reduce both the number of growing follicles and the reserve of primordial follicles.

In animal studies, cyclophosphamide caused a dose-dependent loss of primordial and primary follicles in rodents (Meirow, Lewis et al. 1999, Petrillo, Desmeules et al. 2011), and apoptosis of oocytes (Oktem and Oktay 2007). Similarly, cyclophosphamide caused death of secondary and antral follicles in adult rats, and intrinsic apoptosis of granulosa cells was via caspase 3 and 9 pathways (Lopez and Luderer 2004).

Early studies showed that doxorubicin caused apoptosis in murine oocytes (Perez, Knudson et al. 1997, Jurisicova, Lee et al. 2006), and double-strand DNA breaks in oocytes and granulosa cells of human primordial follicles. Doxorubicin-induced ovarian toxicity has also been associated with the extrinsic apoptotic pathway, via caspase -3, -7 activation in murine granulosa cells (Zhang, He et al. 2017).

Vitamin E consists of at least eight structurally related molecules, 4 tocopherols (α, β, γ, and δ-tocopherol) and 4 tocotrienols (α, β, γ, and δ-tocotrienol) (Iqubal, Khan et al. 2014). α-tocopherol is the most prevalent form of vitamin E in plasma and tissues (Brigelius-Flohe, Kelly et al. 2002) and γ-tocopherol is the most common form in the human diet (Brigelius-Flohe, Kelly et al. 2002).

The potential anticancer activity of tocopherols has been investigated for many years, but the epidemiological evidence is limited and inconsistent, and conclusions regarding the role of vitamin E in breast cancer remain elusive

(Brigelius-Flohe, Kelly et al. 2002, Klein, Thompson et al. 2011, Smolarek and Suh 2011).

γ-tocopherol has demonstrated greater anti-inflammatory and anti-cancer activity than α-tocopherol in a variety of animal cancer models (Klein, Thompson et al. 2011, Smolarek and Suh 2011, Gopalan, Yu et al. 2012). γ-tocopherol induced apoptosis in MCF-7 and MDA-MB-435 breast cancer cells via caspase 8 and 9 (Yu, Park et al. 2008). In another study γ-tocopherol induced apoptosis in breast cancer cells through activation of pro-apoptotic JNK/CHOP/DR5 mediated events (Lee, Ju et al. 2009). Similarly, γ-tocopherol caused cytochrome c release followed by the activation of caspase -9 and -3 in LNCaP prostate cancer cells (Jiang, Wong et al. 2004). In this same study, γ-tocopherol appeared to have no effect of the growth of normal PrEC prostate epithelial cells, which suggested that γ-tocopherol might target cancer cells selectively (Jiang, Wong et al. 2004).

Although the combination of doxorubicin and cyclophosphamide has extensive clinical application, the combined mechanism of action of these two drugs has not been described. Here we evaluate apoptosis and caspase activity in breast cancer cells and ovarian granulosa cells after exposure to chemotherapeutics and/or tocopherols We hypothesized that α and γ-tocopherol would reduce chemotherapeutic-induced apoptosis in ovarian granulosa cells but would induce apoptosis in both breast cancer cell lines.

5.2 Materials and methods

5.2.1 Chemicals and Reagents

All chemicals and reagents used in the study were obtained from Sigma-Aldrich (Australia), unless specified otherwise. The DeadEnd Fluorometric TUNEL system and Caspase Glo 3/7 assay system were purchased from Promega (Australia).

5.2.2 Preparation of solutions

Stock solutions in complete medium (RPMI or DMEM/F12) of doxorubicin (Dox) (100μM) and 4-hydroperoxycyclophosphamide (4-Cyc) (1000μM) (ThermoFisher Scientific, Victoria, Australia) were kept at 4°C and -20°C respectively for a maximum of 3 months. Stock solutions of alpha (αToc) and gamma tocopherol (γToc) were prepared in DMSO to a concentration of 1000μM and stored for a maximum of 3 months at 4°C. Further dilutions in the appropriate medium were prepared before each assay. The 0.5% crystal violet stain was prepared in a 50% methanol (99.9% pure). 100% acetic acid was diluted to 33% with demineralised water, to be used as a destain solution in the crystal violet assay. The TUNEL positive control, DNaseI was prepared in Hank's balanced salt solution supplemented with magnesium to yield a concentration of 1μM. The caspase positive control staurosporine was prepared in complete medium (RPMI or DMEM/F12) to give a final concentration of 1μM. 4% paraformaldehyde (4g) and 2% Triton X-100 were prepared in PBS, 20X of sodium chloride and sodium citrate solution (SSC) was diluted in MilliQ water to yield a working concentration of 2X.

1mg/mL propidium iodide (PI) solution was prepared in MilliQ water, DAPI stain stock solution (1mg/mL) was prepared in sterile water; further dilution was prepared in PBS to a concentration of 1μM/mL

5.2.3 Cell culture

The COV434 human ovarian granulosa cancer cell line was maintained in DMEM/F12 supplemented with 10% FCS and 1% of 10,000 units/mL penicillin + 10mg/mL streptomycin. The OVCAR-3 human epithelial ovarian adenocarcinoma cell line (ATCC, USA) was maintained in RPMI media, supplemented with 20% FCS, 5μg/mL insulin, and 1% v/v of 10,000 units/mL penicillin + 10mg/mL streptomycin. The MCF-7 human epithelial breast adenocarcinoma cell line and the T47D human epithelial breast ductal carcinoma cell line were obtained from the America Type Culture Collection (ATTC) and maintained in RPMI medium supplemented with 10% FCS and 1% v/v of 10,000 units/mL penicillin + 10mg/mL streptomycin. Media in each culture flask were replaced every 2-3 days and each cell line was subcultured twice a week. Cell culture flasks containing 80% confluent cells in exponential growth phase with fewer than 25 passages were used for all experiments.

5.2.4 Effect of doxorubicin, 4-hydroperoxycyclophosphamide, α or γ tocopherol on cell viability

MCF-7, T47D, OVCAR-3 or COV434 cells (20,000 cells per well) were exposed to 100μL of staurosporine, chemotherapeutics or tocopherols for 24h. Concentrations of chemotherapeutics and γToc were chosen based on the effective

concentration that killed 25% of MCF-7 cells (EC25) (Table 5-1). A extended dose response of *αToc* did not yield a cytotoxic concentration that could be used in this experiment; therefore the highest concentration (100μM) was used.

5.2.5 Crystal violet cell viability assay

Cell viability was determined using the crystal violet (CV) assay. Media containing test agents were replaced with 50μL of crystal violet stain (0.5%). Cells were stained for 10min, and then rinsed with demineralised water to remove any excess stain. Cells were left to air dry overnight. 60μL destain solution of 33% acetic acid was added. After 10min, absorbance was read at 570nm with correction at 630nm (Reid, Lang et al. 2015). The numbers of viable cells after exposure to test agents were determined by a comparison with a CV standard curve using densities of 0-80,000 cells per well (R^2 = 0.99) generated for the each replicate experiment.

Single agents	Concentrations (μM)
Dox	1.21
4-Cyc	21.23
αToc	100
γToc	35.1

Combined agents	Concentrations (μM)
Dox + 4-Cyc	1.21 (Dox) + 21.23 (4-Cyc)
Dox + 4-Cyc + αToc	1.21 (Dox) + 21.23 (4-Cyc) + 100 (αToc)
Dox + 4-Cyc+ γToc	1.21 (Dox) + 21.23 (4-Cyc)+ 35.1 (γToc)

Table 5-1Concentrations of chemotherapeutics and tocopherols.

Dox: Doxorubicin, 4-Cyc: 4-hydroperoxycyclophosphamide, αToc: α-Tocopherol, γToc: γ-Tocopherol.

5.2.6 Effect of doxorubicin, 4-hydroperoxycyclophosphamide, α or γ tocopherol on cell death

5.2.6.1 Caspase -3, -7 Activation

MCF-7, T47D, OVCAR-3 or COV434 cells (10,000 cells per well) were added to white 96-well microplates. After a 24h adherence period, cells were exposed to 100μL of chemotherapeutics or tocopherols (Table 5-1) for a further 24h, after which 50μL of test reagents were replaced with 50μL of luminescent Caspase-Glo® 3/7 reagent. Luminescence was recorded after 1h incubation at room temperature. A blank reaction (Caspase-Glo® 3/7 Reagent, vehicle and cell culture medium without cells) was used to measure background luminescence. Blank values were subtracted from experimental values. Negative controls (Caspase-Glo® 3/7 Reagent and vehicle-treated cells in medium) were included for determining the basal caspase activity of the cell culture system. Staurosporine was included as a positive control for caspase activation (Belmokhtar, Hillion et al. 2001). Each concentration of chemotherapeutics was examined in three replicate wells and the experiment was repeated on 3 separate occasions (n=3) for each of the four cell types.

5.2.6.2 Apoptosis detection by DeadEnd fluorometric TUNEL

MCF-7, T47D, OVCAR-3 or COV434 cells (30,000 cells per well) were added to Nunc Lab-Tek II – CC2 chamber slides (Promega, Australia). After an initial 24h adherence period, cells were exposed to 300μL of chemotherapeutics or tocopherols

(Table 5-1) and incubated for further 24h. Test agents were removed and cells were fixed by adding 4% paraformaldehyde in 1X PBS for 25min at 4°C. After rinsing cells with 1X PBS, cells were permeabilized with 0.2% Triton X-100 solution in 1X PBS. The positive control slide was prepared by adding 100µL of DNase I. Cells were treated with 50µL of recombinant terminal deoxynucleotidyl transferase (rTdT) incubation buffer containing: equilibration buffer, nucleotide mix and rTdT enzyme. Cells were incubated in a humidified chamber at 37°C for 1h, protected from light then rinsed in 2X sodium chloride (0.3mM) and sodium citrate (0.03mM) (SSC) solution for 15min at room temperature. 1µg/mL propidium Iodide (PI) solution was used as a counter stain for 15min in the dark. After rinsing with 1X PBS slides were mounted with buffered glycerol.

5.2.6.3 DAPI staining of cells to detect apoptotic nuclei

MCF-7, T47D, OVCAR-3 or COV434 cells (30,000 cells per well) were added to Nunc Lab-Tek II – CC2 chamber slides (Promega, Australia). After an initial 24h adherence period, cells were exposed to 300µL of chemotherapeutics, tocopherols (Table 1) or staurosporine (positive control) and incubated for further 24h. Cells were then fixed by adding 4% paraformaldehyde in 1X PBS for 25min at 4°C. After rinsing cells with 1X PBS, slides were incubated with DAPI stain prepared in sterile 1X PBS at a working concentration of 1µg/mL for 30min in the dark at room temperature. After rinsing with 1X PBS, Slides were mounted with buffered glycerol.

5.2.6.4 Fluorescence microscopy

TUNEL and DAPI labelled cells were examined by fluorescence microscopy using fluorescence microscope (Olympus AX70). TUNEL labelled cells were observed with filter Chroma 31001 at excitation 450-495nm, Dichroic 505 and emission 515-555nm for the green fluorescein of TUNEL, and Chroma 31002 at excitation 515-550nm, Dichroic 565 and emission 575-615nm for the red fluorescence of the PI staining. DAPI labelled cells were examined with filter Chroma 31000 at excitation 340-380nm, Dichroic 400 and emission 435-485nm. Four digital images of each well were taken at 20X magnification, TUNEL positive and negative cells were counted and the percentage of apoptotic cells was calculated. Experiments was repeated in 3 separate occasions (n=3) for each of the four cell types.

5.2.7 Statistical analysis

A one-way ANOVA with Tukey HSD post-hoc was performed to examine the effect of reagent exposure on cell viability and caspase -3, -7 activation. Effect of staurosporine in caspase -3, -7 activation, was assessed in an independent t-test comparison with Levene's test for homogeneity of variance. These statistical analyses were performed using SPSS statistics software (V22.0 IBM, Australia). Statistical significance was set at $p \leq 0.05$. All experiments were performed as three independent replicates, and all data expressed as mean ± standard deviation.

5.3 Results

MCF-7 (20,000 cells per well) were pre-cultured for 24h, and then exposed to 1µM staurosporine for 24h. This inducer of apoptosis significantly reduced MCF-7 cell viability from 36000±3500 to 5800±1900 cells/well but did not induce apoptosis via Caspase -3, -7 activation (Fig 5.1). The same concentration of staurosporine significantly decreased T47D cell viability and increased the levels of caspase -3, -7 activity. Both ovarian cancer cell lines responded to 1µM staurosporine by significantly increasing levels of caspase -3, -7 activity (Fig 5.1). However, the response of OVCAR-3 cells to staurosporine was lower ($p<0.001$) than the response of COV434 or T47D cells.

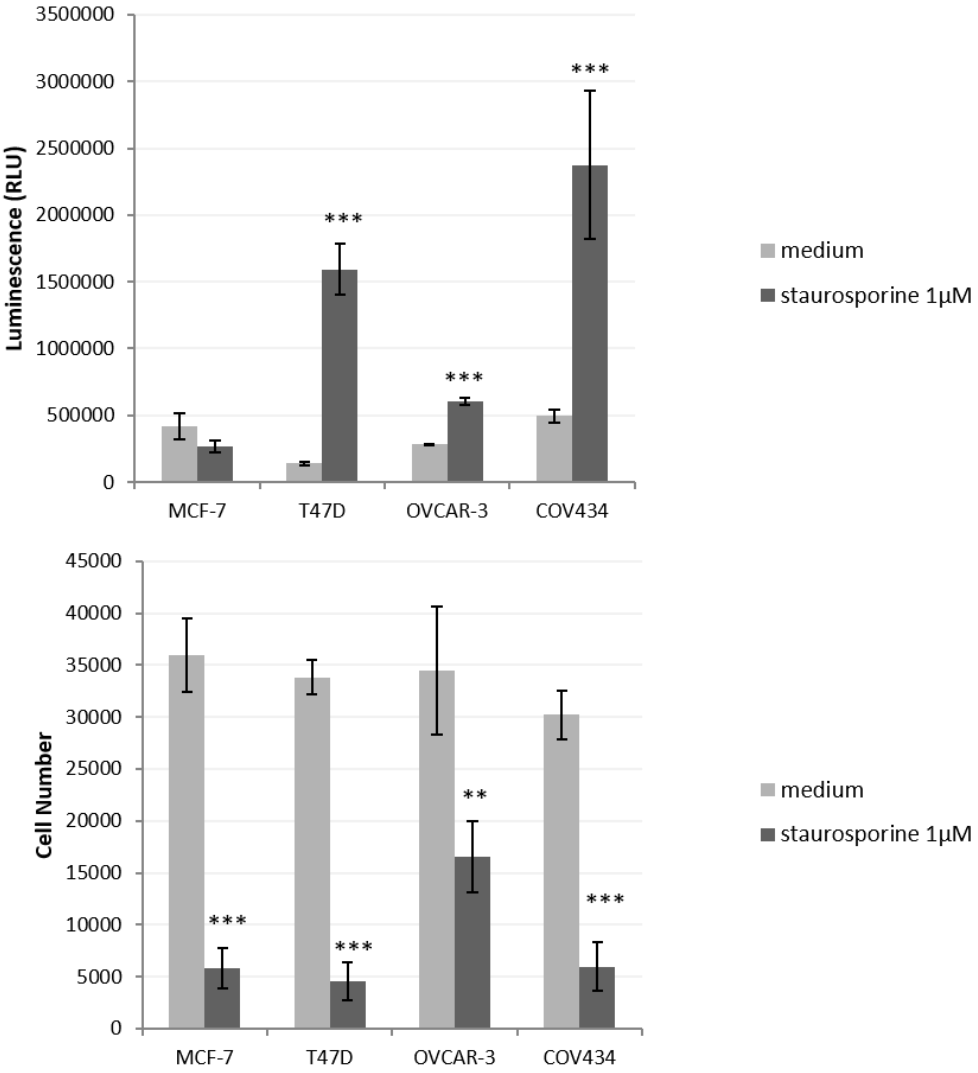


Figure 5.1 Effect of staurosporine on Caspase -3, -7 activity and cell viability.

MCF-7, T47D, OVCAR-3, and COV434 cells were exposed to 1µM staurosporine for 24h then exposed to the luminescent Caspase-Glo® 3/7 reagent. Luminescence was recorded after 1h incubation at room temperature. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data were subjected to independent t-test comparison with Levene's test for homogeneity of variance. Significant difference from medium control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.0001$.

Exposure to the MCF-7 derived EC25 values of Dox or 4-Cyc significantly decreased the cell viability of T47D, OVCAR-3 and COV434 (Fig 5.2). MCF-7 derived EC25 values of Dox reduced the numbers of viable T47D cells to 22500±780

cells/well; COV434 to 20000±3800 cell/well and OVCAR-3 to 25800±4700 cells/well. 4-Cyc was cytotoxic towards COV434 cells (23000±1900 cells/well), and reduced the cell numbers of T47D cells to 24000±1800 cells/well and OVCAR-3 cells to 21000±7100 cells/well.

Exposure to the combination of Dox and 4-Cyc did not significantly increase the cytotoxic effect of the single agents in any of the cell lines tested. Exposure to αToc (Fig 5.3) had no effect on cell viability in any of the cell lines used whereas γToc significantly decreased MCF-7 (25000±4700 cells/well) and T47D (24000±3700 cells/well) cell viability but had no effect on OVCAR-3 or COV434 cells. Although γToc was cytotoxic to breast cancer cells as a single agent, it did not increase the cytotoxicity of the combined Dox + 4-Cyc regimen (Fig 5.3).

No significant increase in caspase -3, -7 activity was observed in either MCF-7 or T47D breast cancer cells after 24h exposure to Dox, 4-Cyc, α or γToc. Similarly, the combination of Dox and 4-Cyc did not significantly increase caspase -3, -7 activation (Fig 5.2 a-b). In OVCAR-3 cells, although Dox had no effect on caspase -3, -7 activity (Fig 5.2c), 4-Cyc and the combination of Dox and 4-Cyc, caused a significant increase (Fig 5.2c). In COV434 cells, each of Dox and 4-Cyc caused a significant increase in caspase -3, -7 activity (Fig 5.2d). Luminescence values after exposure to the combination of Dox and 4-Cyc were not significantly different from Dox as a single agent, but were significantly higher than values obtained after exposure to 4-Cyc alone (Fig 5.2d).

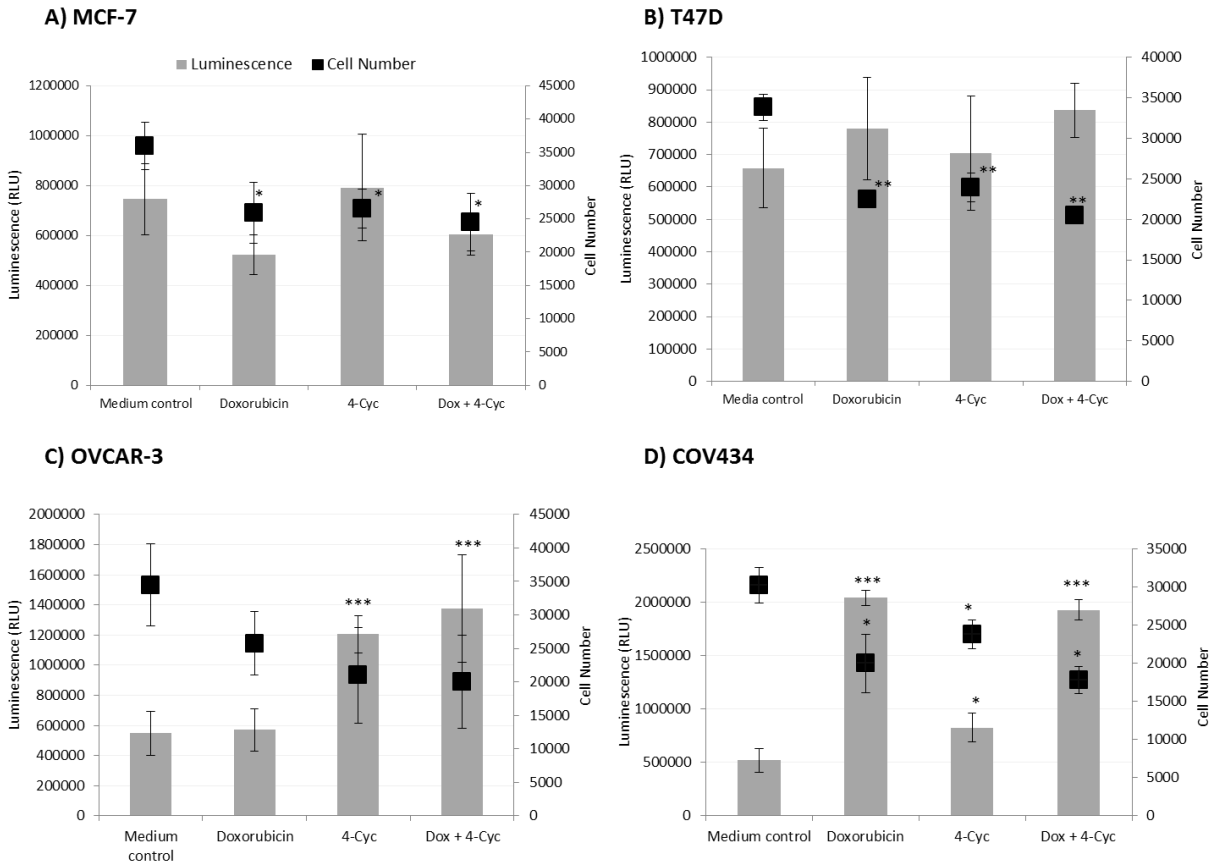


Figure 5.2 Effect of chemotherapeutics on Caspase -3, -7 activity and cell viability.

A) MCF-7, B) T47D, C) OVCAR-3, and D) COV434 cells were exposed to Dox (EC25), 4-Cyc (EC25) or a combination of Dox (EC24) + 4-Cyc (EC25) for 24h then exposed to the luminescent Caspase-Glo® 3/7 reagent. Luminescence was recorded after 1h incubation at room temperature. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data were subjected to ONE-way ANOVA with Tukey post-hoc. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ significant difference from medium control.

Exposure to either α or γToc did not induce caspase -3, -7 activation in OVCAR-3 (Fig 5.3c). In COV434 cells however, γToc but not αToc caused a significant increase in caspase -3, -7 activity compared to vehicle (DMSO) control (Fig 5.3d).

Adding α or γToc to the combination of Dox and 4-Cyc had no effect on caspase -3, -7 activity in any of the cell lines tested compared to the combination of Dox and 4-Cyc.

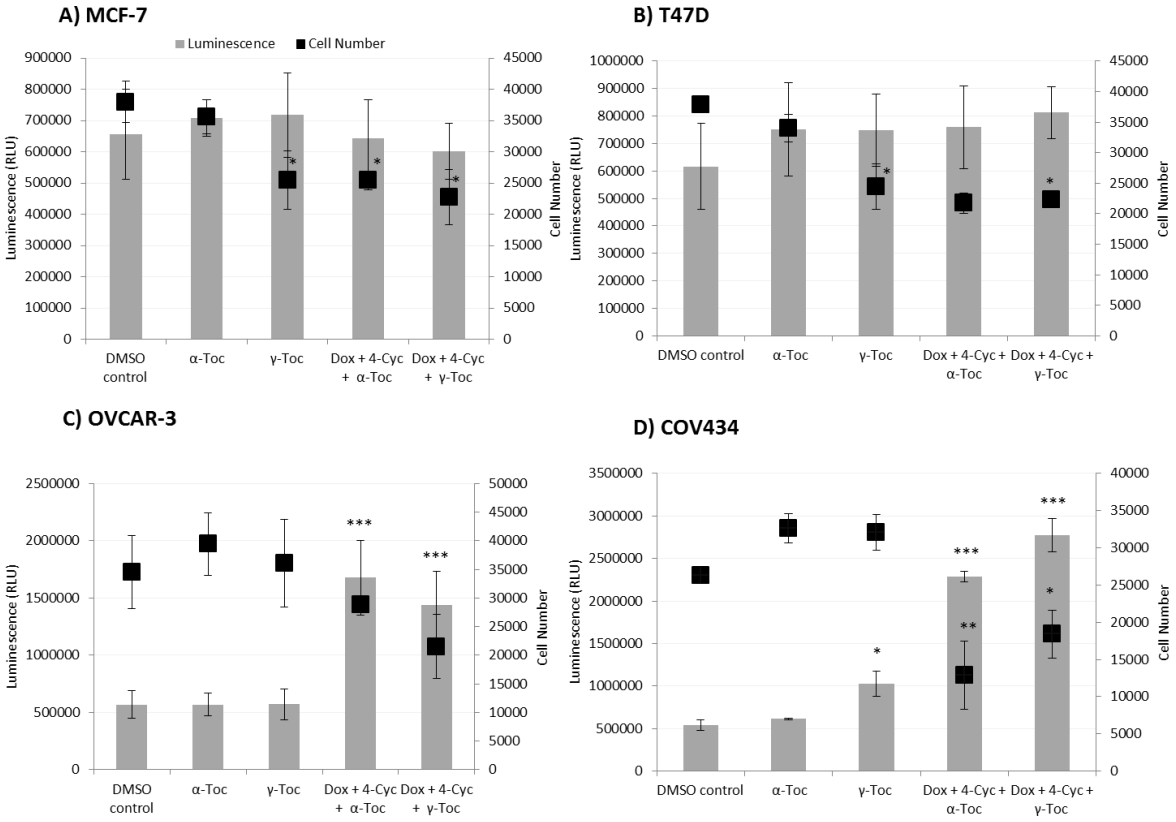


Figure 5.3 Effect of chemotherapeutics and tocopherols on Caspase -3, -7 activity and cell viability.

A) MCF-7, B) T47D, C) OVCAR-3, and D) COV434 cells were exposed to αToc (100μM), γToc (EC25), a combination of Dox (EC25) and 4-Cyc (EC25) and αToc (100μM), or Dox (EC25) and 4-Cyc (EC25) and γToc (EC25) for 24h then exposed to the luminescent Caspase-Glo® 3/7 reagent for 1h. Cell viability was assessed by a crystal violet assay, in which cell number was obtained by comparison with a standard curve. Means ± SD of 3 independent experiments shown. Data were subjected to One-way ANOVA with Tukey and Bonferroni post-hoc. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ significant difference from DMSO vehicle control.

The percentage of TUNEL-positive (apoptotic) cells after exposure to 1μL DNase I (positive control) was 98-100% in all cell lines (Fig 5.4). Cells in medium

alone had fewer than 2% apoptotic cells in all the cell lines tested; similar results were obtained in vehicle (DMSO) – treated cells.

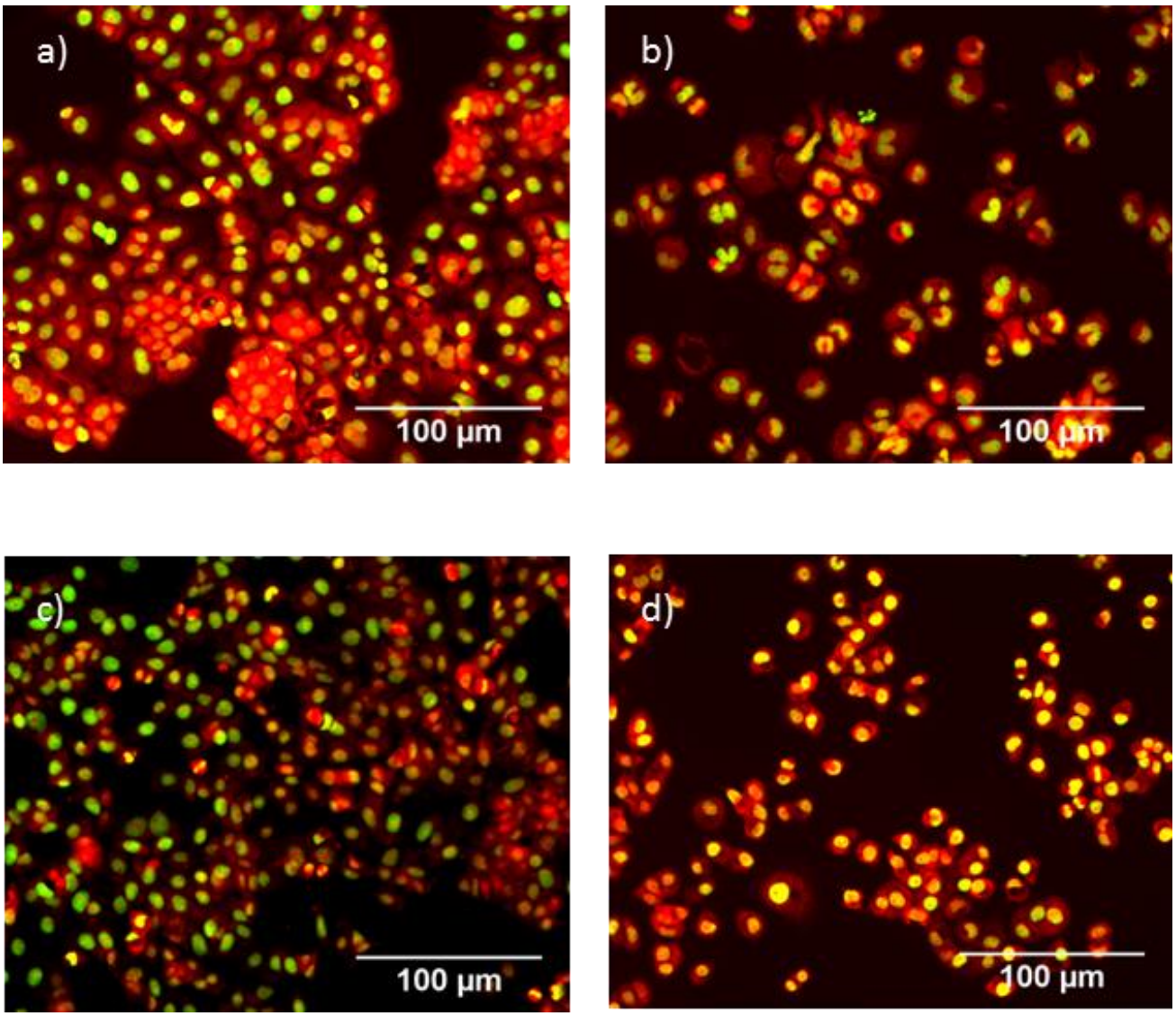


Figure 5.4 TUNEL staining after exposure to 1µM DNase.

Pictures represent overlaid apoptotic stain (green-yellow), and nuclear stain (red) photographed on a fluorescence microscope (Olympus AX70) at 200X magnification. **a)** MCF-7, **b)** T47D, **c)** OVCAR-3, and **d)** COV434 cells were assessed for apoptosis after exposure to DNase I (1µM). TUNEL positive nuclei due to DNA fragmentation (green/yellow spots) in cells indicate apoptosis.

Doxorubicin caused apoptosis, as observed by TUNEL-labelled DNA fragmentation, in 14±2% and 11±3% of MCF-7 and T47D cells, respectively (Fig

5.5). Similarly, increasing levels of DNA fragmentation were observed in OVCAR-3 and COV434 cells when exposed to MCF-7 derived EC25 values of Dox. 4-Cyc – related apoptosis percentages were between 10-25% in all cell lines (Fig 5.5, 5.6).

Comparable results were observed in DAPI stained cells. After exposure to doxorubicin or 4-Cyc, fewer than 20% of the cells showed signs of apoptosis with condensed and fragmented DNA in all cell lines used (Fig 5.7)

Percentage of apoptotic cells (Fig 5.6) after exposure to the combination of doxorubicin and 4-Cyc was not significantly different to the percentage of apoptotic cells after treatment with either doxorubicin or 4-Cyc as single agents as shown by both TUNEL (Fig 5.5, 5.6) and DAPI results (Fig 5.7).

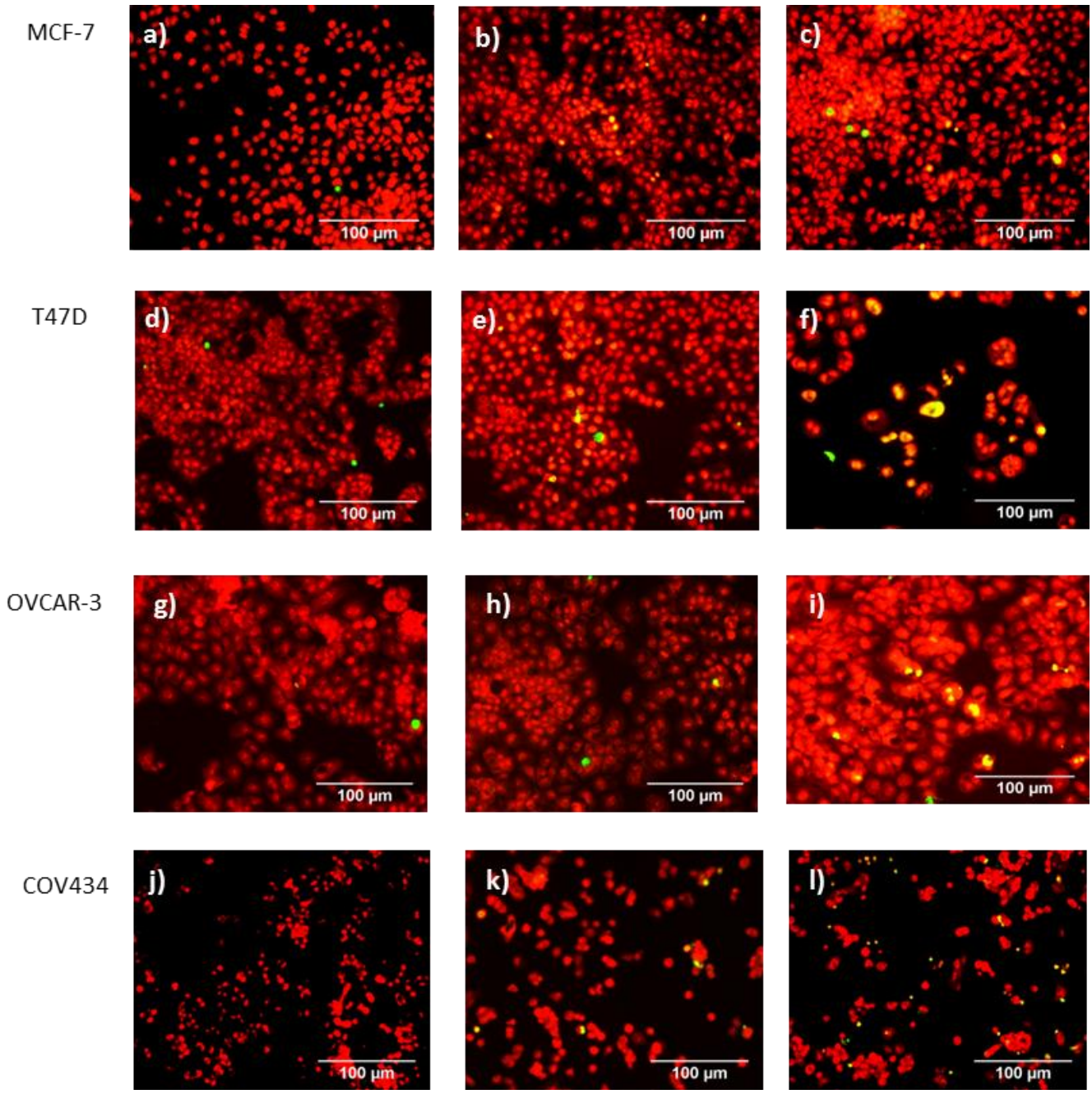


Figure 5.5 TUNEL staining after exposure to chemotherapeutics and/or tocopherols.

Pictures represent overlaid apoptotic stain (green-yellow), and nuclear stain (red) photographed on a fluorescence microscope (Olympus AX70) at 200X magnification. **a)** control MCF-7 cells, **b)** MCF-7 cells after 24h exposure to Dox **c)** MCF-7 cells after 24h exposure to a combination of Dox + 4-Cyc **d)** control T47D, **e)** T47D cells after 24h exposure to 4-Cyc, **f)** MCF-7 cells after 24h exposure to a combination of Dox + 4-Cyc, **g)** OVCAR-3 after 24h exposure to 0.8% DMSO in medium, **h)** OVCAR-3 cells after 24h exposure to αToc, **I)** OVCAR-3 cells after 24h exposure to a combination of Dox + 4-Cyc + αToc, **j)** COV434 after 24h exposure to 0.8% DMSO in medium **k)** COV434 cells after 24h exposure to γToc, **l)** OVCAR-3 cells after 24h exposure to a combination of Dox + 4-Cyc + αToc. TUNEL positive nuclei due to DNA fragmentation (green/yellow spots) in cells indicate apoptosis.

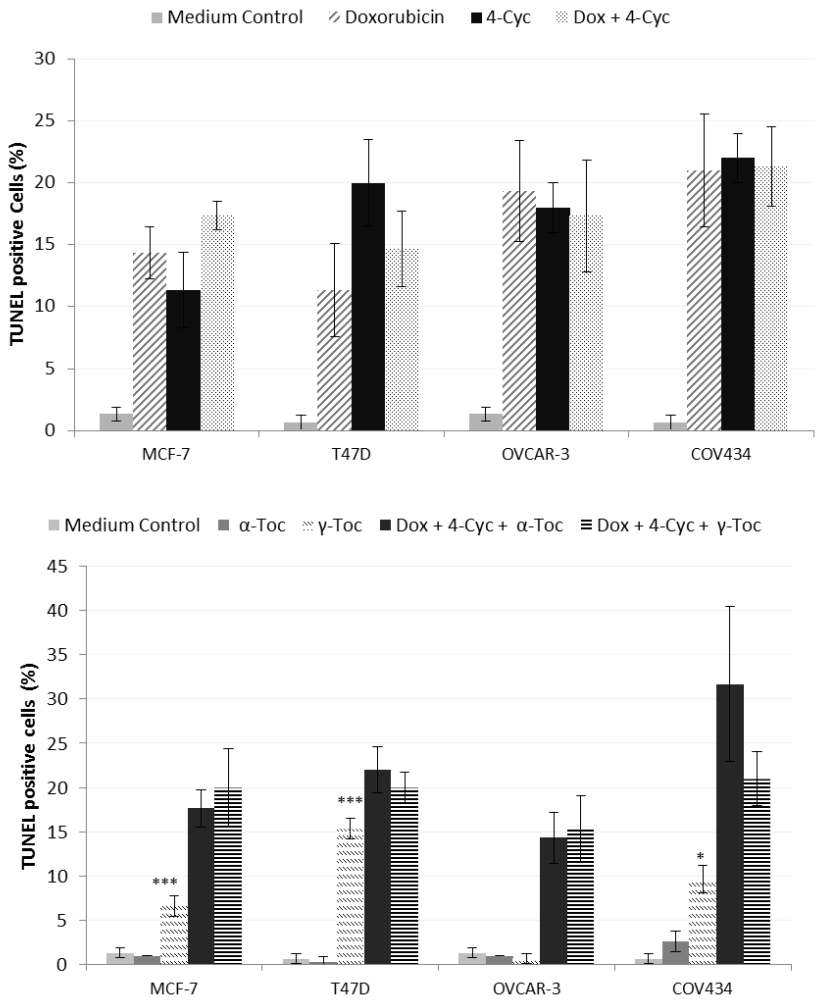


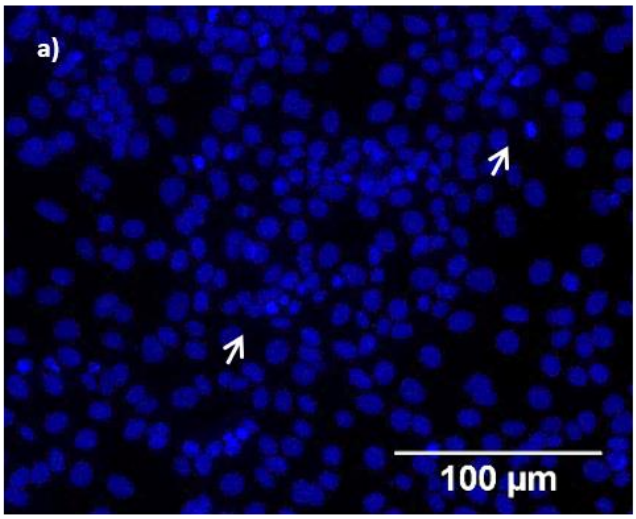
Figure 5.6 Apoptosis caused by exposure to chemotherapeutics and/or tocopherols.

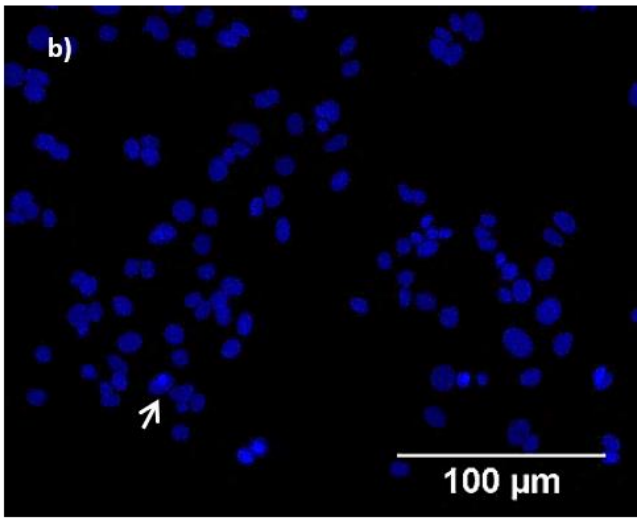
MCF-7, T47D, OVCAR-3, and COV434 cells were exposed to **a)** Dox, 4-Cyc, a combination of Dox and 4-Cyc, or **b)** αToc, γToc, a combination of Dox and 4-Cyc and αToc, or Dox and 4-Cyc (EC25) and γToc for 24h. Cells were stained by TUNEL. Total number of cells and TUNEL positive cells were counted and the percentage of apoptotic cells was calculated. Means ± SD of 3 independent experiments shown. Data were subjected to One-way ANOVA with Tukey post-hoc. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ significant difference from control.

αToc did not induce apoptosis in any of the cell lines and percentages of apoptotic cells after exposure to 100μM αToc were 1-3% in all cell lines (Fig 5.6). On the other hand, γToc induced apoptosis in MCF-7 (7±2%); T47D (15±1%) and COV434 cells (10±2%); but had no effect on OVCAR-3 cells (1±0.5%) (Fig 5.6).

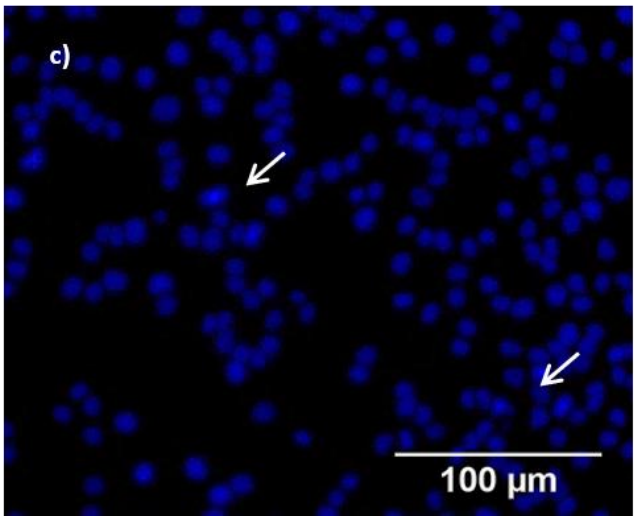
The addition of α or γ-tocopherol to the combination of Dox and 4-Cyc had no effect on the number of TUNEL-positive cells; no significant differences in the percentage of apoptotic cells were found between the combination of doxorubicin and 4-Cyc and α or γ-tocopherol supplemented regimens.

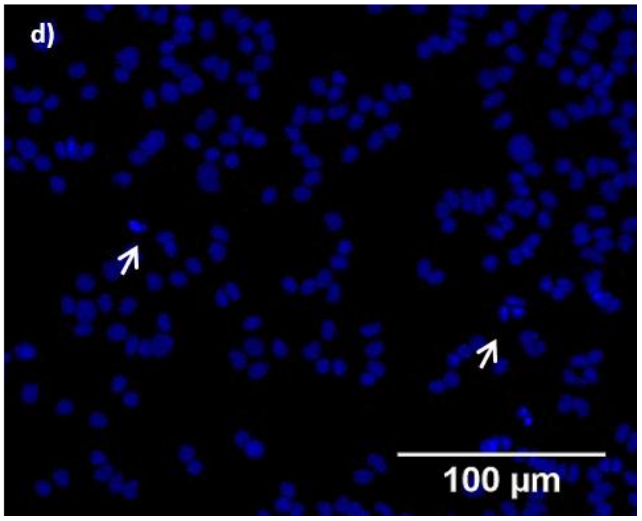
MCF-7





T47D





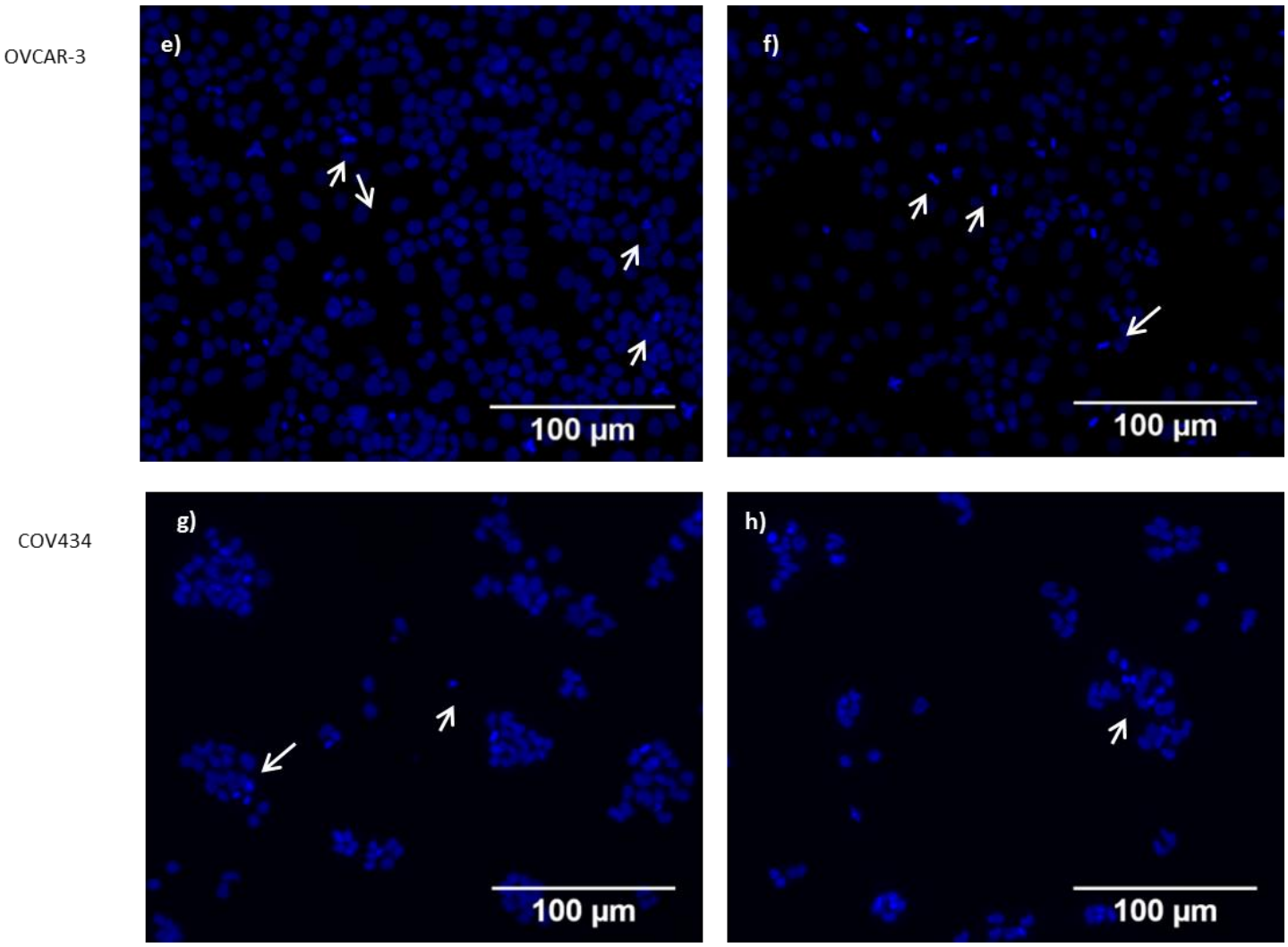


Figure 5.7 DAPI staining after exposure to chemotherapeutics and tocopherols

Staining of DNA with DAPI was examined and photographed on a fluorescence microscope (Olympus AX70) at 200X magnification. **a)** MCF-7 cells after 24h exposure to 0.8% DMSO in media, **b)** MCF-7 cells after 24h exposure to αToc **c)** MCF-7 cells after 24h exposure to a combination of Dox + 4-Cyc + αToc, **d)** T47D cells after 24h exposure to 0.8% DMSO in media. Arrows showing condensed and fragmented DNA.

5.4 Discussion

Apoptosis induction is a key mechanism by which many chemotherapeutic agents kill cancer cells (Debatin 2004). Several reports have found that anticancer drugs target cancer cells by inducing expression of death receptors (Friesen, Herr et

al. 1996, Friesen, Fulda et al. 1997, Kasibhatla, Brunner et al. 1998). Other reports have suggested that chemotherapeutics induce apoptosis by release of cytochrome c (Kaufmann and Earnshaw 2000, Elmore 2007). Although the underlying mechanism of cell death upon exposure to chemotherapeutic is likely to depend on the type of drug, most apoptosis signalling pathways result in the activation of caspases (Debatin 2004). In this study, we evaluated the effect of chemotherapeutics Dox and 4-cyc as single agents and as a combined regimen, on apoptosis-associated caspase activity and DNA fragmentation in breast cancer cell lines and ovarian cancer cell lines. Results showed that the four cells lines displayed different sensitivities to the cytotoxic effect of chemotherapeutics and tocopherols ant that apoptosis signalling pathways may be different in breast cancer and ovarian cells.

Breast cancer cell lines responded to both chemotherapeutic agents by increasing cell death. MCF-7 derived EC25 concentration of Dox reduced MCF-7 and T47D cell viability significantly but no significant caspase -3 or -7 activity (indicative of extrinsic pathway) was detected in either of the breast cancer cell lines. Although neither T47D nor MCF-7 increased caspase 3,-7 activity the presence of DNA fragmentation (TUNEL) and decreases in cell viability after exposure to both Dox and 4-Cyc, suggest that cell death occurred in the absence of caspases -3,-7 and that other caspases or mechanisms may be involved. MCF-7 cells are deficient in caspase-3 expression because of a deletion mutation in their caspase-3 gene (Makin and Dive 2001); however studies have found that T47D cells undergo apoptosis via the extrinsic pathway (Keane, Ettenberg et al. 1999, Mooney, Al-Sakkaf et al. 2002) which suggests that caspase -3, -7 levels should have been detected in these cells. The time required to detect apoptotic events may vary

depending on the cell line (Farfan, Yeager et al. 2004). Jurkat cells exposed to anti-Fas monoclonal antibody (mAb) showed that caspase -8 activity peaked after 3h whereas elevated caspase -3,-7 levels were observed after 7 hour post treatment and DNA fragmentation after 9h (Farfan, Yeager et al. 2004). Therefore, measuring caspase -3,-7 in T47D earlier during exposure might yield different results.

Although caspase-dependant apoptosis plays an essential role in the mediation of cancer cell death via chemotherapy (Debatin 1999, Lowe and Lin 2000, Solary, Droin et al. 2000, Herr and Debatin 2001), other mechanisms have also been taken into consideration. 'Non classical apoptosis', or nonapoptotic mechanisms, such as necrosis could also contribute to chemotherapy-induced cell death. While the signalling pathways and molecules involved in these alternative forms of cell death were not examined in this study, non-caspase proteases such as calpains and cathepsins, Bax or Bax-like molecules, and AIF or endonuclease G may be involved (Borner and Monney 1999, Finkel 1999, Hengartner 2000, Shrubsole, Jin et al. 2001, Wyllie and Golstein 2001).

γToc but not αToc, was cytotoxic to both breast cancer cells as a single agent. γToc increased the percentages of TUNEL-labelled cells but no activation of caspase -3, -7 was observed after exposure. Yu et al., (2008) found that γ-tocopherol induced apoptosis in MCF-7 breast cancer cells through activation of caspase -8 and -9 and in another study γ-tocopherol caused apoptosis on breast cancer cells via stimulation of TNF death receptors (Yu, Park et al. 2008).

Of all the cell lines use this study, COV434 cells were the most sensitive to Dox. In the present study caspase -3,-7 activity was significantly higher in COV434 cells exposed to Dox. The percentage of TUNEL-labelled apoptotic cells after Dox exposure was also higher in COV434 (21±4.6%) than in the other cell lines. COV434

cells were also sensitive to the cytotoxic and apoptosis-inducing effects of 4-Cyc. Zhang et al (2000) showed that COV434 expresses several apoptosis-associated genes, including Nip1, Nip2, Bak, Bad (protein but not mRNA), Bag, Bax, Casp-2 (ICH-1L), Bcl-Xl and Casp-4 (Zhang, Vollmer et al. 2000). Caspase3 activation, PARP cleavage and TUNEL positive staining were also observed after H_2O_2 treatment (Yang, Xie et al. 2017). Similar results were found by Tsai Turton et al. (2007), who described that treatment of COV434 granulosa cells with 4-Cyc was associated with caspase 3 activation (Tsai-Turton, Luong et al. 2007). Exposure to γToc induced DNA fragmentation and Caspase -3 and -7 in COV434 cells, even though the numbers of viable COV434 cells after exposure to γToc were not significantly lower than vehicle control. Apoptosis pathway is a multistep process that occurs by stages (Elmore 2007). Caspase activation is believed to trigger DNA fragmentation and is considered an early apoptotic event, whilst loss of cell adherence is a sign of late apoptosis. The crystal violet assay stains cells attached to the plates, but cannot distinguish viable cells of early apoptotic cells. It is possible therefore that measuring cell viability at different time point could capture γToc cytotoxicity.

The results from the present study suggest that γToc triggers apoptosis in COV434 cells. Jiang et al (2004) found that γToc caused apoptosis via release of cytochrome c in LNCaP prostate cancer cells; but did not affect the viability of normal PrEC prostate epithelial cells (Jiang, Wong et al. 2004). Although COV434 cells are a good *in vitro* model for normal healthy granulosa cells, the cell line was established from a solid primary tumour (Zhang, Vollmer et al. 2000) and it may differ from physiologically normal granulosa cells *in vivo*. It is important to acknowledge that this

cell line has limited extrapolation to *in vivo* setting and therefore we recommend further studies to evaluate the potential of γToc in non-cancer ovarian granulosa.

The MCF-7 derived EC25 values of 4-Cyc increased caspase activity significantly in OVCAR-3 cells, but exposure to Dox did not cause any significant changes in caspase activity. Studies have found that the OVCAR-3 cell line is resistant to Dox and has an abnormal karyotype (Hamilton, Young et al. 1983) It is possible that OVCAR-3 resistance to Dox may have been mediated by changes to caspase genes or expression. Devarajan (2002) found that caspases (Devarajan, Sahin et al. 2002) and d(Shen, Wang et al. 2010) Downregulation of caspases -8 and -10 may contribute to the pathogenesis of choriocarcinoma (Shen, Wang et al. 2010) Hence, the ability of chemotherapeutics to trigger caspase activation and therefore apoptosis may be an important determinant of how sensitive or resistant cancer cells are to the therapy.

Exposure to the combination of Dox and 4-Cyc did not significantly increase the cytotoxic effect of the single agents in any of the cell lines tested. Both Dox and 4-Cyc have multiple mechanisms of action and can induce damage at a number of different loci (Keane, Ettenberg et al. 1999, Sharifi, Barar et al. 2015). The synergistic effect of Dox and 4-Cyc was demonstrated *in vivo* by Corbett et al. (Corbett, Griswold et al. 1975). It has also been hypothesised that combining two drugs with different cytotoxic mechanisms of action could potentially reduce adverse effects without sacrificing anti-cancer efficacy (Pritchard, Lauffenburger et al. 2012). The results from this study however suggest that the two chemotherapeutics may have some cytotoxic mechanisms of action in common. Although γToc was cytotoxic to both breast cancer cell lines and increased the percentage of TUNEL-labelled COV434 cells, the addition of α or γToc to the combination of Dox and 4-Cyc had no

effect on the percentages of TUNEL-labelled cells or caspase activity in any of the cell lines used. The tocopherols neither increase nor decreased apoptosis caused by the combined Dox and 4-cyc regimen. Synergism between two or more drugs is not only dependant on the properties of each agent but also the concentration of each in the combination (Tallarida, Kimmel et al. 1997, Tallarida and Raffa 2010), it is possible therefore that adjusting the dose of γToc changes the drug interaction and the combined effect.

The results from the study showed that MCF-7 cells and T47D cells had different apoptotic cell death pathways than OVCAR-3 and COV434 cells, γToc caused apoptosis in both breast cancer cell lines and in COV434 cells, but the addition of γToc to the combined Dox + 4-Cyc regimen did not increase the cytotoxicity of the chemotherapeutics.

Although we found that the combination of Dox and 4-Cyc induces apoptosis through the activation of caspase –3, -7 in OVCAR-3 and COV434 cells, the *in vitro* role of caspases in breast cancer cell death was not elucidated; therefore, more apoptosis signalling components that measure multiple points in the pathway need to be studied and other non-apoptotic mechanism of cell death must be evaluated. Future studies should also investigate the biology that determines individual responses to cytotoxic therapies in order to assess the interaction between the chemotherapeutic drugs and γToc.

6. CHAPTER VI: DISCUSSION

Clinical relevant doses - 24h Data		MCF-7 cells		KGN cells	
		Mean	Std. Dev	Mean	Std. Dev
Dox	5μM	47.3	22.2	64.6	2.8
	10μM	27.9	8.8	71.7	14.2
	25μM	19.5	5.4	67.5	12.3
4-cyc	0.5μM	101.4	10.0	127.0	14.4
	1μM	104.4	9.1	129.2	14.6
	2.5μM	94.9	13.5	119.7	14.2
αToc	50μM	101.8	11.1	107.9	17.3
	75μM	97.2	11.8	120.8	9.3
	100μM	96.4	10.4	109.2	13.5
γToc	50μM	103.0	4.6	129.6	16.6
	75μM	80.4	8.7	131.2	22.5
	100μM	47.9	0.5	113.9	16.2
Dox + 4-cyc	Dox 10μM + 4-cyc 1μM	30.6	7.4	65.7	4.1
	Dox 25μM + 4-cyc 2.5μM	15.9	5.6	60.6	7.1
Dox + 4-cyc + αToc	Dox 10μM + 4-Cyc 1μM + αToc 75μM	34.7	6.3	85.8	15.0
	Dox 25μM + Cyc 2.5μM + αToc 75μM	22.7	7.5	76.6	9.0
Dox + 4-cyc + γToc	Dox 10μM/Cyc 1μM + γToc 75μM	11.7	3.0	77.4	13.7
	Dox 25μM + Cyc 2.5μM + γToc 75μM	9.3	2.7	69.2	15.5

EC25 values		MCF-7 cells		T24D cells		COV434 cells		OVCAR-3 cells	
		Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev	Mean	Std. Dev
Dox		71.7	8.2	66.7	5.6	57.4	1.9	74.9	5.1
4-cyc		74.1	7.9	70.8	2.2	65.8	5.7	60.6	14.7
αToc		99.2	4.4	100.7	10.0	102.2	8.4	115.3	11.6
γToc		70.6	8.6	72.0	7.8	106.5	10.2	104.4	7.4
Dox + 4-cyc		69.7	13.7	70.0	11.6	50.3	8.4	56.8	11.1
4-cyc+ Dox + αToc		70.9	8.4	64.3	1.7	45.2	8.7	82.4	11.4
Dox + 4-cyc + γToc		62.9	8.6	66.2	4.2	59.3	5.0	61.7	5.9
ROS generation									
Dox	1h	102	7.42	105	9.82	82.5	6.67	149	17.5
	2h	129	8.09	128	14.2	82.7	6.64	156	16.9
	3h	138	13.1	143	18.3	97.3	7.19	173	16.7
	24h	865.13	14.98	772.46	87.43	321.59	44.13	846.17	59.62
4-cyc	1h	460	28	321	20.4	57.1	6.91	243	5.67
	2h	633	30.1	405	6.54	71	6.3	359	33.1
	3h	646	18.6	428	15.7	89.4	7.33	387	29
	24h	1290.3	78.6	1423.8	17.4	518.2	56.2	1159.6	141.1
αToc	1h	43.3	7.04	28.9	7.09	6.72	0.131	65.5	13.2
	2h	69.2	0.34	47.9	2.51	9.43	0.7455	78.5	9.27
	3h	79.3	6.27	65.1	4.07	9.28	2.6662	93.4	4.38
	24h	464.2	37.3	725.6	66.2	233.5	14.1	704.8	53.7
γToc	1h	49.8	7.677	17.2	14.73	8.93	1.469	71.3	29.45
	2h	70.7	10.57	38	14.71	12.8	18.79	72.2	15.68
	3h	79.1	16.12	53.1	14.45	14.4	0.925	88.4	18.65
	24h	446.5	33.3	627.9	62.2	240.9	34.1	681.9	65.4
Dox + 4-cyc	1h	383	29.8	312	50.8	90.4	3.55	200	43.9
	2h	492	45.8	388	48.3	98.7	10.9	291	46.1
	3h	510	68	415	58.7	122	8.97	322	26.6
	24h	1143.0	41.8	1387.3	161.7	550.5	88.7	1049.4	103.1
4-cyc+ Dox + αToc	1h	374	22.4	332	14.8	68	5.21	221	34.9
	2h	505	17.2	418	14.5	83.4	4.56	356	60.8
	3h	528	45.1	455	17.9	91.8	6.78	349	76.9
	24h	1124.0	48.8	1383.8	147.9	559.6	94.2	1115.9	84.3
Dox + 4-cyc + γToc	1h	376	13	277	24.7	57	6.04	183.1	6.96
	2h	512	8.99	350	13.8	58	24.6	276.2	25.4
	3h	535	17.5	374	20.5	72.6	9.93	304.3	35.4
	24h	1102.2	23.5	1190.2	73.5	461.4	24.4	1115.9	84.3
Caspase 3/7 results									
Dox		5.2E+05	4.8E+04	7.8E+05	1.6E+05	2.0E+06	6.9E+04	5.7E+05	1.4E+05
4-cyc		7.9E+05	4.9E+04	7.1E+05	1.8E+05	8.3E+05	1.3E+05	1.2E+06	1.2E+05
αToc		7.1E+05	5.7E+04	7.5E+05	1.7E+05	6.1E+05	7.5E+03	5.7E+05	1.0E+05
γToc		7.2E+05	1.3E+05	7.5E+05	1.3E+05	1.0E+06	1.5E+05	5.7E+05	1.3E+05
Dox + 4-cyc		6.0E+05	2.7E+04	8.4E+05	8.2E+04	1.9E+06	9.5E+04	1.4E+06	3.6E+05
4-cyc+ Dox + αToc		6.4E+05	1.2E+05	7.6E+05	1.5E+05	2.3E+06	6.5E+04	1.7E+06	3.3E+05
Dox + 4-cyc + γToc		6.0E+05	8.9E+04	8.1E+05	9.5E+04	2.8E+06	1.9E+05	1.4E+06	2.9E+05
TUNEL - apoptosis percentages									
Dox		14.3	2.1	11.3	3.8	21.0	4.6	19.3	4.0
4-cyc		11.3	3.1	20.0	3.5	22.0	2.0	18.0	2.0
αToc		1.0	0.0	0.3	0.6	2.7	1.2	1.0	0.0
γToc		6.7	1.2	15.3	1.2	9.7	1.5	0.7	0.6
Dox + 4-cyc		17.3	1.2	14.7	3.1	21.3	3.2	17.3	4.5
4-cyc+ Dox + αToc		17.7	2.1	22.0	2.6	14.3	2.9	14.3	2.9
Dox + 4-cyc + γToc		20.0	4.4	20.0	1.7	15.3	3.8	15.3	3.8

Table 6-1Summary table of results obtained from studies conducted during PhD project

6.1 Background and perspective

The combination of Dox and cyclophosphamide (AC) has been used as an adjuvant chemotherapy regimen for early-stage and advanced breast cancer in both premenopausal and postmenopausal women (Younis, Rayson et al. 2011, Ferlay, Soerjomataram et al. 2015, Yardley, Arrowsmith et al. 2017). However, AC causes premature ovarian failure and infertility (Morgan, Anderson et al. 2012). Clinical studies find reduced levels of reproductive hormones indicative of ovarian failure in cancer survivors, and *in vivo* studies have reported apoptosis in the proliferating follicular granulosa cells of the ovary (Morgan, Anderson et al. 2012), but there are currently no reports describing the cytotoxic effects of the combined regimen that is used to treat breast cancer patients on ovarian granulosa cells *in vitro* or *in vivo*. This PhD research project has examined the effect of the AC regimen on cytotoxicity, apoptosis and ROS production by human breast and ovarian cancer cell lines *in vitro.*

A related research theme in this PhD research project arose from the observation that histological features of doxorubicin-induced cardiomyopathy are similar to those caused by vitamin E deficiency (Myers, McGuire et al. 1977, Legha, Benjamin et al. 1982), and this observation suggested that both were a result of ROS-induced membrane lipid peroxidation. Vitamin E prevents the propagation of ROS (Lu, Xiao et al. 2010) and this led to the proposal that supplementation of chemotherapy with vitamin E might prevent doxorubicin-induced cardiotoxicity.

Early studies suggested that vitamin E decreased the toxicity of doxorubicin without reducing its effectiveness as chemotherapeutic agent (Myers, McGuire et al. 1976, Krivit 1979, Lubawy, Whaley et al. 1979, Herman and Ferrans 1983, Milei, Boveris et al. 1986, Geetha, Sankar et al. 1990). γToc has demonstrated anti-cancer

activity in variety of cancer models, including breast cancer (Klein, Thompson et al. 2011, Smolarek and Suh 2011, Gopalan, Yu et al. 2012), (Yu, Park et al. 2008). Previous studies found that γ-tocopherol increased the levels of pro-apoptotic proteins, whilst inhibiting expression of anti-apoptotic proteins *in vivo* (Smolarek and Suh 2011) and inhibited the proliferation of human breast cancer cells *in vitro* (Lee, Ju et al. 2009, Smolarek and Suh 2011). γ-tocopherol had anti-tumour activity in animal models of colon and prostate cancer (Smolarek and Suh 2011) and delayed the formation of breast cancer tumours in rodent models (Smolarek and Suh 2011). Therefore, this project explored the potential of γToc to increase the chemotherapeutic efficacy of Dox and 4-Cyc against breast cancer cells *in vitro* whilst reducing ROS-induced damage against ovarian granulosa cells.

6.2 Selection of reagent concentrations

Standard AC therapy for breast cancer involves the intravenous administration of Dox to a maximum dose of 60mg/m^2 followed by an infusion of cyclophosphamide up to 600mg/m^2 (Dees, O'reilly et al. 2000, Jones, Savin et al. 2006). These concentrations result in plasma concentrations of Dox 1.8±0.4μM Dox (Swenson, Bolcsak et al. 2003), and serum concentrations of 4-hydroxycyclophosphamide (cyclophosphamide metabolite) that range from 0.23 to 1.08 mg/h/m in a 24h period after administration. The serum concentrations of 4-hydroxycyclophosphamide are highest 2-4h after the administration of cyclophosphamide (Struck, Alberts et al. 1987), approximately 0.5ug/mL (0.02μM). Initially the doses selected for examination bracketed the clinical, *in vivo* therapeutic

serum concentrations of doxorubicin 1.8µM (Swenson, Bolcsak et al. 2003) and 4-hydroxycyclophosphamide 0.02µM (Struck, Alberts et al. 1987)

The cytotoxic effects of clinically relevant doses of Dox and 4-Cyc as single agents and as combined AC regimen were tested on breast cancer (MCF-7) and ovarian granulosa (KGN) cells. The *in vitro* exposure to 4-Cyc had no effect on MCF-7 breast cancer cells or KGN granulosa cells, even though the highest concentration used in the experiment (2.5uM) was two orders of magnitude higher than the plasma concentration of the pharmacologically equivalent metabolite (Struck, Alberts et al. 1987).

The pharmacokinetics of cyclophosphamide have been well characterised (Grochow and Colvin 1979, Moore 1991, de Jonge, Huitema et al. 2005), but much less is known about the kinetics of cyclophosphamide metabolites. The oxidation of 4-hydrocyclophosphamide can produce inactive metabolites (de Jonge, Huitema et al. 2005). Therefore, it was concluded that the clinically relevant dose of cyclophosphamide necessary to treat breast cancer patients might differ from the *in vitro* effective concentration of 4-hydroperoxycyclophosphamide (4-Cyc). The results from this study prompted the design of an experiment to determine the 24h EC25 values of Dox and 4-Cyc against the breast cancer MCF-7 cell line and to evaluate the potential synergistic effect between Dox and 4-Cyc. The EC25 of 4-Cyc against the MCF-7 breast cancer cells was 21.23µM which was much higher than that of its plasma concentration *in vivo* (0.2 µM) (Struck, Alberts et al. 1987); whilst the EC25 of Dox (1.21µM) was similar to the serum concentrations of Dox, 1.8µM.

An extended dose response study in MCF-7 showed that a very high 200µM concentration of αToc was cytotoxic, but only 105µM of γToc was effective at killing

50% of the MCF-7 cell population. This EC50 value was close to the highest concentration tested in the initial KGN study (100µM).

The T47D-derived EC50 values of Dox, 4-Cyc and γToc were not significantly different from the ones obtained for the MCF-7 cells, which led to the conclusion that the concentrations that reduced MCF-7 cell viability were supported by similar EC values obtained for another breast cancer cell line and could therefore be used to model the effects of breast cancer chemotherapy on ovarian cells.

6.3 Cell viability and cytotoxicity

Cell viability and cell death in *in vitro* studies are not absolutes, instead the terms tend to describe opposite ends of a spectrum. Cell viability assays measure different things; The MTT assay measures enzyme activity and the measures cell. These viability assays examine different parts of the spectrum of states a cell might display. It has been suggested that apoptotic bodies may contain intact cell organelles with active enzymes such as succinate dehydrogenase (Zhang, Chen et al. 2018), the enzyme activity measured in the MTT assay. In apoptosis, cells detach from each other and their culture substrates before rounding up and forming apoptotic bodies (Caccamo, Scaltriti et al. 2004). In these circumstances it would be expected that crystal violet viability (which measures adherence) would measure lower levels of viability than the MTT assay, but both might overestimate the number of viable cells, since apoptotic bodies containing active succinate dehydrogenase do not qualify as viable cells capable of undergoing mitosis

Apoptosis is a multistep process that includes early, mid and late-stage cellular events (Elmore 2007). Morphological changes begin with retraction of the cells (Elmore 2007) accompanied by release of cytochrome C and activation of

caspase -8 and -9 (Wong 2011). Mid-stage events involve the activation of caspase -3, 6 and -7 and PARP cleavage, cell shrinkage and activation of nucleases (Bratton, MacFarlane et al. 2000, Adrain and Martin 2001). Caspase-3 activation is believed to trigger membrane blebbing (Andrade, Crisol et al. 2010, Zhang, Chen et al. 2018), which represents the earliest step toward the formation of apoptotic body (Zhang, Chen et al. 2018). At a molecular level, caspase activation has also been linked to DNA fragmentation (Kitazumi and Tsukahara 2011). ICAD is cleaved by several caspases resulting in the release and activation of CAD from the DFF complex (Kitazumi and Tsukahara 2011). CAD is a DNA-specific endonuclease that induces double-stranded breaks and triggers DNA fragmentation.

It therefore appears that caspase -3, -7 activation is crucial for both DNA fragmentation and formation of apoptotic bodies, hence, levels of caspase activity should elevate before signs of further cell damage can be observed by TUNEL or DAPI. The crystal violet assay measures cell viability by detecting the number of cells attached to the well (Feoktistova, Geserick et al. 2016). Loss of cell attachment is a sign of late-stage apoptosis; therefore the percentage of apoptosis by TUNEL can be expected to correlate with the number of viable cells measured in the crystal violet assay. However, cell viability results determined by crystal violet were different from the DNA fragmentation and DAPI stained apoptotic body results. Only 14±2% of MCF-7 cells exposed to EC25 values of Dox showed signs of DNA fragmentation and apoptosis when measured in a TUNEL and DAPI assay, whereas around 28% of cells were dead when measured in a crystal violet assay. Comparison of these two datasets suggests that either a percentage of MCF-7 cells may have been killed by non-apoptotic pathways or that DNA fragmentation followed by loss of cell adherence occurred earlier than the 24h time point examined in the crystal violet

assay. Similar TUNEL and crystal violet results were obtained for T47D, COV434 and OVCAR-3 cells; suggesting that if TUNEL labelling had been applied earlier in the exposure, the number or DNA fragmented cells would have been higher while the percentage of cell death measured using crystal violet would have been lower.

Levels of caspase -3, -7 activity in both breast cancer cell lines did not correlate to the crystal violet cell viability but probably for different reasons as no detectable levels of caspase -3 or -7 were found in MCF-7 or T47D cells. MCF-7 cells are deficient in caspase-3 expression (Makin and Dive 2001) but studies have confirmed that T47D cells undergo apoptosis via the extrinsic pathway (Keane, Ettenberg et al. 1999, Mooney, Al-Sakkaf et al. 2002), suggesting that caspase -3 and -7 activation should have been detected in the T47D cells. Previous studies have shown that the time required to detect different apoptotic events often varies; Jurkat cells treated with anti-Fas monoclonal antibody (mAb) showed elevated levels of caspase 8 after only 3 hours post treatment, whereas caspase -3, -7 activity peaked after 7 hours and DNA fragmentation was observed after 9 hours post treatment (Farfan, Yeager et al. 2004). Measuring caspase activity after of before the optimal time period could have led us to the wrong conclusion that no signal was observed and there was no activation of caspase -3, -7; therefore it is possible that applying the assay earlier in the exposure period might have detected caspase -3, -7 activation in T47D but not in MCF-7 cells.

Apoptosis measured by TUNEL after exposure to γToc was observed in MCF-7 (7±2%); T47D (15±1%) and COV434 cells (10±2%); but had no effect on OVCAR-3 cells (1±0.5%). Caspase -3 and -7 activity was significantly increased after exposure to γToc in COV434 cells but not in the other three cell lines. Caspase -3, -7 activity in COV434 was higher after exposure to Dox than after exposure to 4-Cyc,

on the other hand OVCAR-3 showed a higher caspase -3, -7 activity after exposure to 4-Cyc.

The four cell lines displayed different sensitivities to the cytotoxic activities of the test agents used in this project (Dox, 4-Cyc, αToc, γToc, and the combination of Dox and 4-Cyc with αToc or γToc). Exposure to the MCF-7 derived EC25 values of Dox were expected to cause 25% cell death across all cell lines. As predicted, it reduced MCF-7, T47D and OVCAR-3 cell viability to 72±8, 67±6, and 75±5%, respectively, but it was more cytotoxic to the granulosa cells. Nearly 50% of COV434 cells were killed after exposure to the same concentration of Dox. Like Dox, cell sensitivity to 4-Cyc reduced MCF-7 and T47D to 74±8% and 71±2% respectively, but approximately 40% of OVCAR-3 cells and 35% of COV434 were killed after exposure to the EC25 values of 4-Cyc. The origin and phenotype of the cell line can affect the *in vitro* sensitivity to chemotherapeutics. The OVCAR-3 cell line for example, was obtained from a patient who had been treated with the combination of Dox, cyclophosphamide and cisplatin 8 months prior derivation of the OVCAR-3 cell line (Hamilton, Young et al. 1983). This explains why OVCAR-3 cells were more resistant than T47D and COV434 cells to the cytotoxic activity of Dox. Exposure to γToc affected the two types of cell lines type differently; it significantly decreased MCF-7 and T47D cell viability, but did not affect KGN, COV434 or OVCAR-3 ovarian cells.

6.4 ROS and apoptosis induction

The generation and accumulation of intracellular reactive oxygen species (ROS) (Gomes, Fernandes et al. 2005, Valko, Leibfritz et al. 2007, Fan and Li 2014)

have been described as part of the mechanism of action of both Dox (Doroshow 1986, Gewirtz 1999, Mizutani, Tada-Oikawa et al. 2005) and cyclophosphamide (Luo and Shi 2004, Mythili, Sudharsan et al. 2004, Luo and Shi 2005, Liu, Li et al. 2012).

Many ROS have very short half-lives and OH radicals have a $T_{1/2}$ of approximately 10^{-9} seconds (Valko, Leibfritz et al. 2007, Forkink, Smeitink et al. 2010). This led to the development of a method to characterise ROS generation in the same cells at hourly intervals for 6h (Figueroa, Asaduzzaman et al. 2018) and used to evaluate ROS production by human breast and ovarian cell lines in this PhD project.

ROS generation happened in the first 3h of exposure and it is also likely that the majority of the apoptotic processes occurred before 24h. The time after exposure affects the interrelationship between sets, and so does the concentration of agents as was illustrated by comparing data from the MCF7 and KGN experiment (Chapter III) with the EC25 data (Chapter IV).

Exposure to 4-Cyc induced more ROS faster than exposure to Dox, except in COV434 cells, in which both agents generated the same response. These findings are broadly in agreement with previous reports that ROS mediates 4-Cyc– induced apoptosis in different types of cells (Luo and Shi 2004, Mythili, Sudharsan et al. 2004, Luo and Shi 2005, Liu, Li et al. 2012). To our knowledge this the first time the speed and amount of ROS induced by 4-Cyc is compared with Dox in the same study. A 3h exposure to αToc or γToc had no effect on ROS generation in MCF-7, T47D, OVCAR-3 or COV434 cells; however higher levels of ROS were detected in both breast cancer cell lines after 24h exposure to either αToc or γToc.

ROS have been linked with the regulation of apoptosis via mitochondria, death receptors and endoplasmic reticulum (ER) (Cossarizza, Franceschi et al. 1995, Talley, Dewhurst et al. 1995, Zamzami, Marchetti et al. 1995, Simon, Haj-Yehia et al. 2000). ROS have also been implicated with non-apoptotic mechanisms, such as autophagy and necrosis signalling pathway (Redza-Dutordoir and Averill-Bates 2016). ROS activate the tumour suppressor protein p53 (Kaminskyy and Zhivotovsky 2014). p53 plays a key role in the control of cellular stress responses, inducing either cell cycle arrest in order to promote DNA repair and survival, or cell death by apoptosis, depending on the context (Yoshida and Miki 2010); furthermore, ROS such as H_2O_2 and superoxide can cause cytochrome c release from mitochondria and induction of apoptosis (Chandra, Samali et al. 2000, Madesh and Hajnóczky 2001, Circu and Aw 2012). The relationship between ROS-mediated and caspase reDox regulation and caspase activity appears to involve post-translational modifications of the catalytic sites (Circu and Aw 2012) which are vulnerable to oxidation.

In this PhD study ROS and caspase levels were correlated in OVCAR-3 and COV434 cells. In COV434, the levels of ROS and caspase -3, -7 activity after 24h were higher after exposure to Dox than after exposure to 4-Cyc, whereas ROS and caspase -3, -7 activity in the Dox-resistant OVCAR-3 cells were higher after exposure to 4-Cyc. In the breast cancer cells MCF-7 and T47D although caspase activity was not detected ROS generation was significantly higher after 3h exposure to Dox or 4-Cyc.

While it is clear that ROS can cause apoptosis, and in this study there were correlations between caspase activity and ROS levels, it cannot be concluded that all the observed cell death could be attributed solely to apoptosis.

6.5 Combinations, synergism and additivity

In this PhD study the combined regimen had the same cytotoxicity as the single agent Dox. The combination of two or more therapeutic agents is an important part of cancer therapy (Blagosklonny 2004, Yap, Omlin et al. 2013) that was seen as a way to maximise anti-cancer efficacy by combining agents with different mechanisms of action, whilst minimising adverse effects (Pritchard, Lauffenburger et al. 2012, Bayat Mokhtari, Homayouni et al. 2017). This paradigm rested on the selection of agents that when administered together might display synergism; more anti-cancer activity than that observed by adding the effects of two (or more) single agents. Perceived benefits of this approach were to address tumour heterogeneity, reduce the chances of therapeutic resistance and adverse side effects (Pritchard, Lauffenburger et al. 2012). The benefits of combination chemotherapy were originally derived from *in vivo* studies, one of which found that the combination of Dox and cyclophosphamide was more effective that Dox as a single agent (Corbett, Griswold et al. 1975). The existing paradigm assumes that drug combinations are super additive (synergistic) or additive at the very least. Results from this PhD were therefore surprising because not only was there a lack of synergism between Dox and 4-Cyc *in vitro* but the rates of cell death measured in the crystal violet assay were only 1.3-fold higher than with a single agent. This led to the conclusion that the two chemotherapeutics have some cytotoxic mechanisms of action in common.

γToc was cytotoxic to breast cancer cells as a single agent, but the effect of adding it to the combined Dox + 4-Cyc regimen was not clear. In the initial KGN study, adding γToc (75μM) to the combination of Dox (10μM) and 4-Cyc (1μM) significantly increased the cytotoxicity of the combined regimen after a 24h

exposure; but when the test agents were mixed based on their EC25 values, no additive effect was observed. Previous studies have shown that synergism is not an inherent property of the drug/test agent (Tallarida 2011) and it depends on the doses of each in the combination γToc(Tallarida, Kimmel et al. 1997, Tallarida and Raffa 2010). From these PhD studies it is clear that different concentrations of γToc give rise to different results and that concentration of γToc closer to the EC50 (105μM) might increase the effect of the combined regimen. However, more experiments are needed in order to assess the potential additive or synergistic potential of the combined agents.

The addition of α or γToc to the combination of Dox and 4-Cyc, had no effect on apoptosis or caspase activity in any of the cell lines used. Exposure to γToc did not stimulate ROS generation by human breast and ovarian cancer-derived cell lines; but the addition of γToc to the combination of doxorubicin and 4-Cyc significantly decreased the amount of ROS produced by COV434 granulosa cancer cells. Even though ROS were lower, COV434 cell viability did not increase significantly. It is possible that both tocopherols reduced ROS in KGN granulosa cells that were exposed to Dox and 4-Cyc, and that tocopherol-mediated reduction in ROS affected estrogen production by these cells.

When the EC25 values of the combination of Dox and 4-Cyc were compared to Dox as a single agent, cytotoxicity (crystal violet) in MCF-7 was increased 1.3-fold, ROS production 1.3-fold, and TUNEL labelling by 1.2-fold. The addition of αToc to the combination of Dox and 4-Cyc did not increase cell viability compared to the combined regimen and had no significant effect on ROS production or apoptosis induction. Addition of γToc to the combined increased cytotoxicity in MCF-7 and T47D cells by 1.1 and 1.05-fold, respectively, but had no effect on ROS generation.

6.6 Study weaknesses and future directions

Since apoptosis occurs via a complex signalling cascade that is tightly regulated at multiple points, the results from this PhD project are not enough to draw conclusions about all the potential mechanisms involved; hence, more studies are needed. To capture the optimal period of caspase -3, -7 activation and DNA fragmentation, a time response study could be performed, by assessing cell response at 1,3,6 and 12h. Similarly, apoptosis could also be analysed by detection of phospholipid phosphatidylserine (PS) (Martin, Reutelingsperger et al. 1995, Huerta, Goulet et al. 2007). Annexin-V is a cellular protein that binds and detects PS exposure in apoptotic cells; PS exposure is recognised as an early event in the apoptotic pathway, hence Annexin-V in combination with cell-impermeable dyes, such as propidium iodide, can be used to distinguish apoptotic cells from non-apoptotic cells, and it can also allow for discrimination between early apoptosis and late apoptosis through flow cytometry analysis. Monitoring membrane potential can also provide information about early apoptotic stages (Macho, Decaudin et al. 1996, Huerta, Goulet et al. 2007); a variety of dyes for flow cytometry and fluorescence microscopy can be used to investigate the release of different factors, including cytochrome c, Smac or endoG (Huerta, Goulet et al. 2007).

The results from this PhD study suggest that other mechanisms of cell death may be involved specially in breast cancer cell lines; therefore, other pathways such as necrosis and autophagy should be evaluated in the future. Necrosis can be assessed through specific morphological changes by light or electron microscopy (Krysko, Vanden Berghe et al. 2008); other methods to assess necrosis include detection of high-mobility group box 1 (HMGB1) protein. HMGB1 is an intracellular

that regulates transcription and is passively released during necrosis (Krysko, Vanden Berghe et al. 2008)

The selection of cells for examination *in vitro* is also an important consideration. During this study it was realised the cell lines used offered limited scope to accurately model the complexity of the *in vivo* response. Both MCF-7 and T47D cell lines were derived from pleural effusion of metastatic tissues (Soule, Vazquez et al. 1973, Keydar, Chen et al. 1979), rather than primary breast tumour; therefore, they tend to represent more aggressive types of cancer instead of the primary lesion, which means that most breast cancer subtypes, reflected by the various grades and stages were not well represented by these cell lines. For this reason, it would be more clinically relevant to replace MCF-7 and T47D cell lines with heterogeneous, primary-derived breast cancer cell populations.

On the other hand, KGN cells have been widely used to model steroidogenesis *in vitro* (Nishi, Yanase et al. 2001) and even though they possess many of the characteristics observed in normal granulosa cells, there may be some issues associated with the use of KGN cells to investigate apoptosis mechanisms in normal cells because of their neoplastic origin (Nishi, Yanase et al. 2001). Schrader et al. (2009) found that KGN cells showed a mutation in FOXL2, which is involved in the proliferation and differentiation of granulosa cells and could therefore alter their apoptosis mechanisms. Another source to study the effects of chemotherapeutics with or without tocopherols on the ovary could be 3D primary-derived ovarian follicle culture (Asaduzzaman, Figueroa et al. 2018) since they more closely resemble follicle biology.

It was recognised that non-malignant cell line was needed to evaluate the specific targeting of γToc. Previous studies found that γToc inhibited the proliferation

of prostate cancer cells but had no effect on normal prostate epithelial cells (Jiang et al., 2014). Would γToc affect normal ovarian tissue? Or would γToc show antioxidant activity against chemotherapeutic-induced damage? These questions could be explored in more depth in an *in vivo* study which measures normal ovarian function and response to the treatment of breast tumours. During this PhD project, ovarian follicles from mice were disaggregated and examined in a crystal violet. Results showed primary granulosa cells can be cultured *in vitro* and can be used to generate a linear standard curve (R^2 = 0.98) (Asaduzzaman, Figueroa et al. 2018) and potentially be used to screen the reproductive toxicity of pharmaceutical agents

6.7 Conclusions

At the beginning of this PhD project it was hypothesised that the combination of Dox and 4-Cyc would be more cytotoxic *in vitro* to the human breast cancer cell lines than each chemotherapeutic agent alone but combining both drugs did not significantly potentiate the cytotoxic effect of the single agents. Although it was originally thought that γToc but not αToc would augment the cytotoxic activity of the combination of Dox and 4-Cyc it was discovered that although γToc alone significantly decreased cell viability of both breast cancer cell lines it did not increase cytotoxicity of the combined regimen. However, γToc had a different effect on ovarian granulosa cancer-derived cells. γToc as a single agent was not cytotoxic to COV434 or KGN granulosa cells, and it was also concluded that the antioxidant properties of γToc reduced chemotherapy-related ROS generation and probably supported estrogen hormone production by the KGN cells. The finding that an antioxidant molecule such as γToc can reduce acute chemotherapeutic-generated

ROS production in the ovary but also has cytotoxic effect against breast cancer cells, holds promise for the idea that γToc may have the potential to be developed into a fertility preservation therapy for premenopausal breast cancer patients.

7. APPENDICES

7.1 Mycoplasma results

MycoAlert mycoplasma detection by luminescence
27/06/2017

samples	reading A (Rw/s)	reading A (Rw/s)	Ratio
Caco-2 cells	2630	1401	0.5327
SKOV3 (pass. 2)	852	642	0.753521
SKOV3 (pass. 3)	2252	930	0.412966
FA SK	6226	2024	0.325088
FA CRL	2227	863	0.387517
PC12	3406	1355	0.397827
PC12	3663	1291	0.352443
PC12	2826	1297	0.458953
MCF-7 (pass. 9)	3167	1159	0.365961
KGN (pass. 6)	2900	111809	38.55483
MCF-7 (pass. 23)	3450	1403	0.406667
T47D	2178	759	0.348485
Negative control	**4006**	**378**	**0.094358**
Positive control	**2831**	**183469**	**64.80714**

RATIO	INTERPRETATION
<0.9	negative for mycoplasma
0.9 - 1.2	quarantine cells and retest in 24h
> 1.2	positive for mycoplasma

Table 7-1 Mycoplasma detection by luminescence results

MycoAlert mycoplasma detection by luminescence
19/03/2018

samples	reading A (Rw/s)	reading A (Rw/s)	Ratio	Results
MCF-7	417	180	0.43	NEGATIVE
OVCAR-3	516	239	0.46	NEGATIVE
T47D	839	342	0.41	NEGATIVE
COV434	808	392	0.49	NEGATIVE
HEK	783	364	0.46	NEGATIVE
HT	725	319	0.44	NEGATIVE
HCT	1134	486	0.43	NEGATIVE
BHK	1377	766	0.56	NEGATIVE
HEPG2	1255	617	0.49	NEGATIVE
HEK293	644	318	0.49	NEGATIVE
CACO	639	286	0.45	NEGATIVE
CHO	1617	629	0.39	NEGATIVE
DL1	208	96860	465.67	**POSITIVE**
PC	684	438	0.64	NEGATIVE
Negative control	**1242**	**189**	**0.15**	
Positive control	**555**	**27430**	**49.42**	

RATIO	INTERPRETATION
<0.9	negative for mycoplasma
0.9 - 1.2	quarantine cells and retest in 24h
> 1.2	positive for mycoplasma

Table 7-2 Mycoplasma detection by luminescence results

7.2 STR profiling

REPORT NO. 18-509. Cell Line Sample MCF-7

STR Profile – Sample **– Reference/Repository Sample**

Sample Name	MCF-7
Source	18-509
D3S1358	16
TH01	6
D21S11	30
D18S51	14
Penta E	7,12
D5S818	11,12
D13S317	11
D7S820	8,9
D16S539	11,12
CSF1PO	10
Penta D	12
Amel	X
vWA	14,15
D8S1179	10,14
TPOX	9,12
FGA	23,24,25

Sample Name	MCF7
Source	ATCC HTB-22
D3S1358	
TH01	6
D21S11	
D18S51	
Penta E	
D5S818	11,12
D13S317	11
D7S820	8,9
D16S539	11,12
CSF1PO	10
Penta D	
Amel	X
vWA	14,15
D8S1179	
TPOX	9,12
FGA	

Table 7-3 Certificate of Analysis, Identification Testing (MCF-7 cell line)

Comments:

Full profile generated:

- Out of 9 loci, 28/28 (100%) alleles were identical to MCF7 (ATCC: HTB-22)
- Within the scientific literature samples are usually thought to match if >80% of alleles are identical, so we conclude that this result is consistent with correct identity.

STR Profile – Sample **– Reference/Repository Sample**

Sample Name	**T47D**
Source	**18-510**
D3S1358	**15,17**
TH01	**6**
D21S11	**28,31**
D18S51	**17**
Penta E	**7,14**
D5S818	**12**
D13S317	**12**
D7S820	**11**
D16S539	**10**
CSF1PO	**11,13**
Penta D	**10,12**
Amel	**X**
vWA	**14**
D8S1179	**13**
TPOX	**11**
FGA	**23**

Sample Name	**T-47D**
Source	**ATCC HTB-133**
D3S1358	
TH01	**6**
D21S11	
D18S51	
Penta E	
D5S818	**12**
D13S317	**12**
D7S820	**11**
D16S539	**10**
CSF1PO	**11,13**
Penta D	
Amel	**X**
vWA	**14**
D8S1179	
TPOX	**11**
FGA	

Table 7-4 Certificate of Analysis, Identification Testing (T47D cell line)

Comments

Full profile generated:

- Out of 9 loci, 20/20 (100%) alleles were identical to T-47D (ATCC: HTB-133)
- Within the scientific literature samples are usually thought to match if >80% of alleles are identical, so we conclude that this result is consistent with correct identity

STR Profile – Sample **– Reference/Repository Sample**

Sample Name	**OVCAR-3**
Source	**18-511**
D3S1358	**17,18**
TH01	**9,9.3**
D21S11	**29,31.2**
D18S51	**13**
Penta E	**7,13**
D5S818	**11,12**
D13S317	**12**
D7S820	**10**
D16S539	**12**
CSF1PO	**11,12**
Penta D	**12,13**
Amel	**X**
vWA	**17**
D8S1179	**10,15**
TPOX	**8**
FGA	**21**

Sample Name	**NIH:OVCAR-3 [OVCAR3]**
Source	**(ATCC: HTB-161)**
D3S1358	
TH01	**9,9.3**
D21S11	
D18S51	
Penta E	
D5S818	**11,12**
D13S317	**12**
D7S820	**10**
D16S539	**12**
CSF1PO	**11,12**
Penta D	
Amel	**X**
vWA	**17**
D8S1179	
TPOX	**8**
FGA	

Table 7-5 Certificate of Analysis, Identification Testing (OVCAR-3 cell line)

Comments

Full profile generated:

- out of 9 loci, 24/24 (100%) alleles were identical to NIH:OVCAR-3 [OVCAR3] (ATCC: HTB-161)
- within the scientific literature samples are usually thought to match if >80% of alleles are identical, so we conclude that this result is consistent with correct identity

STR Profile – Sample **– Reference/Repository Sample**

Sample Name	**COV434**
Source	**18-512**
D3S1358	**15,19**
TH01	**9.3**
D21S11	**28**
D18S51	**10,15**
Penta E	**12**
D5S818	**10,11**
D13S317	**8,12**
D7S820	**8**
D16S539	**9,12**
CSF1PO	**10,11**
Penta D	**10,12**
Amel	**X**
vWA	**16,18**
D8S1179	**13,14**
TPOX	**9,11**
FGA	**24,25**

Sample Name	**COV434**
Source	**ECACC 07071909**
D3S1358	
TH01	**9.3**
D21S11	
D18S51	
Penta E	
D5S818	**10,11**
D13S317	**8,12**
D7S820	**8**
D16S539	**9,12**
CSF1PO	**10,11**
Penta D	
Amel	**X**
vWA	**16,18**
D8S1179	
TPOX	**9,11**
FGA	

Table 7-6 Certificate of Analysis, Identification Testing (COV434 cell line)

Comments

Full profile generated:

- Out of 9 loci, 30/30 (100%) alleles were identical to COV434 (ECACC07071909)
- within the scientific literature samples are usually thought to match if >80% of alleles are identical, so we conclude that this result is consistent with correct identity

7.3 Standard curves

7.3.1 Mice primary Granulosa Cells. Crystal Violet Standard curve

	0	312.5	625	1250	2500	5000	10000	20000
rep 1	0.069	0.042	0.0355	0.0435	0.061	0.0865	0.1225	0.2655
rep 2	0.020333	0.021	0.016667	0.021	0.024333	0.046667	0.073333	0.190667
rep 3	0.041333	0.036	0.026	0.029	0.049667	0.072667	0.124	0.311333
average	0.043556	0.033	0.026056	0.031167	0.045	0.068611	0.106611	0.255833
std. dev	0.024409	0.010817	0.009417	0.011405	0.018774	0.020224	0.028829	0.060911

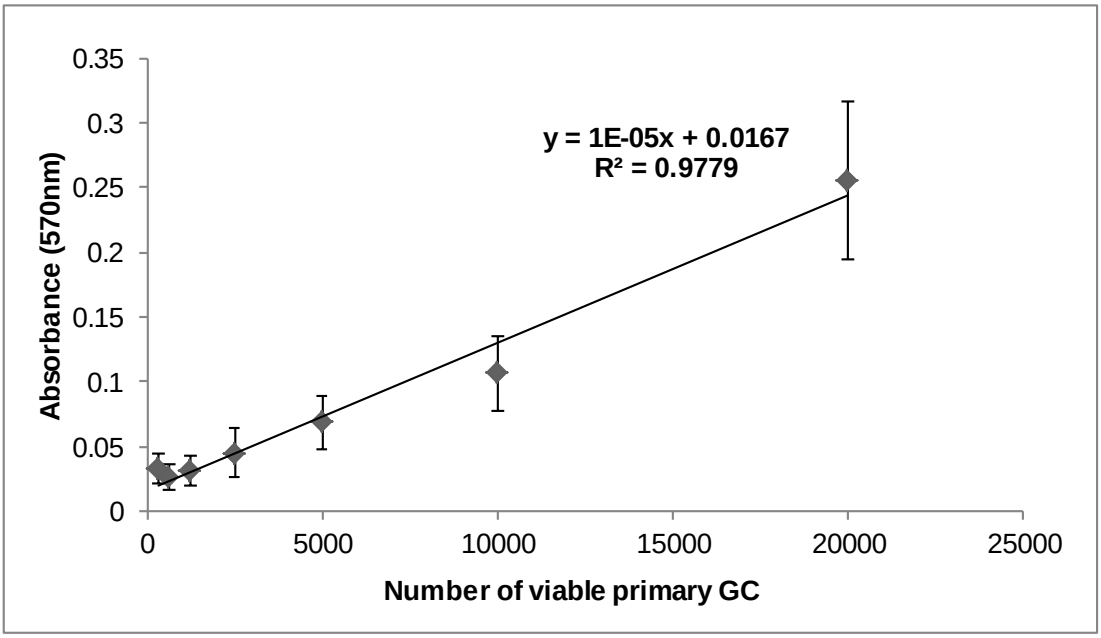


Figure 7.1 Viable primary GC standard curve determined by CV assay.

Primary GC were cultured in wells of 96 well plate at a density of 0 to 25,000 (six replicate wells for each density). Cells were cultured for 24h, viable cell numbers were determined in a crystal violet assay. Mean±SD OD at 570nm with correction at 630nm was plotted against cell densities and fitted to a linear trendline.

7.3.2 KGN cell viability: Standard curve

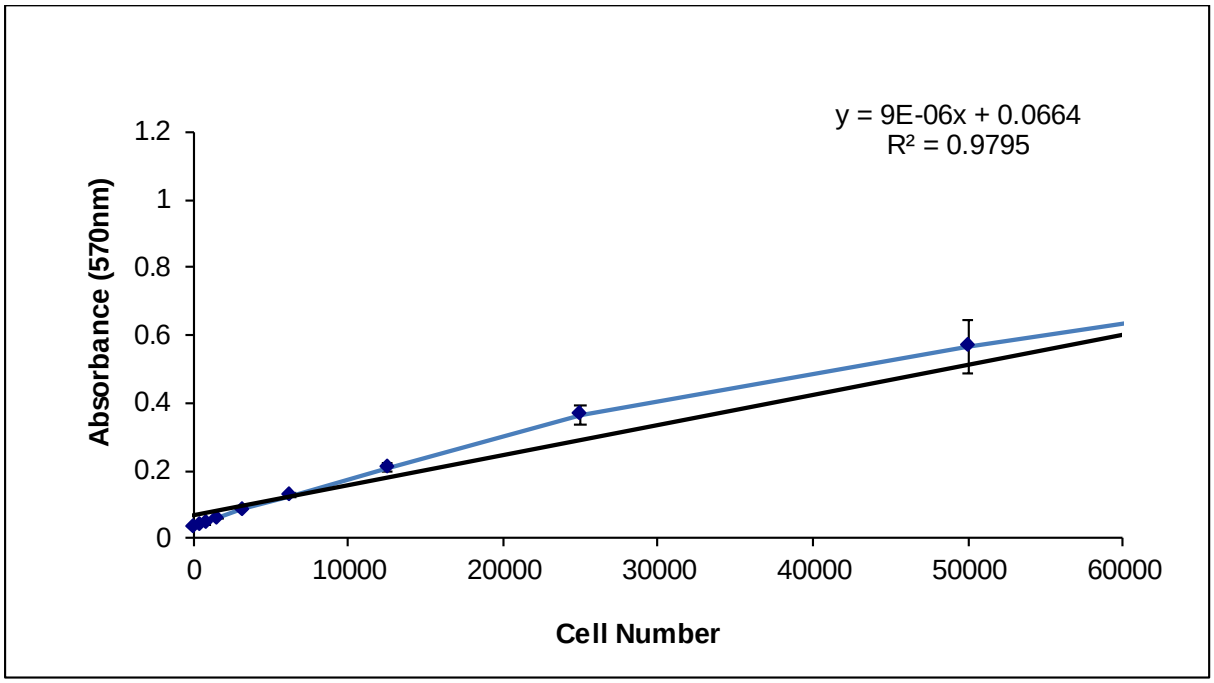


Figure 7.2 Viable KGN cell standard curve determined by CV assay.

KGN cells were cultured in wells of 96 well plate at a density of 0 to 80,000 (six replicate wells for each density). Cells were cultured for 24h, viable cell numbers were determined in a crystal violet assay. Mean±SD OD at 570nm with correction at 630nm was plotted against cell densities and fitted to a linear trendline.

7.3.3 COV434 cell viability: Standard curve

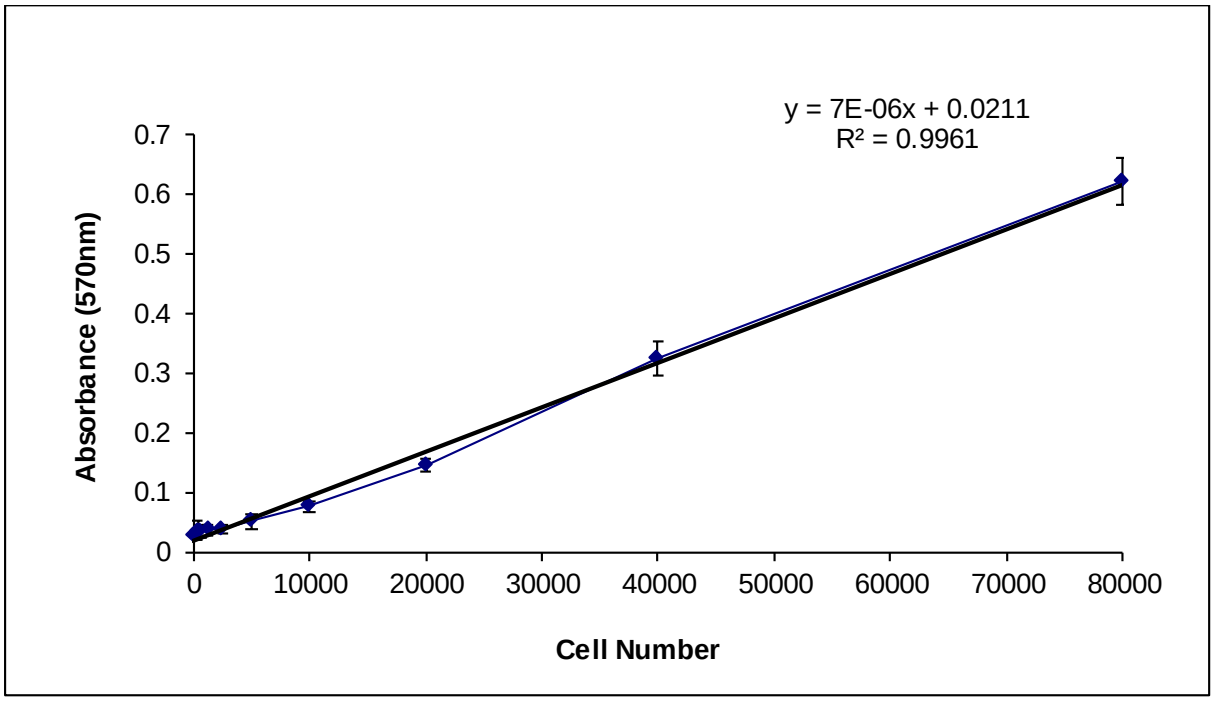


Figure 7.3 Viable COV434 cell standard curve determined by CV assay.

COV434 cells were cultured in wells of 96 well plate at a density of 0 to 80,000 (six replicate wells for each density). Cells were cultured for 24h, viable cell numbers were determined in a crystal violet assay. Mean±SD OD at 570nm with correction at 630nm was plotted against cell densities and fitted to a linear trendline.

7.3.4 OVCAR-3 cell viability: Standard curve

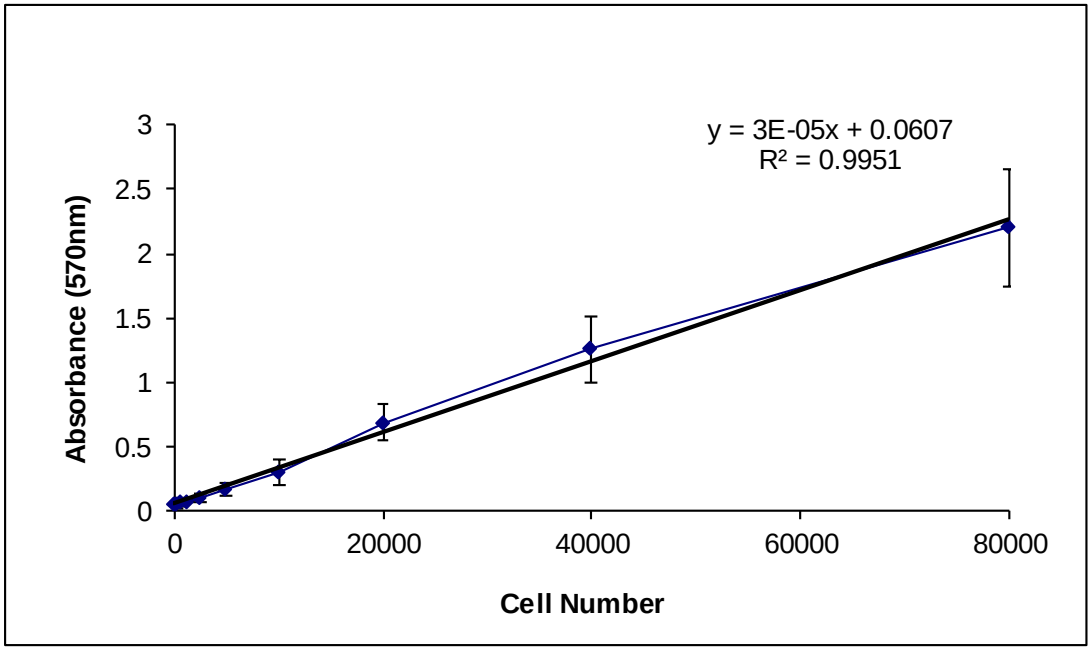


Figure 7.4 Viable -3 cell standard curve determined by CV assay.

OVCAR-3 cells were cultured in wells of 96 well plate at a density of 0 to 80,000 (six replicate wells for each density). Cells were cultured for 24h, viable cell numbers were determined in a crystal violet assay. Mean±SD OD at 570nm with correction at 630nm was plotted against cell densities and fitted to a linear trendline.

7.3.5 MCF-7 cell viability: Standard curve

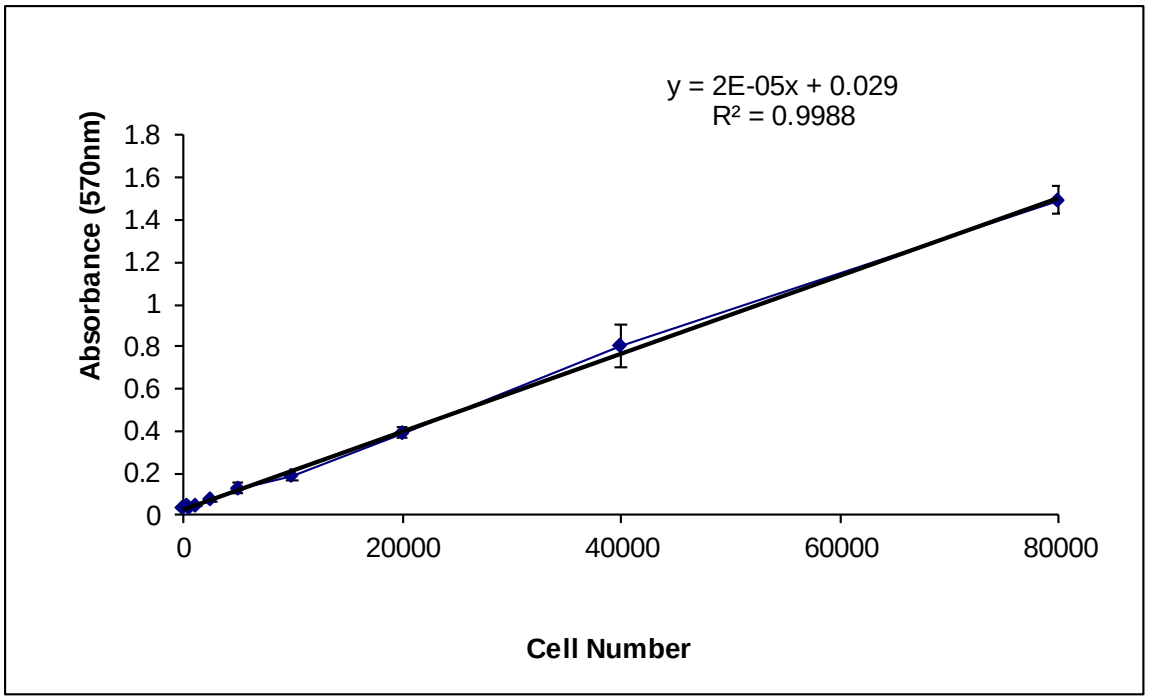


Figure 7.5 Viable MCF-7 cell standard curve determined by CV assay.

MCF-7 cells were cultured in wells of 96 well plate at a density of 0 to 80,000 (six replicate wells for each density). Cells were cultured for 24h, viable cell numbers were determined in a crystal violet assay. Mean±SD OD at 570nm with correction at 630nm was plotted against cell densities and fitted to a linear trendline.

7.3.6 T47D cell viability: Standard curve

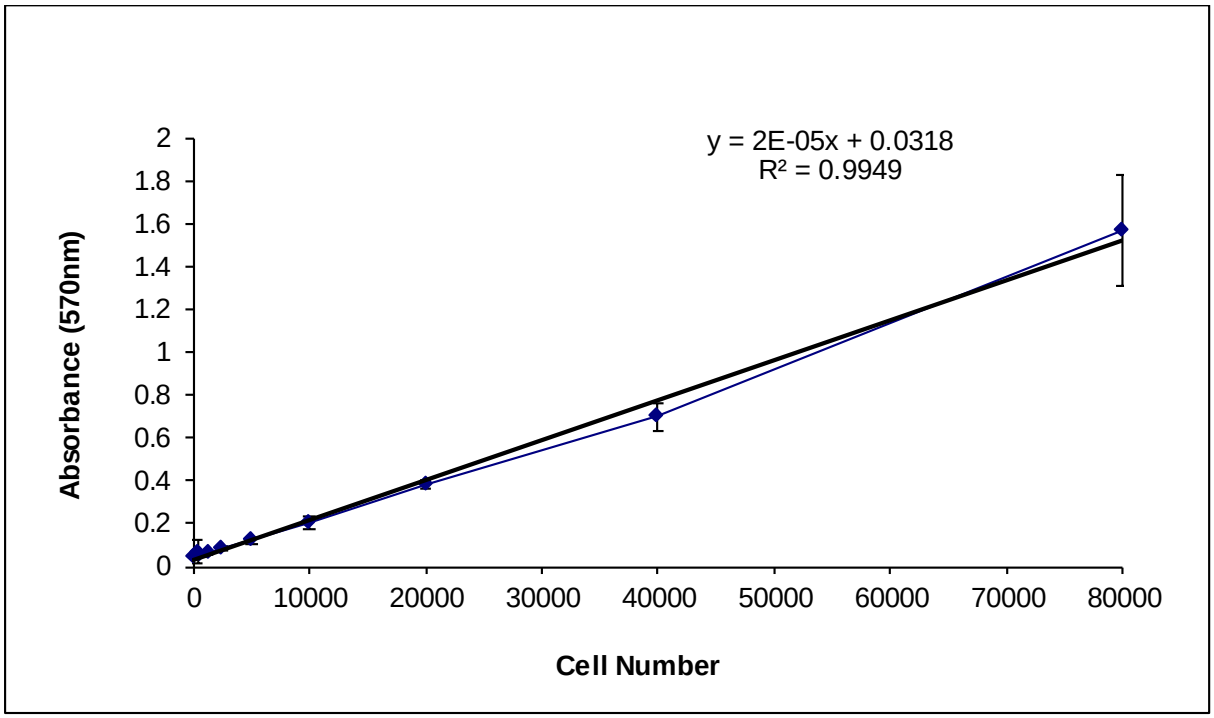


Figure 7.6 Viable KGN cell standard curve determined by CV assay.

KGN cells were cultured in wells of 96 well plate at a density of 0 to 80,000 (six replicate wells for each density). Cells were cultured for 24h, viable cell numbers were determined in a crystal violet assay. Mean±SD OD at 570nm with correction at 630nm was plotted against cell densities and fitted to a linear trendline.

7.3.7 Estrogen standard curve

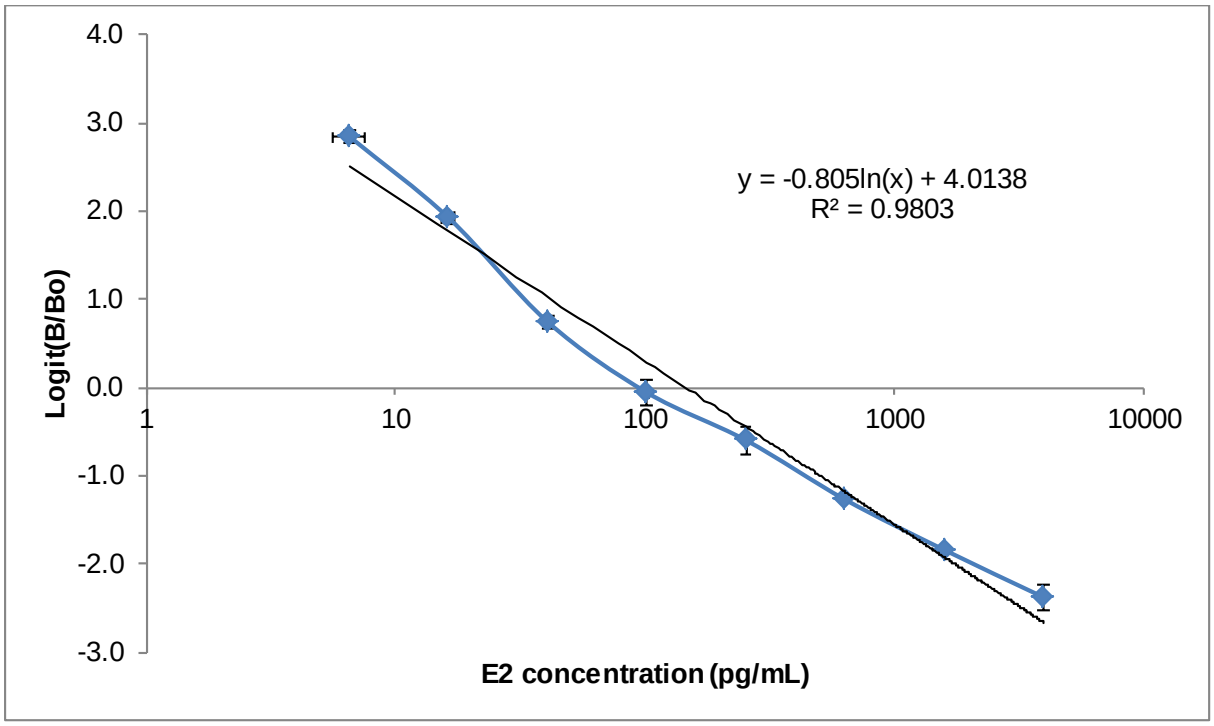


Figure 7.7 Estrogen standard curve.

Estradiol standard was diluted in medium to yield concentrations that ranged from 6.6 to 4000 pg/mL and examined in a competitive Estradiol (E2) EIA (Cayman Chemical ELISA, Ann Arbor, MI, USA). B/B0 denotes the OD value at 412nm of each concentration calculated by subtracting the average OD for non-specific binding wells and then divided by average OD of the media wells. Separate estrogen standard curve was performed for each separate experiment (n=3) and used to calculate the estrogen produced by KGN cells.

7.4 DCFDA – ROS optimisation

ROS microplate assay procedure optimisation for adherent cells

Reagent Preparation		
	Original protocol	**Optimised protocol**
DCFDA solution	1X buffer (Hank's balance salt solution)	1X buffer (Hank's balance salt solution)
TBHP solution	1X supplemented buffer (1X buffer + 5% FCS)	Phenol red free RPMI +1% Pen/Strep + 10% FCS

Original protocol for adherent cells:

1. Grow adherent cells in standard culture media so that $3x10^6$- $4 x10^6$ cells are obtained the day before the experiment.
2. Harvest cells and seed a dark, clear bottom 96-well microplate with 25,000 cells per well. Allow cells to adhere overnight
3. Remove media and add 100µL/well of 1X buffer
4. Remove 1X buffer and stain cells by adding 100µL/well of the diluted DCFDA solution (recommended concentration is 25µM).
5. Incubate cells with the diluted DCFDA solution for 45min at 37°C in the dark.

6. Remove DCFDA solution; add 100µL/well of 1X Buffer or 1X PBS, and measure fluorescence.
7. Remove 1X Buffer/PBS and add 100µL of compound of interest. Treat cells for desired period of time (1-6h).

Optimised protocol for MCF-7 breast cancer cells:

1. Grow adherent cells in standard culture media so that $3x10^6$- $4 x10^6$ cells are obtained the day before the experiment.
2. Harvest cells and seed a dark, clear bottom 96-well microplate with 20,000 cells per well. Allow cells to adhere overnight
3. Remove media and add 100µL/well of complete RPMI (phenol red free)
4. Remove Media and stain cells by adding 100µL/well of the diluted DCFDA solution (optimal concentration for MCF-7 was 10µM).
5. Incubate cells with the diluted DCFDA solution for 45min at 37°C in the dark.
6. Remove DCFDA solution; add 100µL/well of complete RPMI (phenol red free) and measure fluorescence.
7. Add 100µL of compound of interest. Treat cells for desired period of time (1-6h). Reading fluorescence every hour.

ROS microplate assay procedure optimisation for adherent cells		
Parameters	**Original protocol**	**Optimised protocol**
Seeding density	25,000 cells/well	20,000 cells/well
Washing solution	1X buffer (Hank's balance salt solution)	Red phenol free RPMI +1% Pen/Strep + 10% FCS
DCFDA solution concentration	25µM	10µM
DCFDA incubation parameters	45min at 37°C in a humidified 5% CO2	45min at 37°C in a humidified 5% CO2
Toxicity assay		
Incubation period	1-6 hour	1-24 hour
measurement type	Fluorescence end-point	Fluorescence real time monitoring
Fluorescence wavelength	Ex= 485nm Em=535nm	Ex= 475nm Em= 500-550nm
Equipment		
plate spectrofluorometer	VICTOR X multilabel, Perkin Elmer, Australia	GloMax® Explorer, promega, Australia
Reading mode	Bottom-reading	Top-reading
Gain/high sensitivity option	No	Yes

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