

THE USE OF NANOTECHNOLOGY FOR TREATMENT OF MULTIDRUG RESISTANT OVARIAN CANCER CELLS



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By

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DECLARATION

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Summary

The traditional ovarian cancer treatment includes a combination of surgery and chemotherapy. However, the prognosis for ovarian cancer following treatment is very poor in the majority of ovarian cancer patients, especially patients with stage III or IV disease on diagnosis. Many patients respond well initially to chemotherapy but go on to develop recurrent chemoresistant disease. To improve the efficacy of chemotherapy, nanotechnology has been used in the targeting and treatment of ovarian cancer and it has been employed in cancer treatment to overcome some of the limitations seen with traditional therapy.

In addition to their non-toxicity, low cost and commercial availability, nanodiamonds have many other advantages that can be utilized in drug delivery. They can be functionalized with different types of anticancer therapeutic and they can be suspended easily in water, which is important for biomedical applications.

In this thesis, we have developed a novel nanocarrier using NDs coupled with 1st and 2nd line ovarian cancer chemotherapy (PTX, CARBO and DOX) and conjugated with ovarian cancer biomarkers (HE4 and CA125 antibodies) to deliver desired drugs to targeted cancer cells.

The results of our study showed the nanocomplexes developed in our laboratory were successfully taken up by ovarian cancer cell lines and ovarian tumour explants. Cytotoxic effects were evaluated using different biological parameters such as cell viability, mitochondrial membrane changes, cell membrane permeability and cell cycle profiling. The changes of the biological parameters indicated the advantages of functionalised NDs for drug delivery. Moreover, the nanocomplexes induced a greater effect even at lower drug concentrations.

Our developed nanocomplexes improved cytotoxicity in chemosensitive and chemoresistant ovarian cancer cell lines by activation of the apoptosis pathway through caspase-3 upregulation and non-stimulation of the drug transporter protein P-gp.

Therefore, the nanocomplexes developed in our lab are a promising platform to effectively deliver anti-cancer chemotherapeutics to chemoresistant cancer cells that over express drug transporter proteins (P-gp).

We have reported that LDH activity was elevated in ovarian tumour explants following exposure to drugs (PTX, CARBO and DOX) and the nanocomplex treatments, but not in untreated control ovarian tumour explants. We have also found that the nanocomplexes coupled with CARBO and DOX induced caspase-3 upregulation in the ovarian explants compared with the drugs alone.

In summary, *in vitro* and *ex vivo* cytotoxic effects were seen after chemosensitive and chemoresistant ovarian cancer cell lines and ovarian tumour explants were treated with our developed nanocomplexes. The effect appears to be mediated by apoptosis and non-stimulation of P-gp. The use of nanodiamonds complexed to cytotoxic drugs and a specific target offers promise in improving the response of ovarian cancers to treatment and requires further investigation with *in vivo* studies.

Publications

- ❖ **Melad E. Saed**, Sharon O'Toole, Lucy Norris, Steven Gray, Feras Abu Saadeh, John O'Leary, Bashir Mohamed. The Efficacy of Nanocomplexes Complexed with Specific Biomarker Targets in Ovarian Cancer Treatment, An Ex-Vivo Explant Case Study. Laboratory Investigations 2019; 99(1): 98.

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- ❖ **Melad A. Aswisi**, Kieran Crosbie-Staunton, Steven G. Gray, Sharon O' Toole, John J. O'Leary, and Bashir M. Mohamed. *In vitro* targeted delivery and sustained drug release against chemoresistance ovarian cancer cell lines. 2nd Ovarian Cancer forum, South west acute hospital, Enniskillen, Co-Fermanagh, UK, 2015 (Oral presentation).
- ❖ **Melad E. Aswisi**, Steven G. Gray, Jennifer McIntyre, Hugh J. Byrne, Sharon O' Toole, John J. O'Leary, Bashir M. Mohamed. *In-vitro* targeted delivery and sustained toxic effect against sensitive and chemoresistant ovarian cancer cell lines. Irish Association for Cancer Research Annual meeting, Cork, Ireland, 2015. (Poster presentation).
- ❖ **Melad A. Aswisi**, Steven G. Gray, Jennifer McIntyre, Hugh J. Byrne, Sharon O' Toole, John J. O'Leary, Bashir M. Mohamed. Nano-vehicles for targeted sensitive and resistance ovarian cancer chemotherapies. Proceedings of the Irish Society of Gynaecological Oncology 2016, Dublin. (E-poster presentation).
- ❖ **Melad A. Aswisi**, Steven G. Gray, Jennifer McIntyre, Hugh J. Byrne, Sharon O' Toole, John J. O'Leary, Bashir M. Mohamed. *Ex vivo* and *in vitro* targeted delivery and prolonged toxic effect against primary and recurrent ovarian cancer. Irish Association for Cancer Research Annual meeting, Kilkenny, Ireland, 2017. (Poster presentation).

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List of Abbreviations

ABC = ATP-binding cassette

ANOVA = Analysis of variance

CA125 = Cancer antigen 125

CARBO = Carboplatin

CMP = Cell membrane permeability

CT = Computed Tomography

CT TAP = Computed Tomography of Thorax, Abdomen and Pelvis

DLS = Dynamic Light Scattering

DOX = Doxorubicin

DW = Deionised water

EDC = 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide

EOC = Epithelial Ovarian Cancers

EPR = Enhanced Permeation and Retention

FBS = Fetal Bovine Serum

FDA = Food and drug administration

FIGO = International Federation of Gynaecologists and Obstetricians

FITC = Fluorescein isothiocyanate

FNA = Fine needle aspiration

FTE = Fallopian tube epithelium

FTSECs = Secretory epithelial cells of the distal fallopian tube

GP = General practitioner

HCSA = High content screening Analysis

HE4 = Human epididymis protein 4

HGSOC = High-grade serous ovarian cancer

HTHP = High temperature/high pressure

IP = Intraperitoneal

IV = Intravenously

LDH = Lactate dehydrogenase

MDR = Multidrug resistance

MMP = Mitochondrial membrane potential

MMS = Multi-modal screening

MRC-05 = Medical Research Council clinical trial-05

MRI = Magnetic Resonance Imaging
MTX = Mitoxantrone
NACT = Neo-adjuvant chemotherapy
NTA = Nanoparticle tracking analysis
ND-PEG = Nanodiamond pegylation
NDs = Nanodiamonds
NMs = Nanomaterials
OS = Overall survival
OSE = Ovarian surface epithelial
PARP = Poly (ADP ribose) polymerase
PBS = Phosphate-buffered saline
PCS = Primary cytoreductive surgery
PFA = Paraformaldehyde
PFI = Platinum-free interval
P-gp = P-glycoprotein
PLCO = Prostate, Lung, Colorectal and Ovarian
PTX = Paclitaxel
PVDF = Polyvinylidene difluoride
ROCA = Risk of Ovarian Cancer algorithm
ROMA = Risk of Ovarian Malignancy Algorithm
RPMI = Roswell Park Memorial Institute
SiNWs = Silicon nanowire
siRNA = Small RNA interference
TVU = Transvaginal ultrasound
UK = United Kingdom
UKCTOCS = UK Collaborative Trial of Ovarian Cancer Screening
US = United States
USPSTF = US Preventive Services Task Force

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Chapter 1

Introduction

1. Introduction

1.1 Ovarian cancer

1.1.1 Definition

Ovarian Cancer (OC) is a growth that originates from the tissues of the ovary or fallopian tubes to form a malignant tumour or cancer (Lentz 2014). The high death rate associated with OC is not only due to early stage diagnosis challenges or drug resistance development but also due to the uncertainty regarding cancer origin and pathogenesis (Bast Jr, Hennessey et al. 2009, McCluggage 2011, Falconer, Yin et al. 2015, Siegel, Miller et al. 2016, Qiu, Lu et al. 2017). Although this disease is termed ovarian cancer, pathological, epidemiological and mouse models suggest that secretory epithelial cells of the distal fallopian tube (FTSECs) are more likely to be the precursor of a substantial proportion of high-grade serous ovarian carcinoma (HGSOC) (Kindelberger, Lee et al. 2007, Perets, Wyant et al. 2013). Although the traditional model of carcinogenesis has focused on the ovary as a tumour initiation site, recent studies suggest that there may be additional sites of origin outside the ovary, namely the secretory cells of the fallopian tube (Kindelberger, Lee et al. 2007). However, even with improved methods for pathological assessment of fallopian tubes, some HGSOCs seem to arise without fallopian tube involvement (Vaughan, Coward et al. 2011, Bowtell, Böhm et al. 2015, Pinto, Assis et al. 2017). Primary peritoneal cancer is also similar to ovarian epithelial cancer and is treated and staged the same way.

1.1.2 Epidemiology

Incidence and mortality

The incidence of ovarian cancer is more than 240,000 new cases worldwide, and more than 60% of women die from this disease annually (Ferlay, Autier et al. 2007, Ferlay, Shin et al. 2010, Ferlay, Soerjomataram et al. 2015, Siegel, Miller et al. 2016). Ovarian cancer remains the most lethal gynaecologic malignancy because 75% to 80% of all ovarian cancer are diagnosed at an advanced stage and there is a lack of initial symptoms and effective screening tools (Ferlay, Shin et al. 2010, Ferlay, Steliarova-

Foucher et al. 2013, Lowe, Chia et al. 2013, Ferlay, Soerjomataram et al. 2015, Kaldawy, Segev et al. 2016, Siegel, Miller et al. 2016, Lv, Zhuang et al. 2017). The survival rate for ovarian cancer remains low with 5-year overall survival (OS) depending on OC stage (Colombo, Van Gorp et al. 2006). The outcome of patients with advanced ovarian cancer is mainly poor, because of the lack of effective drugs for relapsed diseases, which have developed resistance to current chemotherapeutic agents (Chang, Wang et al. 2017, Wong, Wong et al. 2017). Over the last 50 years, the overall survival of ovarian cancer has only slightly improved, despite the development of more advanced surgical techniques and adjuvant therapies (Chang, Wang et al. 2017). OC is ranked as the fifth leading cause of cancer death in women and it has the lowest survival rate among all gynaecological cancers, with between 10% and 40% 5-year survival rates (Parkin, Bray et al. 2005, Siegel, Miller et al. 2016, Pranjol, Gutowski et al. 2017). Ireland ranks ninth in Europe in terms of OC incidence and fifth in terms of mortality in 2012 (Figure 1.1), (<http://eco.iarc.fr/eucan/CancerOne.aspx?Cancer=27&Gender=2>).

In 2017, the number of new cases of ovarian cancer diagnosed every year was 22,440 in the US, 7,000 in UK and 384 in Ireland. However, there were 272 deaths from ovarian cancer in Ireland (www.ncri.ie/sites/ncri/files/factsheets/ovary.pdf) (Figure 1.2). Historically, > 50% of these cases have been diagnosed in women 65 years and older (Talarico, Chen et al. 2004). Additionally, between 2010 and 2030, the incidence of cancer in older adult patients is expected to increase by 67%, compared with only an 11% increased incidence in younger adult patients (Smith, Smith et al. 2009, Gillen, Gunderson et al. 2017).

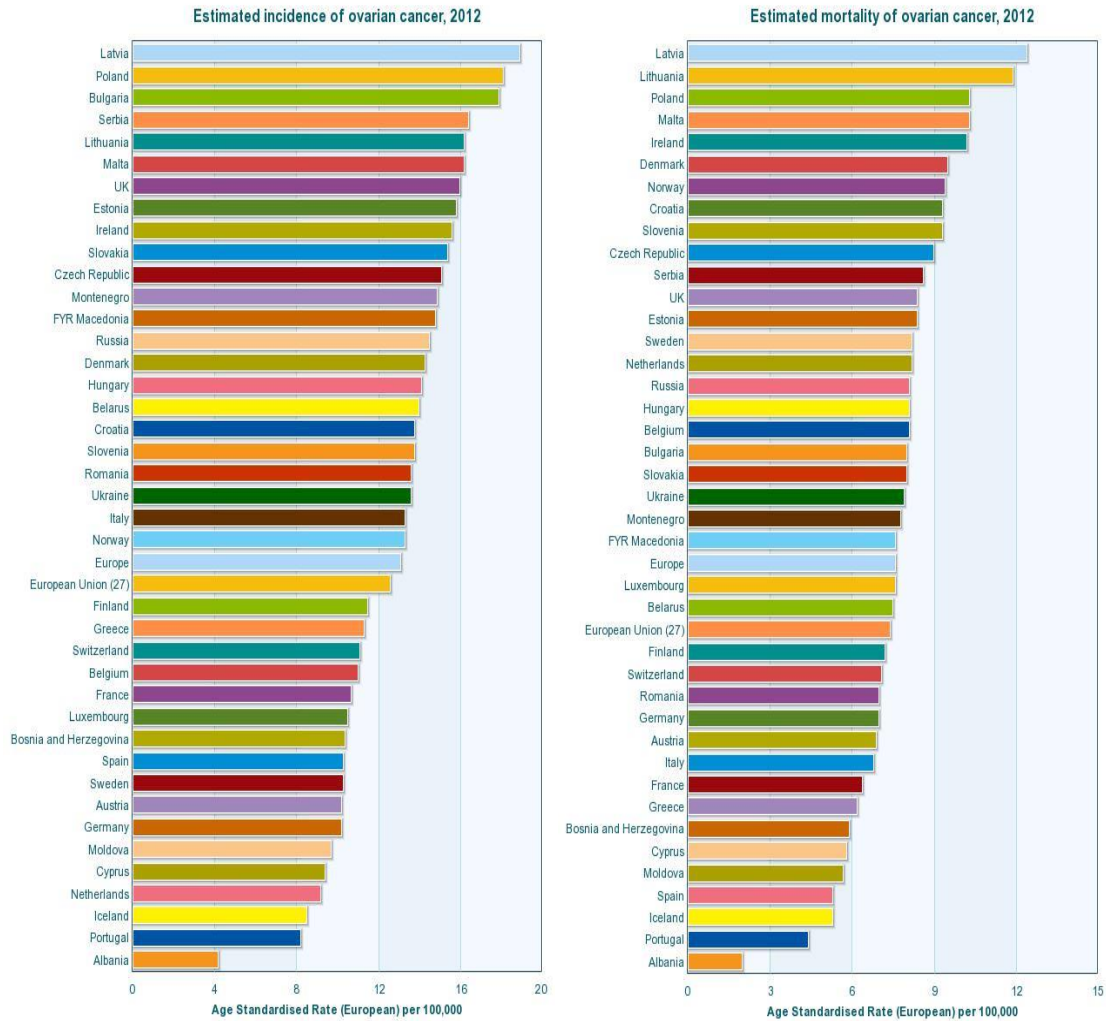


Figure 1.1: Estimate incidence and mortality of ovarian cancer among European countries in 2012. Ireland ranked ninth in estimated incidence and fifth in estimated mortality among European countries.

(<http://eco.iarc.fr/eucan/CancerOne.aspx?Cancer=27&Gender=2>).

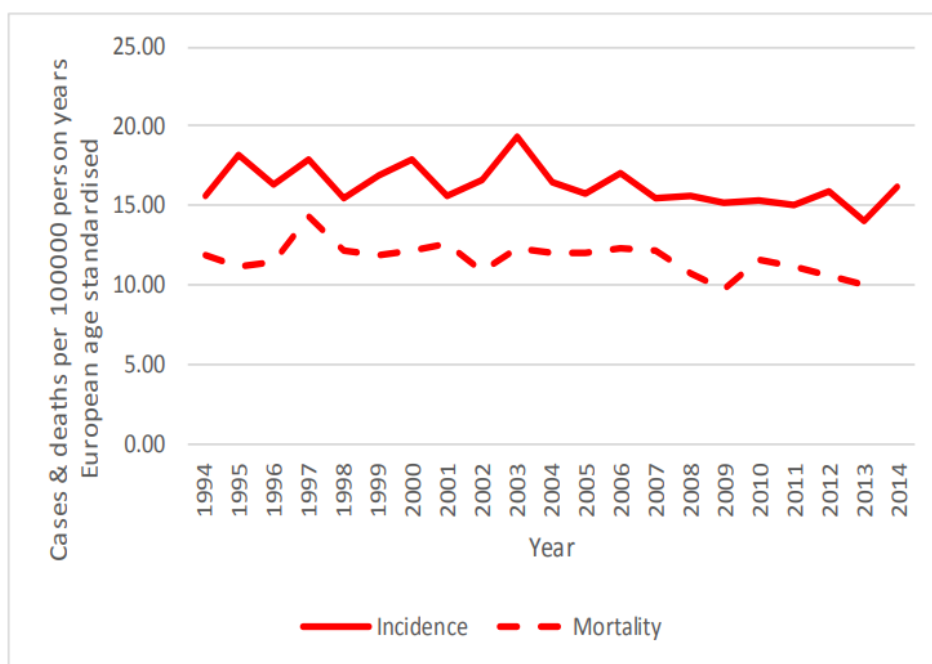


Figure 1.2: Ovarian cancer incidence and mortality rates in Ireland between 1994-2014

During this time the incidence rate was 15.4 cases/100.000 women/year, and the mortality rate was 10.6 death/100.000 women/year (www.ncri.ie/sites/ncri/files/factsheets/ovary).

Aetiology and risk factors

The exact cause of ovarian cancer is still unknown. However, the following factors increase a woman's risk:

- **Age:** the median age of patients with ovarian cancer is 60 years, and the average lifetime risk for women is about 1 in 70. Practically all women over 40 years are considered at higher risk, but it is uncommon before the age of 40 (Cannistra 2004, De Gaetano and Lichtman 2004, Colombo, Van Gorp et al. 2006).
- **Family history:** the strongest risk factor for ovarian cancer is a positive family history of ovarian or breast cancer, and women with 2 or more first-degree relatives (mother, daughter or sister) with ovarian cancer may have a 50% chance of developing the disease (Lessick, Wickham et al. 1997, Godard, Foulkes et al. 1998, Webb and Jordan 2017). Associated BRCA1 and BRCA2 mutations are present in 90% of hereditary cases, and due to BRCA mutations, ovarian cancers occur in younger women with higher grade or advanced serous carcinomas (Decruze and Kirwan 2006). 39% and 21% of women carrying BRCA1 and BRCA2 mutations respectively are more likely to develop an ovarian cancer in their lifetime (Risch, McLaughlin et al. 2001, Modugno 2003, Cannistra 2004, De Gaetano and Lichtman 2004, Colombo, Van Gorp et al. 2006, Kobayashi, Sumimoto et al. 2008, Salehi, Dunfield et al. 2008, Palmirotta, Silvestris et al. 2017).
- **Hormonal features and menstrual history:** the majority of epidemiological studies examining risk factors for ovarian cancer have identified a large number of hormonal and reproductive factors that influence risk (Risch 1998). The risk is possibly triggered specifically among those women with polycystic ovary syndrome and endometriosis (Colombo, Van Gorp et al. 2006, Salehi, Dunfield et al. 2008). The use of exogenous hormones for menopause-related symptoms appear to be associated with an increased risk of ovarian cancer incidence or mortality (Risch 1998, Colombo, Van Gorp et al. 2006). The risk of ovarian cancer is associated with the total time that ovulation is suppressed; oral contraceptive use, pregnancy, and lactation are associated with a reduced risk (De Gaetano and Lichtman 2004).

- Environmental features: the female genital tract cannot prevent the movement of small particulate matter from the outer vagina through the uterus and fallopian tubes to the surface of the ovary, providing a route for external contaminants. Clinical and epidemiological studies suggested additional factors such as the use of talc and fertility drugs might increase the risk of ovarian cancer (Godard, Foulkes et al. 1998, Cannistra 2004, Salehi, Dunfield et al. 2008, Kaldawy, Segev et al. 2016). Researchers have reported talc particles in ovarian tumours, and women with ovarian cancer have reported more frequent use of talcum powder in their genital area than healthy women, but there is little or no evidence that talc is associated with ovarian cancer (Chang and Risch 1997, Kaldawy, Segev et al. 2016).

1.1.3 Symptoms and Signs

Historically, ovarian cancer has been characterized as an asymptomatic disease, because it presents without appreciable signs or symptoms until the disease is advanced (Goff, Agnew et al. 2017). Symptoms can be acute or sub-acute with acute symptoms, such as pleural effusion or bowel obstruction, requiring urgent care. Sub-acute symptoms, such as weight gain, pelvic pain, difficulty eating, bloating, urinary symptoms, adnexal mass or gastrointestinal symptoms, usually can be evaluated in an outpatient setting (Prazak and Gahres 2016, Goff, Agnew et al. 2017). There are several factors that can lead to delays in diagnosis. Some are patient related, such as attributing the symptoms to other factors that can include: menstrual irregularities, menopause, aging, pregnancy, and stress. On average, women wait 2 to 3 months after symptom onset to seek medical attention. Delays occur when women are misdiagnosed initially and treated for another condition such as menopause, aging, dietary changes, stress, depression or functional bowel problems. Occasionally, patients may present with nausea, vomiting or a partial bowel obstruction (Prazak and Gahres 2016).

In general, symptoms that are persistent, more severe or frequent than expected and of recent onset warrant further diagnostic investigation. Women with malignant masses typically experience symptoms 20 to 30 times per month of notable severity (Schorge, Modesitt et al. 2010). The National Cancer Control Programme (NCCP) in Ireland has issued referral guidelines for general practitioners for women presenting with

symptoms that might be suspicious for ovarian cancer. This guideline incorporates a clinical exam, history, CA125 test and then possibly an ultrasound and a referral to a gynaecological oncologist (Figure1.3) (<https://www.hse.ie/eng/services/list/5/cancer/profinfo/resources/gpreferrals/>).

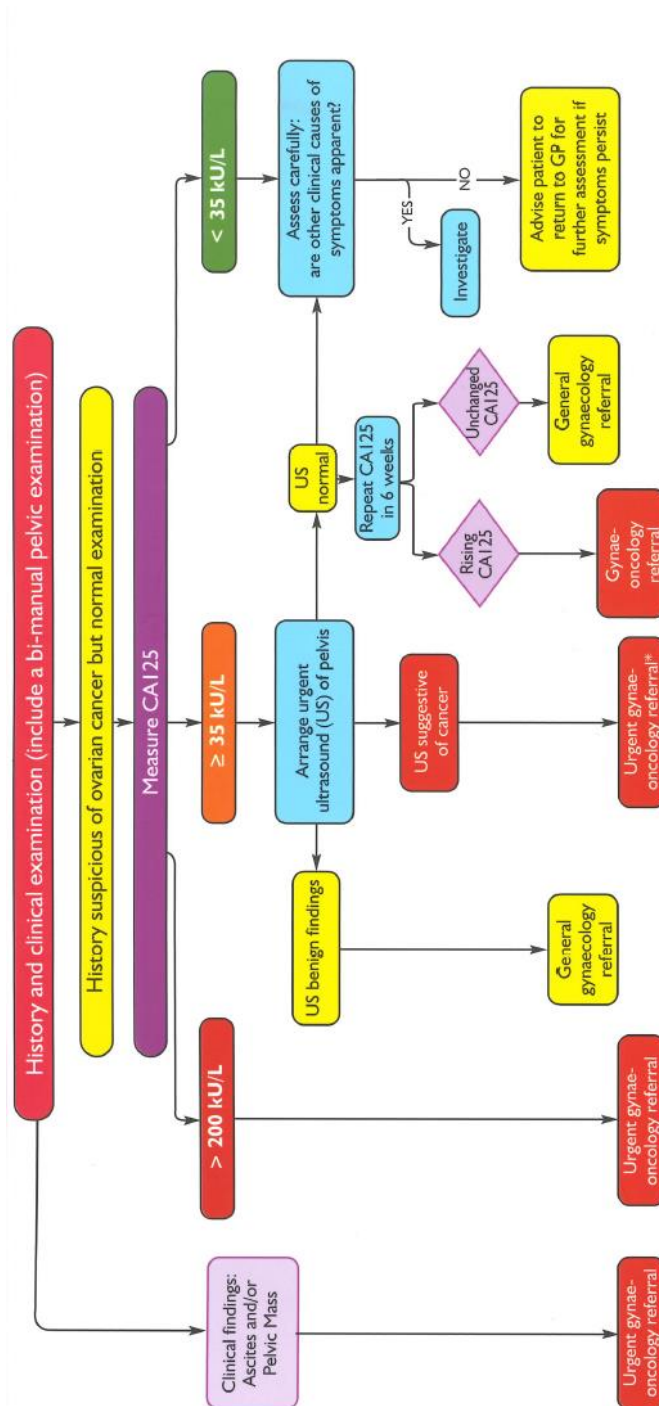


Figure 1.3: GP referral pathway for symptomatic women.
(<https://www.hse.ie/eng/services/list/5/cancer/profinfo/resources/gpreferrals/>).

1.1.4 Screening

Earlier diagnosis of ovarian cancer would lead to improvements in survival rates. The need for a sensitive screening test for early detection of ovarian cancer remains a long-awaited priority (Gupta and Lis 2009, Jayson, Kohn et al. 2014).

Different screening strategies have been assessed to try and improve the early detection of ovarian cancer. The US FDA have approved two biomarkers, Cancer Antigen 125 (CA 125) and Human Epididymis Protein 4 (HE4) and two algorithms, OVA1 and Risk of Ovarian Malignancy Algorithm (ROMA) for risk assessment and management of ovarian cancer (Abraham 2010, Moore, Miller et al. 2011). CA125 has utility in the follow up of patients if it has been elevated on diagnosis means the cancer has progressed or has recurred (Burger 2012).

Two large screening trials have also taken place in order to assess if screening for ovarian cancer can reduce mortality. The results of the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial in the United States reported in 2011 that screening with the biomarker CA-125 along with transvaginal ultrasound (TVU) demonstrated no mortality benefit after a median follow-up of 12.4 years (Pinsky, Yu et al. 2016).

Another large ovarian cancer screening trial, the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) reported its findings in 2015. UKCTOCS had a control arm and two screening arms. One screening arm was based on TVU alone, while the other employed a multi-modal screening paradigm (MMS) that utilized measurement of CA-125 values by the Risk of Ovarian Cancer algorithm (ROCA), which is a simple blood test that assesses a woman's risk of having ovarian cancer. With this initial score, a doctor will be able to determine if further tests such as CA-125 or TVU are needed. Based on the primary endpoint of ovarian cancer specific mortality, the screening arm demonstrated a statistically significant reduction compared to the control arm (Jacobs, Menon et al. 2016). The US Preventive Services Task Force (USPSTF) reviewed the advantages and disadvantages of screening for ovarian cancer and has found acceptable evidence that screening for ovarian cancer does not reduce the mortality (Force 2018).

1.2 Diagnosis

Diagnosis is histopathological, so a biopsy or surgical specimen is needed to make the diagnosis of ovarian cancer. Patients with suspected ovarian cancer may undergo further imaging once referred to a gynaecological oncologist; such as CT-TAP or MRI. Their case is then discussed at a multidisciplinary team meeting to decide the best course of treatment for that patient.

1.3 Histological subtypes

There are many different types of ovarian cancer that can be diagnosed, and the histological subtype determines the course of treatment for the women. Ovarian tumours can be classified according to their embryological cell of origin into three types: (1) surface epithelium (coelomic epithelium) (2) sex cord stroma (mesenchymal) (3) germ cells (mesonephric), and each one of them are divided into five subtypes (Table 1.1). More than 80% of ovarian cancers are epithelial, predominately serous carcinomas (Anagnostopoulos, Taylor et al. 2015).

1.3.1 Histological subtypes classification

Table 1.1: Classification of ovarian tumour

Surface epithelium	Sex cord	Germ cell
High grade serous	Granulosa cell	Dysgerminoma
Low grade serous	Granulosa cell	Dysgerminoma
Endometrioid	Thecoma	Yolk sac
Clear cell	Fibroma	Embryonal carcinoma
Mucinous	Sertoli, Leydig	Choriocarcinoma
Transitional cell	Steroid	Teratoma

- **Epithelial tumours**

Approximately 85% to 90% of ovarian cancer are Epithelial Ovarian Cancers (EOC), which are divided based on tumour behaviour into borderline tumours and invasive epithelial

tumours, and classified based on histological subtype into serous (low and high grade), endometrioid, clear cell, mucinous and transitional cell cancers (Figure 1.4) (Bast Jr, Hennessey et al. 2009). It is becoming increasingly evident that these tumours develop and behave like distinct entities. Serous tumours seem to arise from dysplastic epithelium in the distal fallopian tube (Vaughan, Coward et al. 2011, Bowtell, Böhm et al. 2015, Prazak and Gahres 2016, Dean, Davis et al. 2017, Pinto, Assis et al. 2017). Low grade serous tumours are associated with KRAS, BRAF, NRAS, HER2 mutations, and high grade serous tumours are strongly associated with p53, BRCA 1 & 2 mutations and homologous recombination deficiency (Figure 1.5) (Lawrenson, Ramus et al. 2009, Sudo 2012, Anagnostopoulos, Taylor et al. 2015, Bowtell, Böhm et al. 2015, Patch, Christie et al. 2015).

This kind of ovarian cancer generally presents at advanced stage. Endometrioid tumours are commonly low grade and associated with CTNNB1, PTEN gene mutation and Lynch syndrome. They appear to arise from endometriotic cysts, usually present at early stages, can be bilateral and accompanied by synchronous endometrial cancer. These low-grade tumours behave like endometrioid endometrial cancers, while their high-grade counterparts resemble HGSOc. Clear cell tumours are associated with ARID1A, CTNNB1 and PTEN mutations. They commonly arise on a background of endometriosis and are usually unilateral, respond poorly to chemotherapy and have a poor prognosis. Mucinous ovarian tumours do not usually originate in the ovary, but it is becoming increasingly evident that most of them may be metastatic to the ovary from the gastrointestinal tract, usually the appendix (Anagnostopoulos, Taylor et al. 2015). These tumours are associated with KRAS and HER2 mutations and are largely borderline with a small proportion being malignant and resistant to conventional chemotherapy. Borderline ovarian tumours show histological features that are intermediate between benign and malignant tumours. They are found in younger age women, can spread beyond the ovary to produce non-invasive implants in the omentum or peritoneum and can have late recurrences. It is probable that they represent premalignant disease for low grade ovarian carcinomas. There are major differences in incidence, tumour behaviour, and clinical outcome between each histological subtype. It has been estimated that ~50% of malignant ovarian tumours are serous carcinomas, while ~25%

are endometrioid carcinomas, ~10% are mucinous carcinomas, and ~5% are clear cell carcinomas (Davis, Tinker et al. 2014).

In terms of behaviour, serous carcinomas tend to be aggressive, high-grade neoplasms that spread rapidly throughout the pelvis, while endometrioid and mucinous carcinomas are usually low-grade tumours, confined to the ovary. Clear cell and endometrioid carcinomas, unlike other subtypes, are strongly linked to endometriosis (Djordjevic, Stojanovic et al. 2012). HGSOC is the most lethal type of gynaecological cancer with a high mortality due to late diagnosis (Chene, Chauvy et al. 2017).

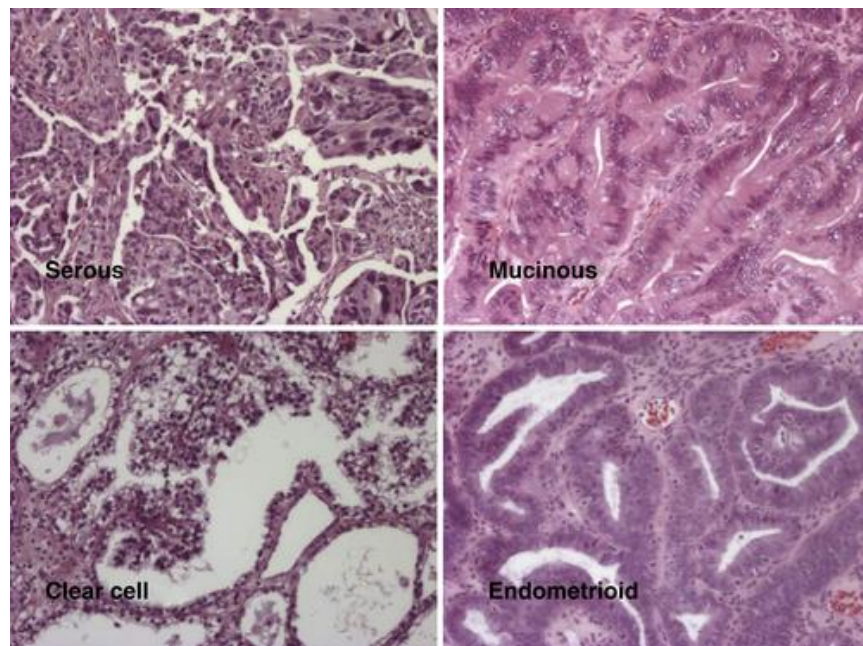


Figure 1.4 : Histology of different epithelial ovarian cancer subtypes. (Bast Jr, Hennessy et al. 2009).

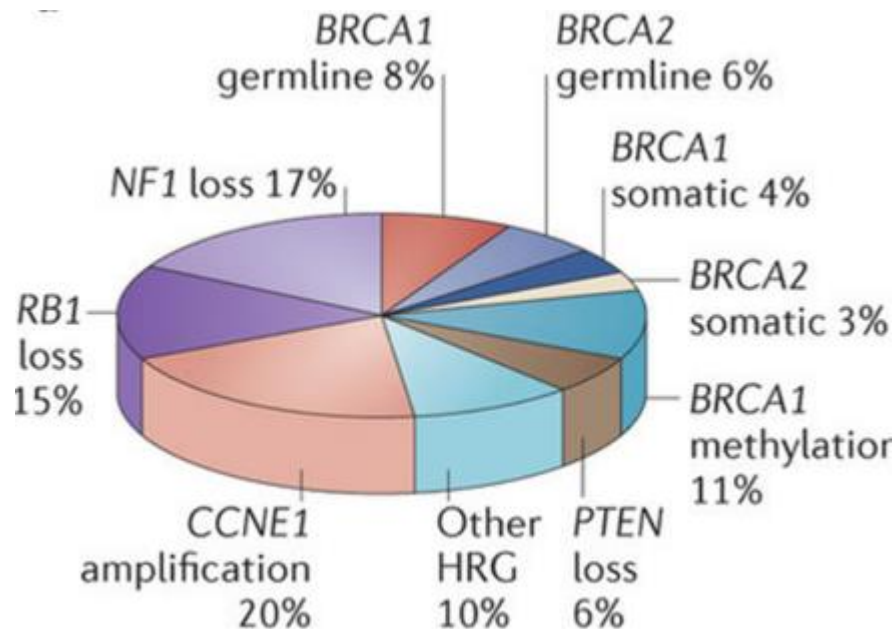


Figure 1.5: The frequency of key driver mutations in HGSOC

Percentage of primary tumours affected by homologous recombination pathway mutations and CCNE1 copy number gains, ranking mutations in BRCA1/2, followed by other germline, somatic, amplification, deletion and methylation events respectively (Patch, Christie et al. 2015).

- **Sex cord-stromal tumours**

Sex cord-stromal tumours represent approximately 7% of all malignant ovarian tumours. They arise from the hormone producing cells of the ovary and stromal fibroblasts. 70% of sex cord-stromal tumours are granulosa cell tumours. They usually present in women older than 60 years, but young or pre-pubertal women are uncommon. They are usually unilateral and can secrete sex hormones, mainly oestrogen. The vast majority of these tumours are diagnosed at an early stage with a favourable prognosis. Late recurrences can also occur. Fertility sparing surgery is suggested for younger women while chemotherapy (bleomycin, etoposide and cisplatin), is used for advanced disease or recurrences. Thecomas, fibromas, Sertoli Leydig cell tumours are rare, usually unilateral and can produce androgen leading to virilisation (Anagnostopoulos, Taylor et al. 2015).

- **Malignant germ cell tumours**

The commonest malignant germ cell tumour is dysgerminoma. Dysgerminomas occur mainly in adolescence and early adulthood and can be bilateral in up to 20%. Since over 60% are confined to one ovary at diagnosis, fertility sparing surgery with unilateral salpingo-oophorectomy or even ovarian cystectomy in selected cases is an option. Dysgerminomas spread through the lymphatic system and though highly radiosensitive, platinum-based chemotherapy is preferable due to fertility preservation. Yolk sac tumour, embryonal carcinoma, polyembryoma, nongestational choriocarcinoma and teratoma tumours are treated with chemotherapy (bleomycin, etoposide and cisplatin). Cure rates approaching 100% in early stage and 75% in advanced disease are usually achieved (Anagnostopoulos, Taylor et al. 2015).

1.3.2 Staging of ovarian cancer

The main purpose of staging systems is to understand the severity of a patient's clinical condition, predict the outcome, and plan a suitable treatment. The steps of staging typically begin by establishing the histopathological diagnosis, according to the tumour cell type, and assessing prognosis based on extent of disease and other known prognostic parameters. The first FIGO ovarian cancer staging was published in 1973. Since that time there have been two other changes including one in 1988 and 2013 (Table 1.2) (Mutch and Prat 2014, Ataseven, Chiva et al. 2016).

Table 1.2: 2014 FIGO ovarian, fallopian tube, and peritoneal cancer staging system and corresponding TNM

FIGO Stage	FIGO Classification	TNM
I	Tumor confined to ovaries or fallopian tube(s)	T1
IA	Tumor limited to one ovary (capsule intact) or fallopian tube, No tumour on ovarian or fallopian tube surface No malignant cells in the ascites or peritoneal washings	T1a
IB	Tumor limited to both ovaries (capsules intact) or fallopian tubes No tumour on ovarian or fallopian tube surface No malignant cells in the ascites or peritoneal washings	T1b
IC	Tumor limited to one or both ovaries or fallopian tubes, with any of the following: IC1 Surgical spill intraoperatively IC2 Capsule ruptured before surgery or tumour on ovarian or fallopian tube surface IC3 Malignant cells present in the ascites or peritoneal washings	T1c
II	Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or peritoneal cancer (Tp)	T2
IIA	Extension and/or implants on the uterus and/or fallopian tubes/and/or ovaries	T2a
IIB	Extension to other pelvic intraperitoneal tissues	T2b
III	Tumor involves one or both ovaries, or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes	T3
IIIA	Metastasis to the retroperitoneal lymph nodes with or without microscopic peritoneal involvement beyond the pelvis	T1, T2, T3aN1
IIIA1	Positive retroperitoneal lymph nodes only (cytologically or histologically proven)	
IIIA1(i)	Metastasis ≤ 10 mm in greatest dimension (note this is tumour dimension and not lymph node dimension)	T3a/T3aN1
IIIA1(ii)	Metastasis > 10 mm in greatest dimension	
IIIA 2	Microscopic extra pelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes	T3a/T3aN1
IIIB	Macroscopic peritoneal metastases beyond the pelvic brim ≤ 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes	T3b/T3bN1
IIIC	Macroscopic peritoneal metastases beyond the pelvic brim > 2 cm in greatest dimension, with or without metastases to the retroperitoneal nodes (Note 1)	T3c/T3cN1
IV	Distant metastasis excluding peritoneal metastases Stage IV A: Pleural effusion with positive cytology Stage IV B: Metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of abdominal cavity) (Note 2)	Any T, Any N, M1 T3c/T3cN1)

(Note 1: includes extension of tumour to capsule of liver and spleen without parenchymal involvement of either organ). (Note 2: Parenchymal metastases are Stage IV B).

1.4 Treatment

Treatment for ovarian cancer involves a combination of surgery and chemotherapy and it should be done in a specialist centre by a gynaecological oncologist. This combination has been found to increase survival for women with advanced stage ovarian cancer (Colombo, Van Gorp et al. 2006, Decruze and Kirwan 2006). Some patients may receive chemotherapy before having surgery to remove their tumours; this is known as neoadjuvant chemotherapy and is often followed by interval debulking surgery. Chemotherapy plays a key role in the management of women with epithelial ovarian, fallopian tube and primary peritoneal cancers (Colombo, Van Gorp et al. 2006, Prazak and Gahres 2016).

1.4.1 Surgical treatment

Surgery is the mainstay of treatment for women with early and advanced stage ovarian cancers (Tew and Fleming 2015, Tew 2016, Webber and Friedlander 2017). These patients will require a combination of cytoreductive surgery and chemotherapy to give them their best chance of long-term survival (Fruscio, de Haan et al. 2017). The purpose of cytoreductive or debulking surgery is to provide a histopathological diagnosis and to remove as much cancer as possible and then to establish the International Federation of Gynaecological Oncologists (FIGO) staging (Jayson, Kohn et al. 2014, Webber and Friedlander 2017).

1.4.2 Chemotherapy for ovarian cancer

Following surgical staging for ovarian cancer, most patients with advanced stage disease receive chemotherapy. This is referred to as adjuvant chemotherapy (Tew and Fleming 2015, Tew 2016, Fruscio, de Haan et al. 2017). Studies have shown that a combination of both a platinum-based chemotherapy and a Taxol (typically paclitaxel and carboplatin) improves the survival of women with ovarian cancer. Platinum-based therapy is an alkylating agent that kills cancer cells by binding to DNA and interfering with cell repair, leading to cell death (Gordon and Butler 2003, Januchowski, Zawierucha et al. 2014, Prazak and Gahres 2016).

Chemotherapy can be given by Intravenous (IV) or Intraperitoneal (IP) routes. Common side effects related to chemotherapy include fatigue, alopecia, myelosuppression,

neuropathy, and nausea and vomiting. Supportive medication is given to help alleviate symptoms of chemotherapy to allow for less toxicity related to treatment (Fruscio, de Haan et al. 2017, Webber and Friedlander 2017).

In patients who are unable to consider upfront surgical debulking, usually because complete cytoreduction is not possible at time of surgery, then it would be appropriate to treat with neoadjuvant chemotherapy.

1.4.3 Histology and treatment selection

HGSOC is generally very sensitive to platinum-based chemotherapy, with response rates of 80% or more. In contrast, clear cell carcinomas have poor response rates to platinum-based chemotherapy, with reports that up to 52% will progress on first-line therapy, and response rates ranging from 45% to as low as 11% (Webber and Friedlander 2017). A large international phase III study recruited 667 patients with stages I-IV clear cell ovarian cancer and compared cisplatin/irinotecan with paclitaxel/carboplatin as adjuvant chemotherapy (GCIIG/JGOG3017). The results were recently reported and showed that 2-year platinum-free interval (PFS) rates were no different and approximately 75%. Almost 65% of the patients had stage I disease and had an 89% 2-year PFS compared to a 40% - 50% 2-year PFS in stages II – IV (Sugiyama, Okamoto et al. 2016, Webber and Friedlander 2017).

Mucinous tumours also have a very poor response rate of 23%, and up to 63% of the patients show tumour progression on platinum based-chemotherapy [49]. As these tumours have histopathological similarities to gastrointestinal tumours, approaches utilising chemotherapy regimens conventionally used for colorectal cancer have been used, e.g. fluoropyrimidines in combination with oxaliplatin. However, a randomised phase II trial designed to compare a conventional ovarian cancer regimen of carboplatin/paclitaxel to capecitabine/oxaliplatin as first-line therapy for mucinous ovarian cancers was closed early because of poor recruitment (Parmar, Torri et al. 1998).

Low-grade serous tumours are biologically distinct from high-grade serous cancers and should be considered separately. These tumours are typically indolent and slow-growing

tumours and have very low response rates to chemotherapy. Maximal cytoreductive surgery is the mainstay of their management, whereas strategies targeting pathways such as MEK and KRAS/BRAF remain under investigation (Davis, Tinker et al. 2014, Webber and Friedlander 2017).

1.4.4 Neo-adjuvant chemotherapy treatment (NACT)

Neo-adjuvant chemotherapy (NACT) is typically considered for patients who are not good candidates for surgery at the time of diagnosis because of the extent of disease, poor performance status or medical comorbidities. This assessment to not proceed with surgical debulking must be made by a gynaecological surgical oncologist prior to starting neoadjuvant chemotherapy. Confirmation of disease can be made by a biopsy, fine needle aspiration (FNA) or a paracentesis. Most patients receive three cycles of chemotherapy, and provided there is evidence of response, they are offered interval debulking surgery followed by an additional three cycles of chemotherapy. The choice between primary cytoreductive surgery (PCS) and NACT remains controversial (Meyer, Cronin et al. 2016, Webber and Friedlander 2017).

1.4.5 Treatment of recurrent ovarian cancer

The development of resistance to chemotherapy represents another significant problem in treatment of ovarian cancer. The period of time between the end of primary chemotherapy and relapse is an indicator of resistance. The longer this period, the better chances of responding to chemotherapy (Markman and Bookman 2000, Armstrong 2002, Brigulova, Cervinka et al. 2010, Herzog, Krivak et al. 2010).

Despite a high response rate to first-line chemotherapy, most women with advanced stage ovarian cancer will relapse with a median PFS of 16 months after initial diagnosis. Recurrent disease can be detected biochemically based on a rising CA125; however, a rising tumour marker typically pre-dates clinical or radiological evidence of recurrence of cancer by about 3-6 months. The MRC-05 landmark trial demonstrated that there was no survival advantage associated with early initiation of chemotherapy in asymptomatic patients with recurrent disease based on GCIG CA125 progression alone. Early initiation of chemotherapy was also shown to have a negative impact on the quality of life.

Retreatment with chemotherapy should not be routinely commenced in asymptomatic patients with CA125 progression alone unless there are other reasons such as evidence of ascites or large-volume disease on radiological imaging as it would be expected that these patients would soon develop symptoms. For patients who are diagnosed with recurrent disease after an initial response to platinum-based chemotherapy, treatment approaches are based on the time from completion of previous therapy [also referred to as the PFI] to the date of recurrence. Patients with a PFI of six months or longer are considered to have platinum-sensitive disease with response rates to platinum-based chemotherapy ranging from 27% - 65% and a median survival of 12 - 24 months. Conversely, those with a PFI of <6 months are classified as having platinum-resistant disease, whereas patients who progress on chemotherapy or within 4 weeks of stopping chemotherapy are platinum refractory. Platinum sensitivity may be a function of the method used for detection of recurrence, with asymptomatic CA125 progression clearly representing a very different situation to a patient presenting with large-volume ascites and peritoneal disease (Friedlander, Trimble et al. 2011, Davis, Tinker et al. 2014, Webber and Friedlander 2017).

1.4.6 Targeted treatments in ovarian cancer

Understanding the molecular mechanisms of tumour angiogenesis led to the identification of potential angiogenic targets and the development of novel anti-vascular agents to treat ovarian cancer and other cancers, and many of these agents are being evaluated in clinical trials and have shown promising antitumor activity such as bevacizumab and PARP inhibitor (Folkman 1990, Hicklin and Ellis 2005, Lu, Thaker et al. 2008, Spannuth, Sood et al. 2008).

Bevacizumab was the first anti-vascular endothelial growth factor (VEGF) monoclonal antibody found to be active in EOC and has shown in several clinical trials. The combination of bevacizumab and paclitaxel plus carboplatin-based regimens offers a new treatment option for women with EOC, especially in those with a high risk of progression (Monk, Choi et al. 2005, Monk, Han et al. 2006, Burger, Sill et al. 2007, Mabuchi, Terai et al. 2008, Graybill, Sood et al. 2015, Ruan, Ye et al. 2018).

Poly (ADP ribose) polymerase (PARP) inhibitors are among the most exciting new classes of oncology drugs and obtained very promising results in EOC PARP inhibitors exert their anticancer effects by inhibiting the functions of PARP enzymes, which are instrumental in binding to damaged DNA and initiating repair (Plummer, Jones et al. 2008, Audeh, Carmichael et al. 2010, Domchek, Aghajanian et al. 2016, Ledermann 2016).

Mutations in one of the BRCA genes result in impaired homologous recombination in DNA repair, which causes genetic abnormalities that promote ovarian carcinogenesis. Interestingly, earlier reported randomized trials confirmed that PARP inhibitors provide a substantial clinical benefit to BRCA mutant patients and this defect has been exploited by the introduction of PARP inhibitors to provide specific cancer cell cytotoxicity (Jayson, Kohn et al. 2014, Suh, Kim et al. 2017, Cortesi, Toss et al. 2018, Morgan, Clamp et al. 2018).

Recently, immunotherapy has been used to treat patients with ovarian cancer by using immune checkpoint inhibitors which have demonstrated clinical response only in a small subpopulation of patients with ovarian cancer (Gaillard, Secord et al. 2016, Suh, Kim et al. 2017).

1.4.7 Chemoresistance of ovarian cancer

Mortality rates for ovarian cancer have not improved drastically in the last 40 years. In a clear majority of cases, this failure can be attributed to the development of Multidrug Resistance (MDR) and it is a term used to describe the broad-spectrum resistance to chemotherapy in human cancer (Dong, Mattingly et al. 2009). In chemoresistance, cancer cells develop the potential to survive in the presence of single or multi cytotoxic drugs. Therefore, exploring the mechanism of chemoresistance in ovarian cancer is not only beneficial for understanding disease progression, but also helpful to develop targeted treatment options to reverse this phenomenon (Hami, Rezayat et al. 2017). Common drugs used in the 1st and 2nd line treatment of ovarian cancer include

carboplatin, paclitaxel and doxorubicin and their mechanisms of resistance are detailed below.

Paclitaxel

The first extraction and characterization of Paclitaxel (PTX) was in 1971 from the Pacific yew tree. Various studies have described paclitaxel as an effective anticancer agent (Cheng, Jiang et al. 2002). Paclitaxel is a chemotherapeutic drug used to treat different kinds of cancer including OCs and kills cancer cells by inducing cell cycle arrest at G2/M phase by binding to the β -tubulin subunit and inhibiting their disassembly stimulating the apoptosis pathway by inducing mitochondrial stress (Sarisozen, Abouzeid et al. 2014).

Mechanisms of resistance to PTX

Several mechanisms have been demonstrated in the development of drug resistance to chemotherapy such as an increase in the expression of P-glycoprotein (P-gp) which is one of the ABC proteins family responsible for the MDR phenotype by activation of the efflux pathway to pump drugs outside the cancer cells. Altered accumulation of drugs and intracellular distribution or increases in the metabolism of drugs increase the repair level of damaged DNA and reduce apoptosis (Dumontet, Duran et al. 1996, Xue and Liang 2012, Xie, Cao et al. 2013).

Carboplatin

Carboplatin is a derivative of cisplatin and has a similar mechanism of action, differing only in terms of structure and toxicity. Carboplatin (1,1-cyclobutyldicarboxylate) is one of the main platinum-based drug used as an antitumor drug. It is intended specifically for the treatment of cancer of the testis, ovary, head, neck, and small cell lung cancer (Fuentes, Alonso et al. 2003). The main target of carboplatin is DNA, to which it binds efficiently, thereby inhibiting replication and transcription and inducing cell death (Brabec and Kasparkova 2005). The nature of these DNA adducts affects many transduction pathways and triggers apoptosis or necrosis in tumour cells. The adducts formed by this compound can be mono-adducts or intra and interchain di-adducts (Hah, Stivers et al. 2006).

To be activated, carboplatin must cross the cell membrane. Inside the cell, the molecule undergoes hydrolysis of 1,1-cyclobutanedicarboxylate, becoming positively charged. This allows carboplatin to interact with nucleophilic molecules within the cell, including DNA, RNA and protein, generating the formation of adducts of platinum. This process occurs through covalent binding of carboplatin to sites of purine bases, forming DNA-protein or DNA-DNA interactions (Hah, Stivers et al. 2006, Sousa, Wlodarczyk et al. 2014).

The linkage between DNA and carboplatin can produce lesions in DNA, inhibiting the process of DNA replication, causing changes that generate errors in replication, with the accumulation of cells in G2/M phase and the induction of apoptosis (Tapia and Diaz-Padilla 2013). Alkylation of a single strand of DNA can be repaired easily, but cross-linked inter strands; such as those produced by bifunctional alkylating agents require more complex mechanisms of repair (Siddik 2003, Sousa, Wlodarczyk et al. 2014).

Mechanism of resistance to carboplatin

The development of resistance to platinum-based chemotherapy is a major clinical challenge in cancer treatment, since the cellular response which confers resistance to carboplatin is multifactorial and poorly understood (Davis, Tinker et al. 2014). It has been observed that the most common intracellular mechanisms by which cells become resistant to carboplatin include decreases in drug accumulation in the cell, loss or decrease in mismatch repair, inhibition of apoptosis, and mutations in p53 (Mistry, Kelland et al. 1991, Helen HW and Macus Tien 2010, Sousa, Wlodarczyk et al. 2014). Decreased accumulation of the drug in the cell can be attributed to decreased influx, increased efflux or both. However, copper transporters could play a role in intracellular carboplatin accumulation. The major copper influx transporter is CTR1 which regulates carboplatin intake and ATP7A and ATP7B which are copper efflux transporters involved in efflux of the drug from the cells (Holzer, Katano et al. 2004, Safaei and Howell 2005). P-gp and MRP proteins are members of the adenosine triphosphate-binding cassette (ABC) family which are responsible for the transport of drugs from the cancer cells and

have been linked with MDR in ovarian cancer and other cancer types (Kool, de Haas et al. 1997, Choudhuri and Klaassen 2006, Korita, Wakai et al. 2010).

Doxorubicin

Doxorubicin (DOX) is an anthracycline antibiotic that is widely used against several solid tumours, such as ovarian cancers. One important disadvantage of DOX is the development of MDR in cancer cells due to the overexpression of P-gp, which can significantly influence the therapeutic effect of DOX (Lei, Srinivasan et al. 2011).

Mechanisms of resistance to DOX

There are two proposed mechanisms by which doxorubicin acts in the cancer cell.

- Intercalation into DNA and disruption of topoisomerase-II-mediated DNA repair.
- Generation of free radicals and their damage to cellular membranes, DNA and proteins.

In brief, DOX is oxidized to semiquinone, an unstable metabolite, which is converted back to DOX in a process that releases reactive oxygen species. Reactive oxygen species can lead to lipid peroxidation and membrane damage, DNA damage, oxidative stress, triggering apoptotic pathways of cell death. Candidate genes that may modulate this pathway involve those capable of the oxidation reaction (NADH dehydrogenases, nitric oxide synthases, xanthine oxidase), and those capable of deactivating the free radicals such as glutathione peroxidase, catalase, and superoxide dismutase. Alternatively, doxorubicin can enter the nucleus and poison topoisomerase-II, resulting in DNA damage and cell death. Candidate pharmacogenes for this part of the pathway include the enzymes involved in the DNA repair mechanisms and cell cycle control (TOP2A, MLH1, MSH2, TP53, and ERCC2 genes) (Thorn, Oshiro et al. 2011, JG Marin, Briz et al. 2012, Nielsen, Ejlersen et al. 2014).

1.4.8 Multiple strategies to overcome chemoresistance in cancer

In chemotherapy, one of the major issues is chemoresistance to different anticancer drugs such as the taxanes (paclitaxel), anthracyclines (doxorubicin) and platinum (carboplatin). However, there are some strategies that have been investigated to overcome chemoresistance such as:

- Modulation of multidrug resistance efflux pumps by using MDR inhibitors, MDR modulators, MDR reversal agents or chemosensitizers (Lei, Srinivasan et al. 2011, Miao, Du et al. 2013).
- Using anticancer drugs which are not substrates of ABC transporters (Miao, Du et al. 2013).
- Using small RNA interference (siRNA) therapy to overcome MDR, where the activity of MDR genes involved in drug efflux is suppressed (Miao, Du et al. 2013, Saraswathy and Gong 2013, Mariño, Blyuss et al. 2017).

Nanotechnology also offers a promising role to treat and overcome chemoresistance in ovarian cancer. Functionalised nanoparticles can enhance the therapeutic efficacy of anticancer agents at the target site by their passive and active tumour targeting abilities (Lei, Srinivasan et al. 2011, Mochalin, Shenderova et al. 2011, Alshatwi, Vaiyapuri Subbarayan et al. 2012, Arora, Jensen et al. 2012, Delie, Allemann et al. 2012, Perevedentseva, Hong et al. 2013, Narvekar, Xue et al. 2014, Boverhof, Bramante et al. 2015, Kathiravan, Ravi et al. 2015, Passeri, Rinaldi et al. 2015, Vijayanthimala, Lee et al. 2015, Wang, Lee et al. 2015, Miles, Greenwood et al. 2016, Chen and Zhang 2017, Khan, Saeed et al. 2017, Kumar, Mutreja et al. 2017, Turcheniuk and Mochalin 2017) and will be discussed in more detail below.

1.5 Nanotechnology

A small particle can be named as “nano” if it meets a set of predefined physical conditions as described by the European Commission (Kolitsi 2001, Commission 2011). A natural manufactured material containing particles, in an unbound state or as an aggregate or agglomerate can be considered nanosized if 1 – 50% of the sample population falls within the 1 nm – 100 nm size range as outlined by the United States Environmental Protection Agency (Usepa 2007, Boverhof, Bramante et al. 2015).

The term nanomaterial (NM) refers to an extensive and highly varied population of nano sized objects that have been extensively researched over the past few decades.

1.5.1 Types of Nanoparticles

Nanoparticles can be grouped into different classes depending on source of origin and composition (Figure 1.6) (Ponnappan and Chugh 2015).

1- Biological Nanoparticles

Biological NPs are synthesized naturally in a biological system, which can form part of intracellular structures such as exosomes or extracellular molecules. Some of these NPs are used in nanoparticle-based pharmaceuticals such as viruses, albumin and lipoproteins (Ponnappan and Chugh 2015, Singh, Kim et al. 2016).

2- Metal-Based Nanoparticles

Various nanoparticles for pharmaceutical applications have been designed with metals and metal oxides which include gold, iron, silica and silver nanoparticles. Metal-based nanoparticles have been of considerable interest as pharmaceutical agents as they cause *in vivo* cytotoxicity when administered at high concentrations (Schrand, Rahman et al. 2010, Saha, Agasti et al. 2012, Ponnappan and Chugh 2015).

3- Polymer-Based Nanoparticles

Several polymer-based nanoparticles have been used for biomedical applications including chitosan, gelatine, polylactic acid (PLA), polyglycolic acid (PGA),

polyethyleinimine (PEI) and copolymers such as poly (lactic-co-glycolic acid) (PLGA) (Vrignaud, Benoit et al. 2011, Ponnappan and Chugh 2015) .

4- Lipid-Based Nanoparticles

Liposomes, nanostructured lipid carriers (NLCs) and solid lipid nanoparticles (SLNs) are the lipid-based nanopharmaceuticals that find applications as nanocarriers. A few liposome-based nanoparticulate drugs have been already approved by the US FDA, and some of them are in clinical development. Examples include Doxil, a liposomal doxorubicin for treatment of metastatic breast cancer and ovarian cancer (Li and Szoka 2007, Ponnappan and Chugh 2015).

5- Carbon Nanoparticles

Carbon Nanoparticles include carbon nanotube (single-walled CNTs (SWCNTs) and multiwalled CNTs (MWCNTs)), carbon nanowires and nanodiamonds (Bondar' and Puzyr' 2004, Ponnappan and Chugh 2015).

6- Dendrimers

Dendrimers are synthetic, immensely branched nanoscopic macromolecules that form a tree-like structure, which include chitin, propylene imine, and melamine (Ponnappan and Chugh 2015).

1.5.2 Classification of NPs

NPs are classified according to chemical composition, structure, phase composition, or dimensionality.

1- In terms of chemical composition, nanomaterials are classified into:

- Organic structures which include polymer nanoparticles such as Poly ethylenimine (PEI), poly (lactic-co-glycolic acid) (PLGA), polylactide acid (PLA), and polyglycolic acid (PGA) or dendrimers such as Chitin, Propylene imine, and Melamine.
- Inorganic structures which include metal oxide nanoparticles, semimetal oxides, metal nanoparticles, semiconductor quantum dots or carbon structures

(nanotubes, graphene, fullerenes) (Figure 1.1) (Chan, Shiao et al. 2006, Zolnik and Sadrieh 2009, Fadeel and Garcia-Bennett 2010, Zhang, Zhi et al. 2010, Cai and Xu 2011, Geszke, Murias et al. 2011).

2- In terms of structure, nanomaterials are divided into:

- Quantum dots.
- Nanotubes.
- Nanowires.
- Dendrimers.
- Micelle formations such as spherical micelle, globular micelle, rod like micelle, and spherical bilayer vesical (Zolnik and Sadrieh 2009).

3- In relation to phase composition, nanomaterials can be classified as follows;

- One-dimensional nanoparticles; the one-dimensional system (thin film or manufactured surfaces) has been used for decades. Thin films (sizes 1–100 nm) or monolayers are now common place in the field of solar cells offering different technological applications, such as chemical and biological sensors, information storage systems, magneto-optic and optical device, and fiber-optic systems.
- Two-dimensional nanoparticles such as carbon nanotubes
- Three-dimensional nanoparticles such as dendrimers, quantum dots and fullerenes (Carbon 60) (Nikalje 2015, Bhatia 2016).

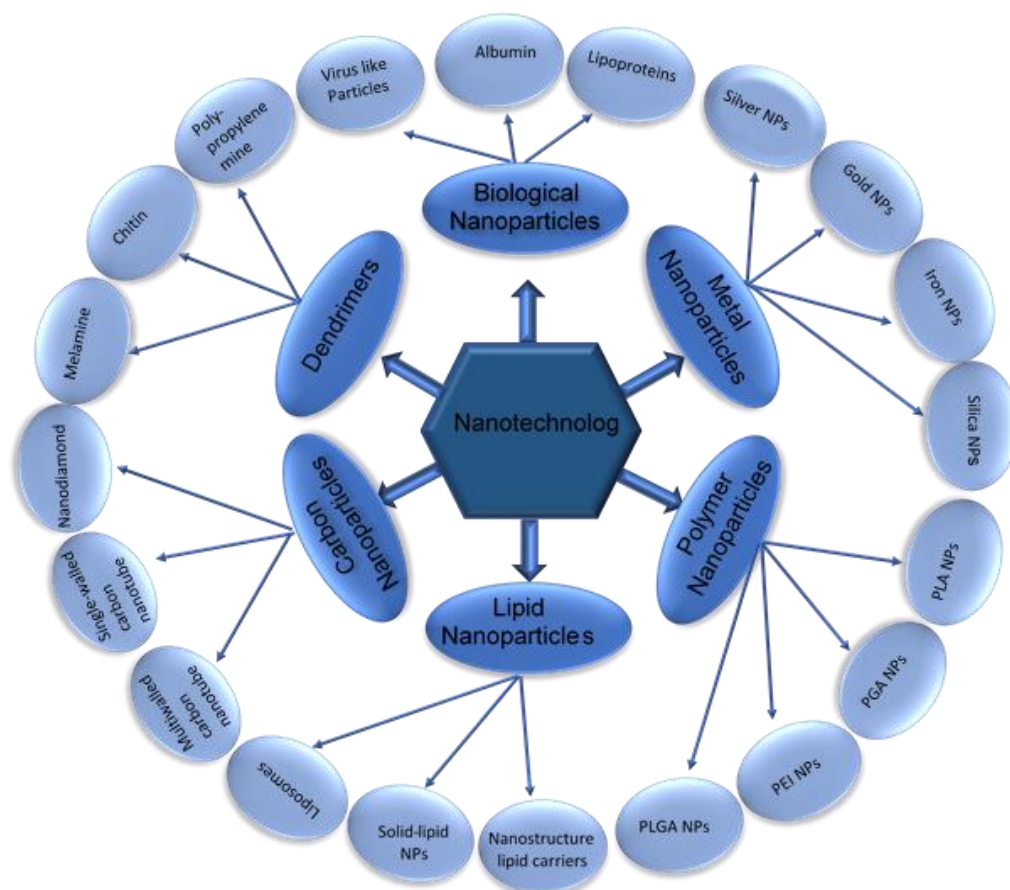


Figure 1.6: Different types of nanoparticles being used in the diagnosis and therapy of several human diseases, including cancer.

1.5.3 Synthesis of nanoparticles

Two main methods are employed for the synthesis of NPs

1- Top-down synthesis

In this method, a destructive approach is employed which reduces the size of larger molecules into smaller units. These are converted into suitable nanosized molecules by grinding/milling, and other decomposition techniques such as chemical and physical methods (figure 1.7) (Wang and Xia 2004, Iravani 2011, Mogilevsky, Hartman et al. 2014, Bello, Agunsoye et al. 2015).

2- Bottom-up synthesis

This approach is known as a building up approach and is employed in reverse as NPs are formed from relatively simpler substances. Examples of this method include chemical vapour deposition (CVD), physical vapour deposition (PVD), atomic or molecular condensation, spinning, and biochemical synthesis (figure 1.7)(Wang and Xia 2004, Iravani 2011, Mogilevsky, Hartman et al. 2014, Bello, Agunsoye et al. 2015).

Both top-down and bottom-up methods use synthetic polymers / monomer, stabilizers and organic solvents. Researchers are trying to find alternatives for NP synthesis by using natural polymers and synthesis methods with less toxic solvents (Hussain, Singh et al. 2016).

Green synthesis (bottom up synthesis) of NPs is an alternative route to physico-chemical methods. It refers to the formation of green nano-products and use of these products to achieve sustainable development and plays important roles in medicines, clinical applications and *in vitro* diagnostic applications (Gunalan, Sivaraj et al. 2012, Khan and Ahmad 2013, Jayalakshmi 2014, Arumugam, Karthikeyan et al. 2015, Kargar, Ghasemi et al. 2015).

The NPs produced by the green synthesis method are different from those produced by using physical and chemical approaches where the physical and chemical materials are replaced by natural products extracted from plants or microorganisms (Singh, Mishra et al. 2010, Hussain, Singh et al. 2016).

The use of natural materials for production of NPs is sustainable and eco-friendly as there is no harsh or toxic chemical used. It is inexpensive and free of chemical contaminants which is advantageous for biological and medical applications where purity of NPs is the main goal. Furthermore, NPs synthesised via the green route are more stable and effective in comparison with those produced by physico–chemical methods (Yeo, Chen et al. 2004, Mogilevsky, Hartman et al. 2014, Needham, Arslanagic et al. 2016, Parveen, Banse et al. 2016).

Different NPs are synthesized by biological methods (green synthesis) such as silver, gold, cadmium sulphide, iron, iron oxides, zinc oxide, cerium oxide, copper, copper oxide, and titanium dioxide (Sanghi and Verma 2009, Sangeetha, Rajeshwari et al. 2011, Xiong, Wang et al. 2011, Rajakumar, Rahuman et al. 2012, Balamurugan, Saravanan et al. 2014, Priya, Kanneganti et al. 2014, Kathiravan, Ravi et al. 2015).

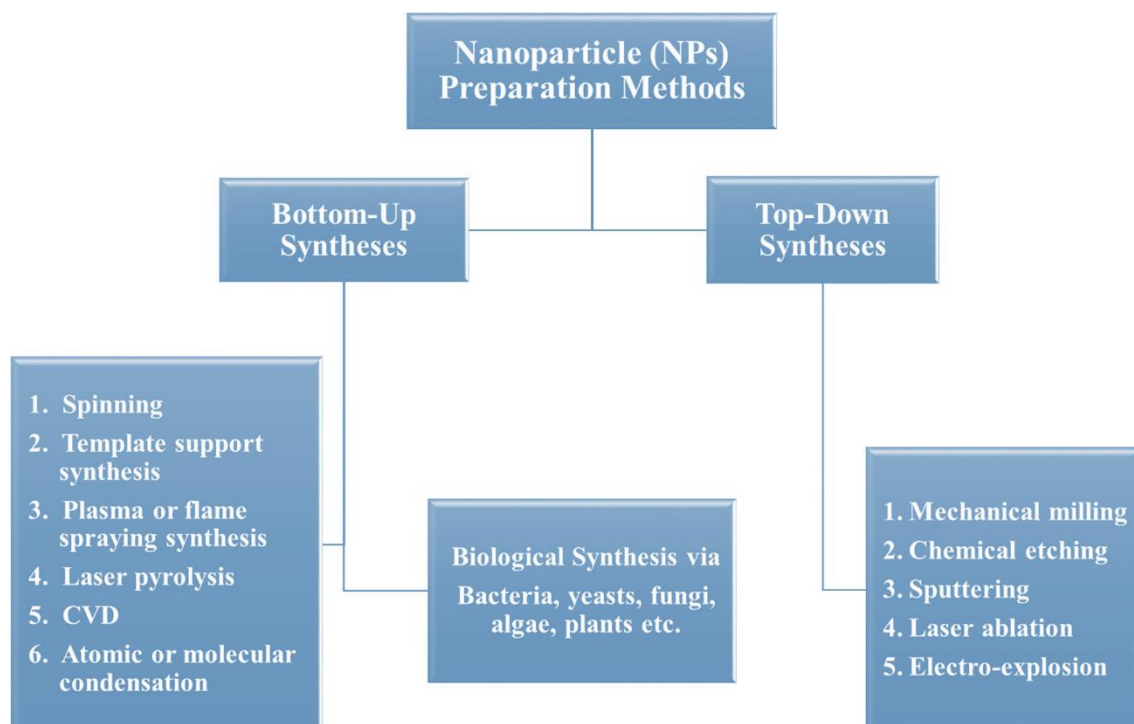


Figure 1.7: Typical synthetic methods for NPs for the top-down and bottom-up approaches (Khan, Saeed et al. 2017).

1.5.4 Characterisation of Nanoparticles (NPs)

After NPs are synthesized, the conformational details about shape, size, dispersity, and surface property are determined by using various techniques such as UV–Vis absorption spectroscopy, X-Ray Diffraction (XRD), Fourier Transmission Infrared (FTIR) spectroscopy, Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), Energy Dispersive X-ray Analysis (EDAX), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) (Fedlheim and Foss 2001, Raut Rajesh, Lakkakula Jaya et al. 2009).

FTIR spectroscopy is used to determine the functional groups present on the surface of NPs which might be responsible for reduction and stabilization of NPs (Sankar, Rizwana et al. 2015). The DLS and NTA are used to analyse the size distribution dispersed in liquid and the EDAX is used to determine the main component of NPs (Jiang, Oberdörster et al. 2009, Strasser, Koh et al. 2010).

1. Morphological characterisation

The morphology of the NP influences most of the properties of the NP and plays an important role in NP characterisation. There are different characterisation techniques for morphological studies, but microscopic techniques such as polarized optical microscopy (POM), SEM and TEM are the most important of these (Schaffer, Hohenester et al. 2009, Khan, Saeed et al. 2017).

2. Structural characterisation

The structural characteristics provide different information about NP properties. There are many techniques used to study structural properties of NPs such as XRD, energy dispersive X-ray (EDX), XPS, IR, Raman, BET, and Zeta size analyser. XRD is one of the most important characterisation techniques to explore the structural properties of NPs and it gives enough information about the crystallinity and phase of NPs (Sun, Murray et al. 2000, Fedlheim and Foss 2001, Raut Rajesh, Lakkakula Jaya et al. 2009, Emery, Saal et al. 2016, Khan, Saeed et al. 2017).

3. Particle size and surface area characterisation

Different techniques can be used to estimate the size of NPs. These include SEM, TEM, XRD, Atomic force microscopy (AFM), NAT and DLS. SEM, TEM, XRD and AFM can give more information about the particle size, but the zeta potential size analyser/DLS can

be used in the case of extremely small sized NPs (Kestens, Roebben et al. 2016, Sikora, Shard et al. 2016, Khan, Saeed et al. 2017).

In general NPs are composed of three layers; (the surface layer, the shell layer and the core). The surface layer can be functionalised with different small molecules such as drugs, antibodies and/or biological ligands (Shin, Cho et al. 2016).

The physical stability and the *in vivo* distribution of the nanoparticles are affected by different properties such as size distribution, particle diameter and charge, these determine the interaction of nanoparticles with the biological environment as well as their electrostatic interaction with bioactive compounds (Scholes, Coombes et al. 1999, Bhatia 2016).

1.5.5 Nanomaterials and biomedical applications

Nanotechnology in the field of medicine may revolutionise the way that we detect and treat cancer in the human body in the future, with most nano-techniques making remarkable progress towards becoming realities. Most of these techniques have been used for diagnostic purposes to detect cancer at an early stage by attaching antibodies with fluorescent molecules. They then attach to cancer cells allowing their identification using imaging systems. In addition, NMs have taken a new direction to specifically treat the cancer cells without causing any damage to the normal cells, minimising the side effect of treatments and increasing drug efficacy (Subbiah, Veerapandian et al. 2010, Yallapu, Jaggi et al. 2010, Narvekar, Xue et al. 2014, Gharpure, Wu et al. 2015). This can reduce systemic toxicity of anticancer drugs and potentially circumvent the problem of drug resistance. Table 1.3 summarises different nanoparticles being used as anticancer treatments and the mechanism of action of these particles. Nanoparticles can bypass drug efflux by ABC transporters as they are internalized by non-specific or specific endocytosis mechanisms which ultimately leads to higher intracellular drug levels (Xue and Liang 2012, Khan, Khan et al. 2017). Different mechanisms of intracellular uptake of various nanocarriers have been reported including: (a) macropinocytosis, (b) clathrin mediated endocytosis, (c) caveolae-mediated endocytosis, and (d) clathrin and caveolae-independent endocytosis (Shahzad, Khan et al. 2014, Khan, Khan et al. 2017). A successful nanocarrier should ideally meet the following requirements:

- Formulation with biocompatible/biodegradable/ bio-excretable materials,
- High drug and cargo loading capacity,
- Site-specific delivery mechanism to avoid normal cells and tissues,
- Zero or negligible premature drug release, and
- Controlled release mechanism to provide an effective dose to the target site
(Passeri, Rinaldi et al. 2015).

The use of NMs in medicine offers great possibilities to be a major tool for overcoming obstacles such as MDR cancer cells. The carbon-based materials include diamond nanoparticles (Cademartiri and Ozin 2009), which have shown great promise in biomedical applications and are the focus of this thesis.

Table 1.3 Summary of nanoparticles being evaluated as anti-cancer treatments

Type of nanoparticle	Mechanism	Tumour type	Ref
Polymer-oil nanostructured carrier	Apoptosis	Ovarian cancer	(Narvekar, Xue et al. 2014)
Folate-conjugated CAP-loaded lipid nanoparticles	Apoptosis	Ovarian cancer	(Lv, Zhuang et al. 2017)
Mesoporous silica nanoparticles	Apoptosis	Ovarian cancer	(Zhang, Guo et al. 2015)
Poly (lactic-co-glycolic acid) nanoparticles	Apoptosis	Breast cancer	(Sharma, Sharma et al. 2017)
Gold nanoparticles	Apoptosis	Human cervical cell lines	(Tomoaia, Horovitz et al. 2015)
Polymeric micelles	Apoptosis	Ovarian cancer	(Gou, Liu et al. 2015)
Micelles	Apoptosis	Glioblastoma	(Sarisozen, Dhokai et al. 2016)
Platinum nanoparticles	Apoptosis	Human adenocarcinoma (lung, ovarian, pancreatic and normal peripheral blood mononucleocyte)	(Bendale, Bendale et al. 2017)
Polymeric micelles	Apoptosis	Cervical and ovarian cancer	(Luong, Kesharwani et al. 2017)
NDs	Apoptosis	Cervical cancer cells	(Mytych, Lewinska et al. 2014)

Nano-sized layered double hydroxide (LDH)	Apoptosis	Non-small cell lung Cancer	(Zhu, Wang et al. 2016)
Dendrimer nano-architectures	Apoptosis	Cervical and ovarian cancer	(Luong, Kesharwani et al. 2016)
APRPG conjugated PEG-PLGA nanoparticles	Apoptosis	Ovarian cancer	(Wang, Liu et al. 2014)
Nanodiamonds	Apoptosis and P-gp downregulation	Recurrent mammary tumours and liver cancer	(Chow, Zhang et al. 2011)
Nanoscale metal-organic frameworks	Apoptosis and P-gp downregulation	Ovarian cancer	(He, Lu et al. 2014)
Lipid nanoparticles	Overcoming P-gp-mediated drug resistance	Human ovarian carcinoma cell line	(Dong, Mattingly et al. 2009)
Lipid-functionalised dextran-based biocompatible nanoparticles	Suppressed P-glycoprotein expression	Osteosarcoma and Ovarian cancer	(Kobayashi, Iyer et al. 2013)
Iron oxide-titanium dioxide core-shell nanocomposites	Evaded P-gp-mediated drug export	Ovarian cancer	(Arora, Jensen et al. 2012)
Nanodiamonds	Overcoming P-gp	Breast cancer	(Toh, Lee et al. 2014)
Gold nanoparticles	Overcoming P-gp	Human hepatoma cell lines	(Gu, Cheng et al. 2012)
Silicon nanowires	Overcoming P-gp	Breast cancer cells	(Peng, Su et al. 2014)
Nucleic acid nanoparticles	Overcoming P-gp	Ovarian cancer	(Pi, Zhang et al. 2017)

D-α-tocopherol polyethylene glycol succinate	Inhibit P-gp and induce apoptosis and necrosis	Ovarian cancer cells and Mouse sarcoma tumour cell lines	(Bao, Guo et al. 2014)
Nanodiamonds	Necrosis	Cervical and lung cells	(Lim, Jung et al. 2016)
Ormosil nanoparticles	Necrosis	Ovarian cancer	(Nagesetti, Srinivasan et al. 2017)
Poly (lactic-co-glycolic acid) nanoparticles	Limiting expression of chemo-resistant genes and apoptosis	Ovarian cancer stem cells	(Abou-ElNaga, Mutawa et al. 2017)
Self-assembled nanoscale coordination polymers	Downregulating the expression of MDR genes	Ovarian cancer	(He, Liu et al. 2015)
Mesoporous silica nanoparticles	Knocks down gene expression of a drug exporter by delivering siRNA	Cervical adenocarcinoma	(Meng, Liong et al. 2010)

1.5.6 Diamond Nanoparticles (Nanodiamond)

Nanodiamonds (NDs) are regarded as a new class of nanoparticle in the carbon family, with remarkable physical and chemical properties. The first production of nanoscale diamond particles was in the 1960s. NDs are produced primarily via two mechanisms through top down synthesis pathway: (1) High temperature/high pressure (HTHP) (2) Detonation methods. As a result, this simple material exhibits adaptable applications in physics, chemistry, materials science and electronics, among others. Recently, the low cytotoxicity of NDs has indicated their potential for medical and biological applications, hence the interest in nanodiamond applications in biology and medicine is on the rise over recent years (Turcheniuk and Mochalin 2017). Several important breakthroughs have led to a wider interest in these particles, including:

- The size distribution of single diamond particles with a small diameter of 4–5 nm (single-digit nanodiamonds) became available.
- Low cost and flexible production.
- Researchers started to use fluorescent nanodiamonds due to its non-toxicity and as a result, it is currently being considered for applications in biomedical imaging, drug delivery and other areas of medicine.
- Nanoscale magnetic sensors based on nanodiamonds were developed.
- The surface properties of NDs allows a variety of drugs and biomolecules to be attached.
- Rich chemistry of nanodiamond surface, as well as bright and robust fluorescence resistant to photobleaching are the distinct parameters that render nanodiamonds superior to any other nanomaterial such as gold nanoparticles, when it comes to biomedical applications (Mochalin, Shenderova et al. 2011, Passeri, Rinaldi et al. 2015, Turcheniuk and Mochalin 2017).

The surfaces of diamond nanostructures are crucial when determining their utility and biocompatibility. In particular, the surface of NDs is important for biological and medical applications when assessing targeted drug delivery, selective bio-sensing and effective therapy. The diamond surface must first undergo harsh treatment with strong chemicals or plasma irradiation in order for functional groups to be introduced onto the surface.

Then various linker molecules or biomolecules, including biomarkers and drugs can be linked onto the surface (Chen and Zhang 2017).

In order to approve a new material for biomedical applications, it is essential to investigate biocompatibility and toxicity, and to understand the absorption, distribution, metabolism and excretion characteristics of the NDs. Many experiments have been performed in which various concentrations of diamond nanostructures have been incubated with various cancer cell lines and the resulting viabilities of the cells have been tested. All results indicate that cultured cancer cell viabilities are not negatively affected at reasonably high concentrations of up to 200 $\mu\text{g/ml}$ of nanodiamonds after 10 days incubation (Perevedentseva, Hong et al. 2013, Perevedentseva, Lin et al. 2013, Chen and Zhang 2017). Moreover, it was shown that in the presence of NDs, the process of cell division was not impaired. In addition, the ND particles did not alter the cell cycle or affect total cell number, these findings proved that endocytic ND particles were noncytotoxic for cell division and differentiation (Perevedentseva, Lin et al. 2013).

Fluorescent NDs are NDs which display a fluorescent centre in their cores providing more advantages for *in vitro* and *in vivo* imaging. Most NDs are very small (5 nm or less in diameter) with the potential to penetrate into the smallest pores in the body. These specific properties of NDs are unmatched by any other carbon or non-carbon nanomaterial making NDs a superior nanomaterial for theranostics (Figure 1.8). NDs offer additional benefits as they can be relatively easily and inexpensively produced by detonation on a large industrial scale and are available commercially for quite an affordable price. All these factors contribute to a growing interest in using the hardest material (diamond) in its nanoscale form to fight some of the most difficult problems (cancer) faced by human society (Turcheniuk and Mochalin 2017).

The size of the nanoparticles (NPs) plays an important role in determining their behaviour in the biological system. For example, passive transport through nucleolemma is only possible for NPs of 5 nm or less. Single-digit detonation NDs meets this requirement and potentially can passively deliver drugs not just into the cytoplasm,

but can deliver it further into the nucleus (Martín, Alvaro et al. 2010, Turcheniuk and Mochalin 2017).

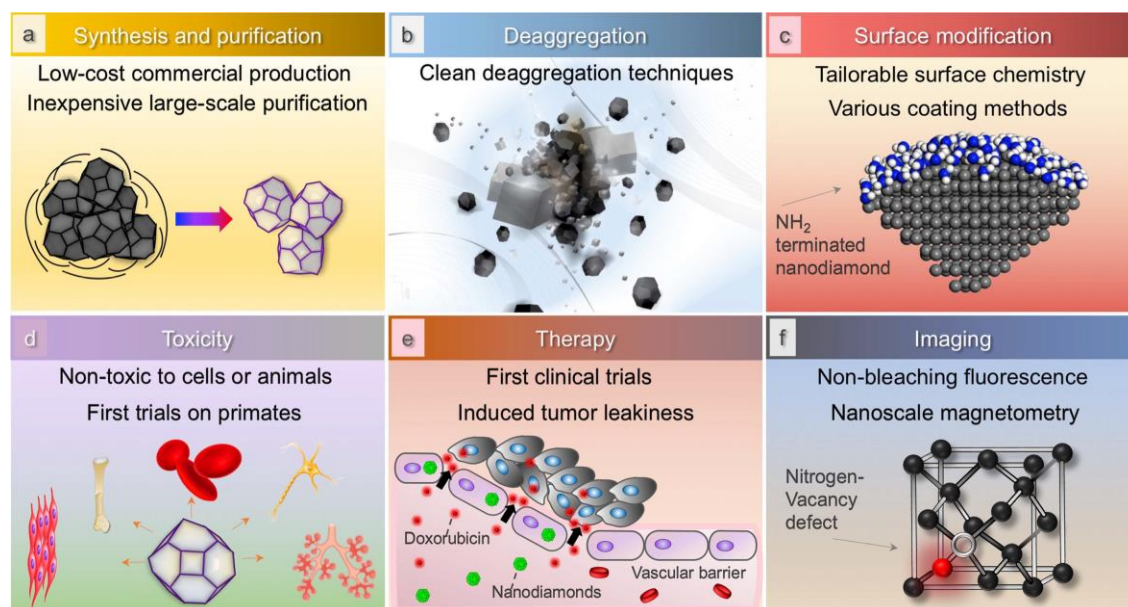


Figure 1.8: Steps towards nanodiamonds as theranostic

(a) synthesis and purification, (b) deaggregation, (c) surface modification, (d) toxicity evaluation, (e) nanodiamond-based therapy, (f) biomedical imaging. The schematic figure is adapted from (Turcheniuk and Mochalin 2017).

1.5.7 Nanodiamond and Drug Delivery Approach

Chemotherapeutic drugs in solution or in a polymer solution which are delivered orally or intravenously have poor pharmacokinetics with a narrow therapeutic window (Yallapu, Jaggi et al. 2010). These drugs reach a maximum tolerated concentration immediately and then are eliminated from the body. An ideal drug formulation with maximum benefits for patients should release at a minimum effective concentration over a period of time. NMs have the potential to address this issue. In general, NM delivery systems are known to carry the incorporated drugs into cells and improve the intracellular concentration of the drug. Studies have shown that intracellular entry of drug loaded NPs (functionalised NPs) delivery systems can be via endocytosis, followed by release of loaded drugs in the cytoplasm. This is an alternative route of drug entry that enables bypassing or inhibition of P-gp-mediated efflux (Yallapu, Jaggi et al. 2010, Narvekar, Xue et al. 2014, Gharpure, Wu et al. 2015).

The specificity of the functionalisation can be further enhanced by the conjugation of antibodies to the ND formulations, and these immune conjugated formulations will have a better therapeutic efficacy over other drug formulations (Narvekar, Xue et al. 2014).

It has been reported that NDs were complexed with anti-ovarian cancer drugs such as cisplatin for ovarian cancer treatment, which induced higher cytotoxicity effects than drugs alone (Huynh, Pearson et al. 2013, Ho, Wang et al. 2015). Table 1.4 lists examples of different anti-cancer drugs complexed to NDs.

In addition to facilitating the delivery of water-insoluble drugs, NDs can also be used to enhance drug efficacy (Perevedentseva, Lin et al. 2013). NDs have been used to assist the delivery of DOX (ND/DOX) to liver and breast cancer cells and were more effective than the drug alone. In addition, the blood circulation time of the ND-DOX complexes were 10 times longer than that of free DOX molecules. As a result, the ND-DOX complexes could efficiently induce damage and shrink the size of xenograft mice tumours (Chow, Zhang et al. 2011, Chen and Zhang 2017). It has also been demonstrated that NDs can improve DOX accumulation in murine liver tumour models and thus inhibit metastasis to different parts of the body (Chow, Zhang et al. 2011, Man

and Ho 2012). In parallel with DOX, many other drugs have been delivered with the assistance of ND. In addition to single-drug therapies, NDs can also be used to deliver a combination of several drugs in a single treatment for a synergistic effect against multiple cancer cell lines (Wang, Lee et al. 2015, Chen and Zhang 2017).

NDs have improved cellular internalisation and therapeutic efficacy when linked with paclitaxel (NDs-PTX) for drug delivery and cancer therapy (Liu, Zheng et al. 2010). The successful conjugation was confirmed using atomic force microscopy, nuclear magnetic resonance spectroscopy and infrared spectroscopy. The complex was shown to reduce cell viability, and induce mitotic arrest and apoptosis in breast and lung human carcinoma cells (Perevedentseva, Lin et al. 2013).

To date, NDs have been functionalised either covalently or electrostatically with a vast array of therapeutic molecules, including DNA, RNA, hormones, proteins, vitamins, and small-molecule therapeutics (Kostarelos, Lacerda et al. 2007, Mochalin, Shenderova et al. 2011, Moore, Gatica et al. 2012, Huynh, Pearson et al. 2013, Hsiao, Hui et al. 2016, Whitlow, Pacelli et al. 2017).

NDs have been studied for adsorption and triggered release of many anticancer drugs such as doxorubicin, cisplatin and paclitaxel. When entering the cell in the form adsorbed on NDs, the drugs cannot be easily ejected by cell efflux mechanisms. On the other hand, the drugs are slowly desorbed from the NDs inside the cancer cells maintaining the level of therapeutic concentrations, an effect that has been used to reverse the drug resistance of cancer cells to traditional chemotherapeutics (Turcheniuk and Mochalin 2017).

As tumours have a leaky vasculature, this promotes the uptake of nanocomplexes, and they become trapped inside. The poor lymphatic drainage promotes the enhanced permeability and retention (EPR) index. In addition, the size and charge of nanoparticles allows for passive targeting into tumours, passive targeting takes place through the diffusion into tumours or angiogenic tumour vasculatures. The nano formulations (drug loaded nanoparticles) have more interstitial access to the tumour and enhance the

retention in tumours. In comparison, active targeting involves the conjugation of nanoparticles to immunogens (antibodies or targeting moieties) which improves the delivery of the drug. This improved delivery is due to increased uptake as a result of longer circulation because of the EPR effect. In addition, the targeting moiety assists in endocytosis of nanoparticles which, in general, increases internalisation of nanoparticles (Yallapu, Jaggi et al. 2010).

Improving drug delivery systems using drug complexed to NPs and conjugated with antibody has the potential to advance cancer treatment and improve quality of life for patients. NDs are now playing an integral role in drug delivery systems. NDs are being used to manipulate not only the size of the drug particles but the physical characteristics and thus the extent and location of drug delivery (Subbiah, Veerapandian et al. 2010, Ho, Wang et al. 2015). The ability of NDs to transform cancer treatment will be assessed in this thesis.

Table 1.4: Different anti-cancer drugs complexed to NDs.

Composition	Drug	Results	References
NDs	Purvalanol A, 4-hydroxytamoxifen	NDs enhance drug dispersion in water, and maintain drug cytotoxicity	(Chen, Pierstorff et al. 2009, Passeri, Rinaldi et al. 2015)
Nanodiamond-embedded polymeric microfilms	Doxorubicin	Slow release; DOX-NDs patch in pre- and postoperative therapies; localized therapeutic release and tumour apoptosis prior to surgical intervention, or reduce the recurrence of cancer after surgery	(Lam, Chen et al. 2008, Passeri, Rinaldi et al. 2015)
Novel covalently bonded ND-PTX conjugate	Paclitaxel	Mitotic arrest and apoptosis of cancer cells. Inhibition of tumorigenesis in human lung cancer cells in xenograft SCID mice	(Liu, Zheng et al. 2010, Passeri, Rinaldi et al. 2015)
DOX and TAT conjugated to NDs surface through carbodiimide coupling	Doxorubicin	TAT-ND–DOX enhance translocation across C6 glioma cells membrane and exhibit a higher cytotoxicity effect	(Li, Shao et al. 2011, Passeri, Rinaldi et al. 2015)
NDs covalently conjugated with PEG-4000	Doxorubicin	Delivery of Dox into HepG2 via a clathrin-dependent endocytosis, increase of uptake half-life of ND-PEG-DOX in comparison to free DOX.	(Wang, Tong et al. 2013, Passeri, Rinaldi et al. 2015)
NDs embedded in PEG diacrylate gels	Daunorubicin and Doxorubicin	Sequential elution	(Xiao, Duan et al. 2013, Passeri, Rinaldi et al. 2015)

DSPE-PEG 2K coating ND DNX	Doxorubicin	Enhancement of DOX cytotoxicity, cell apoptosis, inhibition of lung metastasis of breast cancer, reduction of systemic toxicity	(Xiao, Duan et al. 2013, Passeri, Rinaldi et al. 2015)
ND with cisplatin incorporated via direct attachment to ND surface, physical adsorption POEGMEMA surface coating, or complexation to MAETC groups of a POEGMEMA-st-PMAETC surface layer	Cisplatin	All ND systems displayed lower IC50 values than free cisplatin in A2870 and A2870cis ovarian cancer cells. POEGMEMA-coated particles the most cytotoxic,	(Huynh, Pearson et al. 2013, Passeri, Rinaldi et al. 2015)

1.6 Aims and Hypotheses

1.6.1 Hypotheses

We hypothesise that multi-functionalised nanomaterials with specific biomarkers and/or drugs might provide a greater advantage in regards to:

- Improving the treatment strategy.
- Overcoming chemoresistance.
- Reducing the side effects of treatment.

1.6.2 Aims

The aim of this project was to establish a novel strategy for ovarian cancer therapy by developing “nanocarrier” complexes. A functionalised diamond-based nanomaterial loaded with chemotherapeutic agents such as Paclitaxel (PTX), Carboplatin (CARBO) and Doxorubicin (DOX), and coupled with HE4 or CA125 to direct and deliver a chemotherapy payload would be evaluated to:

- Assess the ability of the nanocomplex to enhance the treatment of ovarian cancer in a cell line model.
- Assess the mechanism of action of the nanocomplex.
- Assess the ability of the nanocomplex to improve the efficacy of chemotherapy in an ovarian cancer explant model.

Chapter 2

MATERIALS AND METHODS

2 Materials and Methods

2.1 Materials

Table 2.1: Chemotherapy drugs

Drugs	Manufacturer
Paclitaxel (PTX)	Teva Pharma B.V., The Netherlands
Carboplatin (CARBO)	Teva Pharma B.V., The Netherlands
Doxorubicin (DOX)	Teva Pharma B.V., The Netherlands

Table 2.2: Chemicals and reagents for cell culture and cytotoxicity

Product	Manufacturer	Cat #
RPMI media	Life technologies UK	179140
Phosphate-buffered saline	Life technologies, UK	1880350
Fetal Bovine serum	Sigma-Aldrich, UK	BCBV8238
TrypLE™ solution	Life technologies, UK	1896988
Penicillin and Streptomycin	Corning, USA	30001132
Paraformaldehyde (PFA)	Sigma-Aldrich, UK	P6148
Hoechst	Thermofisher scientific, USA	62249
Mitochondrial Membrane Potential tracker	Thermofisher scientific, USA	1892089
Cell Membrane Permeability dye	Thermofisher scientific, USA	1860267

Table 2.3: Materials used for western blots

Product	Manufacturer	Cat #
Trizma Base	Sigma Aldrich, UK	T1503
Trizma-HCl	Sigma Aldrich, UK	T5941
Glycine	Sigma Aldrich, UK	G8898
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, UK	L3771
Sodium chloride (NaCl)	Sigma Aldrich, UK	S7653
Methanol	Sigma Aldrich, UK	32213
Ammonium persulfate	Sigma-Aldrich, UK	A3678
2-Mercaptoethanol	Sigma Aldrich, UK	M3148
Bromophenol blue	Sigma Aldrich, UK	114391
Ponceau S	Sigma Aldrich, UK	P3504
Tween 20	Amresco, USA	M147
Luminol western reagent	Santa Cruz Biotechnology, Germany	SC-2048
Milk powder	Marvel	
Restore Plus Western Stripping Buffer	Thermofisher scientific, USA	46430
Gel Loading tips	Sigma Aldrich, UK	CLS4853
Blotting Paper	Sigma Aldrich, UK	GB003
Whatman westran 0.2µm PVDF membranes 20cm x3m roll	Sigma Aldrich, UK	Z613835
Bradford reagent	Sigma-Aldrich, UK	SLBB8733V
Pierce ® RIPA buffer	Thermofisher scientific, USA	89900
Protease inhibitor	Thermofisher scientific, USA	1862209
Bovine Serum Albumin (BSA)	Sigma-Aldrich, UK	A2153

Table 2.4: Antibodies used for western blots

Antibodies	Manufacturer	Cat #
HE4 Antibody (2.5mg/ml)	Fujirebio Diagnostics Ab, Sweden	33457
CA125 Antibody (2.4mg/ml)	Fujirebio Diagnostics Ab, Sweden	34827
PGP (E-10)	Santacruz Biotechnology, Germany	F0916
CAS-3 (E-8)	Santacruz Biotechnology, Germany	C2514
B-ACTIN	Santacruz Biotechnology, Germany	J1916
Goat Anti-Mouse Immunoglobulins HRP	Dako, USA	P044701-2
Goat Anti-Rabbit Immunoglobulins HRP	Dako, USA	P044901-1

Table 2.5: Materials used for nanodiamond functionalization

Product	Manufacturer	Cat #
Diamond nanoparticles	Nanodiamond Products Limited, Ireland	
2-methoxy (poly ethyleneoxy)-propyl trimethoxy saline	Gelest, USA	SIM6492.7
NaOH	Sigma-Aldrich, UK	71687-100G
1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC)	Sigma-Aldrich, UK	E7750-100G
PBS pH 6.4	Sigma-Aldrich, UK	40-0201 SAJ
Paclitaxel (PTX)	Teva Pharma B.V., The Netherlands	
Carboplatin (CARBO)	Teva Pharma B.V., The Netherlands	
Doxorubicin (DOX)	Teva Pharma B.V., The Netherlands	
HE4 Antibody	Fujirebio diagnostic inc, Sweden	33457
CA125 Antibody	Fujirebio diagnostic inc, Sweden	34827

2.2 Methods

2.2.1 Cell lines

Chemoresistant ovarian cancer cell lines (OAW42, SKOV-3 and A2780-cis), chemosensitive ovarian cancer cell lines (OVCAR-8 and A2780-sen) and the immortalised human ovarian surface epithelial cell line (HIO-80) (which was used as control) were used in this study. OAW42 was purchased from Sigma Aldrich, SKOV-3, A2780-sen, A2780-cis, and HIO-80 cell lines were received as a kind gift from the Discovery consortium and OVCAR-8 was received as a gift from S. Gray, Trinity College Dublin. Cell lines had been fingerprinted by Source Biosciences (Nottingham, UK) to confirm identity by examining standard DNA microsatellite short tandem repeat (STR).

2.2.2 Cell culture

1. Cell lines were cultured in 75ml flasks using RPMI-1640 media supplemented with 10% Fetal Bovine Serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin antibiotic. Cells were cultured under standard cell culture conditions (37°C and 5% CO₂). Cells were cultured until 80% confluent (3-4 days).
2. Cells were passaged using 2 ml 1X TrypLE™ solution to detach cells for 5min in 37°C, then vortexed and centrifuged for 4 min at 3000 rpm.
3. 10ml of complete RPMI medium was added and cells split in a ratio of 1:4 to 1:10 depending on the characteristics of each cell line.
4. Finally, the flasks were incubated at 37°C again. A2780cis cells were maintained in complete RPMI media supplemented with 1 µM cisplatin every 3-4 passages to maintain cisplatin resistance.

2.2.3 Cell Seeding and Scanning

1. Cells were seeded in 96 well plates, 100 µl/well at a cell density $3-4 \times 10^3$ cell/100µl.
2. Cells were allowed to attach overnight at 37°C for 24h.
3. The next day cells were treated according to the experiment design procedure (Cytotoxicity experiment, chapter 3, section 3.2) then the plates were incubated at several time points (24h, 48h, and 72h).
4. Following incubation, cells were stained with Mitochondria Membrane Potential (MMP) tracker (1:2000) and/or Cell Membrane Permeability (CMP) (1:1000) dye and

Hoechst dye (1:1000) to measure the cell viability, mitochondrial changes, cell membrane damage and cell cycle, then cells were incubated for 1h at 37°C.

5. Cells were fixed with 3% paraformaldehyde (PFA) for 30 min at 37°C.
6. Cells were washed three times 5 min each using PBS.
7. The plates were scanned using the HCS and Cytell [™] imaging system and data was collected and analysed using IN Cell Investigator software (version 1.6).
8. The cell cycle was measured using cell cycle BioApp in the Cytell imaging system which provides a complete single colour assay workflow to identify cells in all stages of the cell cycle according to DNA content (GE Healthcare Life Sciences).

2.3 Nanodiamond Pegylation (ND–PEG)

1. Nanodiamond powder (100µg) (provided as a gift from Nanodiamond Products Limited, Ireland) was re-suspended in deionised water (DW) to form nanodiamond solution and autoclaved.
2. NDs (100µl) were dispersed in 0.2 mM 2-methoxy (poly ethyleneoxy)-propyl trimethoxy saline.
3. The mixture was stirred at 37°C overnight.
4. The resulting colloidal suspension containing methoxy-PEG-coated nanoparticles (ND–PEG) was washed three times and then re-suspended in DW.
5. Pegylated NDs were stored at 4°C until further use.

2.3.1 Preparation of the Nanodiamond-Drug Complex.

To form the ND-drug complex

1. ND-PEG solution (100 µg/mL) and DOX (10 µM/mL), CARBO (14µM/ml) and PTX (2.5nM/ml) were mixed.
2. NaOH solution was added until the final concentration of NaOH was 2.5mM. Following reaction at room temperature (RT),
3. the mixture was centrifuged for 15 min at 1,000 rpm.
4. the pelleted ND/Drugs were washed five times with DW and then re-suspended in DW.

2.3.2 Conjugation of HE4 and CA125 to ND/drugs

1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) reagent was used to facilitate the conjugation either of HE4 (2.5mg/ml) or CA125 (2.4mg/ml) onto the ND/Drugs surface.

1. 50mg of EDC was dissolved in 14ml of PBS pH 6.4. This was added to a mixture containing 100µg of ND/Drugs and 50µl of HE4 or CA125 (Figure 2.1).
2. The reaction mixture was mixed gently overnight at RT.
3. The ND-drug-HE4 complex (ND/Drug/HE4) and/or ND-drug-CA125 complex (ND/Drug/CA125) was dispersed in 1000µl of DW.
4. Two controls were run in the same manner as the reaction mixture; one lacked EDC and the other lacked both EDC and antibody.

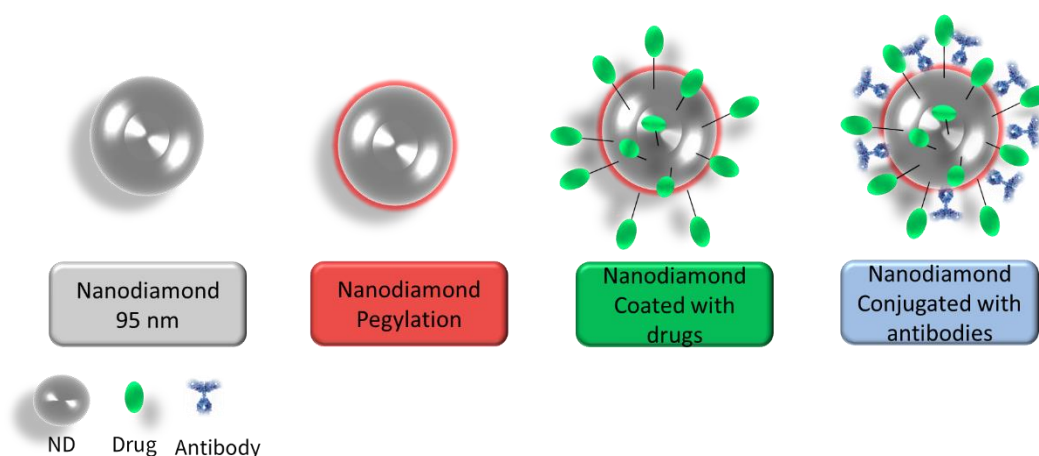


Figure 2.1: Nanodiamond functionalization diagram

NDs were pegylated, coupled with drugs (PTX, CARBO, and DOX) and then conjugated with antibodies (HE4 and CA125).

2.4 Cytotoxicity and biological parameters measurement

Multi parametric cytotoxicity testing (Cell viability (CV), MMP, CMP, and Cell Cycle) was carried out following 24h, 48h, and 72h exposure to treatments with three different doses of each nanocomplex. Experiments were performed in triplicate for each condition and 3 independent experiments were performed.

1. ND/PTX/HE4 and ND/PTX/CA125

Each plate was set up with 100 μ l (in triplicate) of media containing three different concentrations (1.25nM/ml, 2.5nM/ml, and 5nM/ml) of nanocomplexes, 10 μ M/ml of ND and 2.5nM/ml of PTX, then this mixture was added to cells in each well.

2. ND/CARBO/HE4 and ND/CARBO/CA125

Each plate was set up with 100 μ l (in triplicate) of media containing three different concentrations (7 μ M/ml, 14 μ M/ml, and 28 μ M/ml) of nanocomplexes, 10 μ M/ml of ND and 14 μ M/ml of CARBO, then this mixture was added to cells in each well.

3. ND/DOX/HE4 and ND/DOX/CA125

Each plate was set up with 100 μ l (in triplicate) of media containing three different concentrations (5 μ M/mL, 10 μ M/mL, and 20 μ M/mL) of nanocomplexes, 10 μ M/ml of ND and 10 μ M/ml of DOX, then this mixture was added to cells in each well.

Cytotoxicity was determined by measuring the variation in cell count, MMP, CMP and cell cycle using Hoechst dye, MMP tracker and CMP dye (Table 2.2). Images were acquired using the Cytell [™] imaging system (GE Healthcare, UK) and analysed using the IN Cell Investigator software (version 1.6). An example of a typical image from these experiments is shown in Figure 2.2, where the blue colour indicates the nucleus, red colour indicates the mitochondria and the green colour indicates the cell membrane.

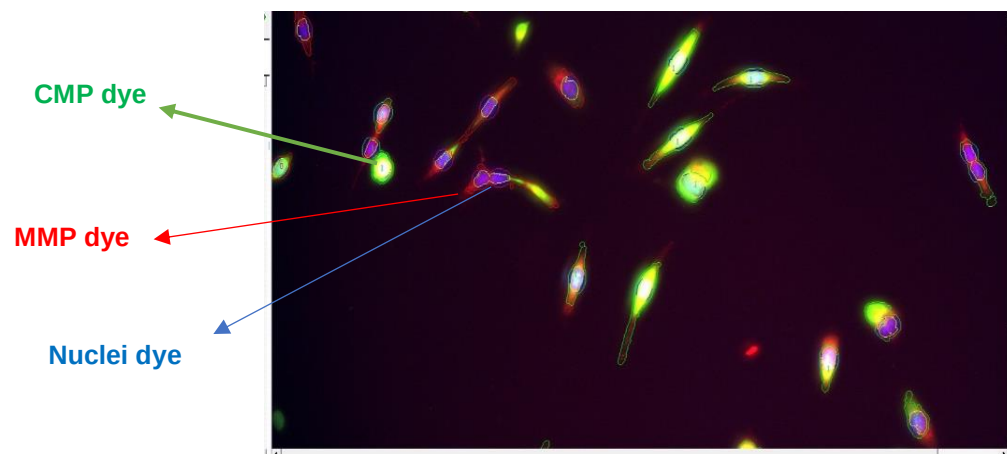


Figure 2.2: Representative image of SKOV-3 cells scanned by HCSA and analysed by IN Cell software.

Nucleus stained with Hoechst (blue colour), mitochondria stained with MMP tracker dye (red colour), and cell membrane stained with CMP dye (green colour).

2.5 Protein analysis

Cells were seeded in T25 flasks and incubated for 24h at 37°C and 5% CO₂, and the following day cells were exposed to the desired drugs and functionalised NDs for 48h at 37°C and 5% CO₂ (the time point 48h was chosen as the optimum time point to see the effect of the nanocomplexes at the protein level). Cells were harvested in cold PBS and centrifuged at 400 g for 10min at 4°C, the supernatant was discarded, and the pellet was suspended in 200ul of RIPA buffer with 0.1% of protease inhibitor and kept on ice for 15 min. The suspension was centrifuged at 400g for 15 min at 4°C, the supernatant was transferred to another tube and kept at – 80°C.

2.5.1 Protein Concentration Measurement (Bradford assay)

1. Serial concentrations were performed from the protein standard (BSA) (1500, 750, 375, 188, 94, and H₂O).
2. 5ul of protein samples were added in duplicate in a 96 well plate.
3. 250ul of Bradford reagent was added to each well, and the plate was incubated on a shaker at RT for 15 min.
4. The plate was read at 595nm using microplate reader.
5. Linear regression and interpolation of protein concentration from the standard curve was performed using GraphPad prism 5 software.
6. The protein samples were normalised to load equal amount of protein concentration of each sample into each well.

2.5.2 Western Blotting

Western blotting is a membrane immunoblotting technique used to detect the presence of specific target proteins. Following SDS-PAGE proteins were electrophoretically transferred to a thin membrane composed of nitrocellulose or PVDF (polyvinylidene difluoride). The membrane was placed on top of the gel, overlaid with blotting paper or sponges, this stack or transfer sandwich was then loaded into an electrophoretic apparatus. The transfer sandwich was immersed in buffer and an electric current was applied. This applied electric current will cause separated proteins contained within the gel to migrate and bind to the membrane. The membrane then was probed with specific

antibodies for target proteins of interest. Individual protein bands can be visualised using a HRP labelled secondary antibody and a suitable chemiluminescence reagent.

2.5.3 Sample Preparation

1. Protein samples were thawed on ice for 30 minutes.
2. Protein samples were diluted to 30ug in a total volume of 30ul with RIPA buffer in fresh Eppendorf tubes.
3. Loading dye was added to each tube in 1:5 ratio of the protein sample.
4. 4ul of loading dye was added to each sample, then all protein samples were kept in boiled water for 30 secs and transferred onto ice for 3 mins.
5. 5ul of a loading protein marker was loaded in the first well.
6. 30ul of protein samples were loaded into each well.
7. The rig was connected to the power supply and run at 120v for 60 mins.

2.5.4 Coomassie Blue Staining

1. Gel was removed from the transfer sandwich and placed in a small container filled with deionised water and briefly rinsed.
2. Gel was covered with Coomassie blue and placed on an orbital shaker for 30min.
3. Coomassie stain was poured off and gel was rinsed with deionised water.
4. The gel was covered with the de-stain solution (40% methanol and 10% glacial acetic acid) for 10mins.

2.5.5 Western Blot

1. Transfer buffer was kept at 4°C prior to use.
2. Blotting paper and membrane were cut to the required size.
3. Blotting paper was immersed in transfer buffer.
4. The PVDF membrane was immersed in 70% methanol for 1 minute to be activated and then immersed in transfer buffer and allowed to equilibrate for at least 15 mins.
5. The top and bottom portions of the gel were cut, and the top right-hand corner was removed for orientation.

6. The gel was carefully removed from the gel cassette and placed in a tray of deionised water.
7. The transfer sandwich was prepared as outlined:
 - a. Two pieces of filter paper soaked with transfer buffer.
 - b. PVDF Membrane.
 - c. The gel.
 - d. Two pieces of filter paper soaked with transfer buffer.
8. Air bubbles were removed by rolling the surface of the sandwich.
9. The rig was connected to the power supply and run at 32 mA for 1 hour to transfer protein samples.

2.5.6 Protein Detection

1. The transfer sandwich was de-assembled.
2. The membrane was carefully peeled away from the gel and the side which was in contact with the gel was facing upwards. The notched corner should now be on the left-hand side.
3. Next the membrane was immersed in appropriate blocking solution such as 5% w/v Marvel milk protein and incubated at room temperature for 1 hour on an orbital shaker at 50revs/min to block non-specific binding sites.
4. The membrane was immersed in 10mls of diluted primary antibody (1:1000 of Caspase 3, or 1:1000 of P-gp), then incubated at 4°C overnight on an orbital shaker.
5. Following overnight incubation with the primary antibody, the membrane was rinsed quickly with 1X TBST to remove most of the residual unbound antibody, then three washes were performed (1 x 15min and 2 x 5min washes in 1x TBST) on an orbital shaker.
6. Afterwards, the membrane was transferred to a new tube containing the suitable secondary antibody (1:1000 of Goat Anti-Mouse Immunoglobulins HRP) and incubated on an orbital shaker at room temperature for 1 hour, then three washes were performed (1 x 15min and 2 x 5min washes in 1x TBST) on an orbital shaker.

2.5.7 Developing Blots

1. Luminol detection reagent was prepared by adding an equal volume of detection solution A (Luminol enhancer solution) with an equal volume of detection solution B (Peroxide solution) in a tinfoil covered tube and then mixed thoroughly.
2. The membrane was placed between two plastic strips and the excess moisture was removed by pressing down with a piece of tissue paper.
3. The membrane was placed within the plastic sheet inside the LAS-4000 luminescent Image analyser.
4. The chemiluminescence option was selected and the images were taken at appropriate times.
5. The black and white balance was adjusted to obtain the optimum image, then the image was saved.

2.5.8 Stripping blots

1. Blots were stripped in order to examine expression of multiple proteins.
2. The membrane was activated using 70% ethanol for 5 mins at RT.
3. The membrane was placed in a 50 ml tube and the stripping buffer was added as following:
 - a. 3.12ml of Tris-HCL (62.5mM, pH 6.8).
 - b. 5ml of 20% SDS.
 - c. 0.415ul of β -mercaptoethanol.
 - d. The volume was made up to 50 ml.
4. The tube was incubated in a water bath for 45 mins at 50 °C.
5. Three washes were performed for 5 mins each with TBST.
6. The membrane was blocked with 5% milk for 1 hour at RT.
7. The new primary antibody (β -Actin) was applied and incubated for two hours at RT.
8. The developing steps were repeated as described in section 2.5.7.

2.6 Tissue culture (*Ex vivo*)

2.6.1 Ethical approval

Tissue samples were obtained from patients recruited through the DISCOVERY bioresource. Having given full and informed consent, patients attending the Gynaecological Oncology Service and scheduled for surgery at St James's Hospital (by Dr Feras Abu Saadeh and his team) were invited to donate tissue samples. Fresh tumour biopsies were obtained following pathologist examination. The study was approved by the St. James's Hospital Ethics committee.

2.6.2 *Ex vivo* culture

1. Tumour tissue samples were cut into small pieces of 1 - 2mm in size in sterile conditions.
2. These small pieces were cultured in 96 well plates with RPMI supplementary media (10% fetal bovine serum, 1% L-glutamine, and 1% penicillin and streptomycin antibiotics) and incubated for 7 days at 37°C and 5% CO₂. Media was changed every 2 days.
3. Tumour tissue samples were treated with the nanocomplexes (ND/DOX/HE4 and ND/DOX/CA125, ND/CARBO/HE4 and ND/CARBO/CA125, ND/PTX/HE4 and ND/PTX/CA125) and incubated for 48h at 37°C and 5% CO₂.
4. The protein was harvested from treated tumour tissue samples and controls and then protein concentrations were measured and prepared for western blot.

2.6.3 Measurement of Lactate Dehydrogenase release and Caspase-3 expression in ovarian tumour explants

The cytotoxic effect of the drugs (PTX, CARBO and DOX) and nanocomplexes on ovarian tumour explants was evaluated by measuring lactate dehydrogenase (LDH) release into the supernatant after 48h of treatment using the LDH Cytotoxicity Detection Kit (Cat. # MK401, Takara Bio, USA) and 2% Triton® X-100 was used as positive control. The LDH assay measures cellular enzyme activity as damaged cells release LDH into the supernatant.

2.7 Optimisation Experiments

Several experimental aspects of this project required optimisation for drug concentrations and time points, ND characterization and baseline expression levels of HE4 and CA125 antibodies and these are outlined below.

2.7.1 Drugs response curve

In order to select the optimum starting doses of chemotherapy drugs (paclitaxel, carboplatin and doxorubicin) used in the cytotoxicity study, the doses of drugs that were selected to be loaded on the pegylated ND were based on clinically relevant doses of these drugs. Dose selection strategies were evaluated for all cells. Doses ranged from 0.37 – 10 nM/ml, 1.75 – 40 μ M/ml, and 0.65 – 25 μ M/ml for paclitaxel, carboplatin and doxorubicin respectively. All these ranges were selected from the results of cytotoxic assays of chemosensitive and chemoresistant ovarian cancer cell lines and benign ovarian cells initially encompassing inhibitory concentration (IC) values ranging from IC₁₀ – IC₉₀ (Table 2.6, Table 2.7 and Table 2.8).

All cells were seeded in 96 well plates at a concentration of 1×10^4 , plates were incubated at 37°C and 5% CO₂ for 24h. Cells were then treated in triplicate with selected test doses and drug-free media was added to the control and incubated for 24h, 48h and 72h. After each time point cells were counted using HCSA. Percentage cell survival was calculated as detailed below:

$$\frac{\text{Average cell number of treated cells}}{\text{Average cell number of control cells}} \times 100$$

The selected doses for ND/PTX/HE4 and ND/PTX/CA125 were 1.25, 2.5 and 5 nM/ml, selected doses for ND/CARBO/HE4 and ND/CARBO/CA125 were 7, 14, and 28 μ M/ml, and selected doses for ND/DOX/HE4 and ND/DOX CA125 were 5, 10, and 20 μ M/ml, all these doses were selected according to the IC₅₀ of all drugs (Figures 2.3, 2.4 and 2.5).

Table 2.6: IC₅₀ of paclitaxel for all ovarian cells.

Selected dose of PTX used in this study based on the average of IC₅₀ for three-time points. SEM are provided for all cell lines after the treatment with PTX at three time points.

Drug Cells	PTX (2.5 nM/ml)			Average (nM/ml)	Average (ng/ml)	Clinical dose
	24h SEM	48h SEM	72h SEM			
HIO-80	4.5 ± 1.2	1.2 ± 0.8	1.0 ± 1.2	2.2	1.92 ng/ml	1-100ng/ml
OAW42	0.0 ± 0.7	2.5 ± 0.7	2.2 ± 0.9	2.3	1.96 ng/ml	1-100ng/ml
SKOV-3	1.8 ± 1.3	1.0 ± 1.2	0.8 ± 1.2	1.2	1 ng/ml	1-100ng/ml
OVCAR-8	1.5 ± 2.0	1.1 ± 0.9	1.0 ± 1.5	1.2	1 ng/ml	1-100ng/ml
A2780	5.0 ± 1.5	1.2 ± 1.3	0.8 ± 1.0	2.3	1.96 ng/ml	1-100ng/ml
A2780-cis	5.0 ± 1.2	1.1 ± 1.5	0.7 ± 1.2	2.3	1.96 ng/ml	1-100ng/ml

Table 2.7: IC₅₀ of carboplatin for all ovarian cells.

Selected dose of CARBO used in this study based on the average of IC₅₀ for three-time points. SEM are provided for all cell lines after the treatment with CARBO at three time points.

Drug Cells	CARBO (14 µM/ml)			Average (µM/ml)	Average (µg/ml)	Clinical dose
	24h SEM	48h SEM	72h SEM			
HIO-80	0.0 ± 0.1	14.1 ± 1.3	10 ± 1.0	12	4.4 µg/ml	1-20µg/ml
OAW42	28 ± 3.1	14 ± 2.0	8 ± 1.5	16.6	6.1 µg/ml	1-20µg/ml
SKOV-3	26 ± 4.1	15 ± 1.9	10 ± 2.0	17	6.3 µg/ml	1-20µg/ml
OVCAR-8	0.0 ± 0.2	26 ± 2.2	14 ± 2.1	20	7.4 µg/ml	1-20µg/ml
A2780	20 ± 2.3	14 ± 1.3	10 ± 1.5	14.6	5.4 µg/ml	1-20µg/ml
A2780-cis	0.0 ± 0.3	0.0 ± 0.6	25 ± 3.1	25	9.2 µg/ml	1-20µg/ml

Table 2.8: IC₅₀ of doxorubicin for all ovarian cells.

Selected dose of DOX used in this study based on the average of IC₅₀ for three-time points. SEM are provided for all cell lines after the treatment with DOX at three time points.

Drug Cells	DOX (10 µM/ml)			Average (µM/ml)	Average (µg/ml)	Clinical dose
	24h SEM	48h SEM	72h SEM			
HIO-80	15 ± 1.2	11 ± 2.5	6 ± 1.4	10.6	5.7 µg/ml	0.6 – 5.4 µg/ml
OAW42	14 ± 2.0	10.1 ± 1.6	6.1 ± 0.8	10	5.4 µg/ml	0.6 – 5.4 µg/ml
SKOV-3	17 ± 3.1	10 ± 2.8	7.5 ± 2.0	11.5	6.2 µg/ml	0.6 – 5.4 µg/ml
OVCAR-8	12 ± 2.5	10 ± 1.2	6 ± 2.3	9.3	5 µg/ml	0.6 – 5.4 µg/ml
A2780	13 ± 1.2	9.8 ± 0.6	6 ± 1.7	9.6	5.2 µg/ml	0.6 – 5.4 µg/ml
A2780-cis	12 ± 2.3	9.8 ± 2.0	6 ± 1.4	9.2	5 µg/ml	0.6 – 5.4 µg/ml

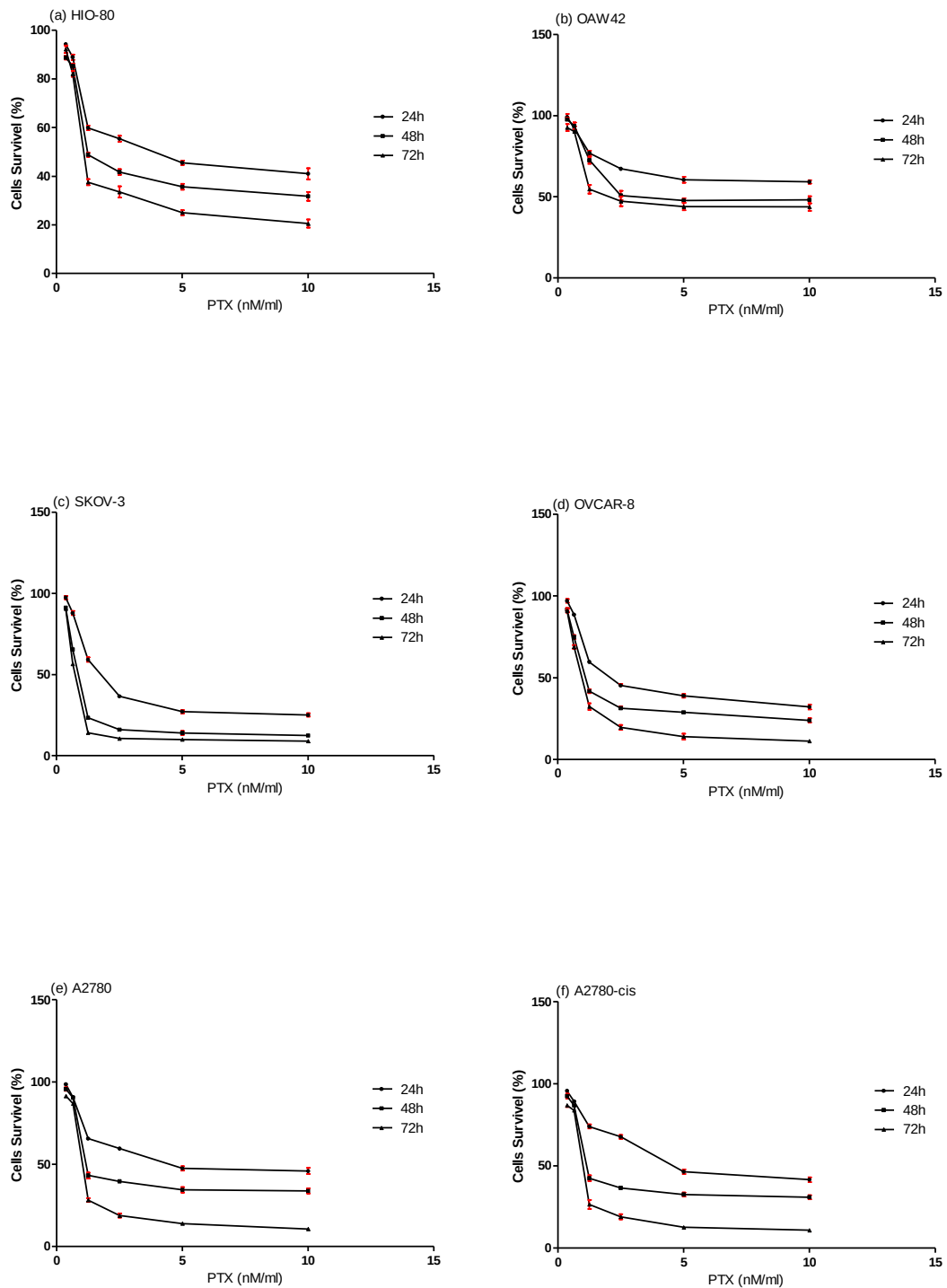


Figure 2.3: Dose optimisation of PTX

Cells were treated with different doses of PTX ranging from 0.37 – 10 nM/ml.

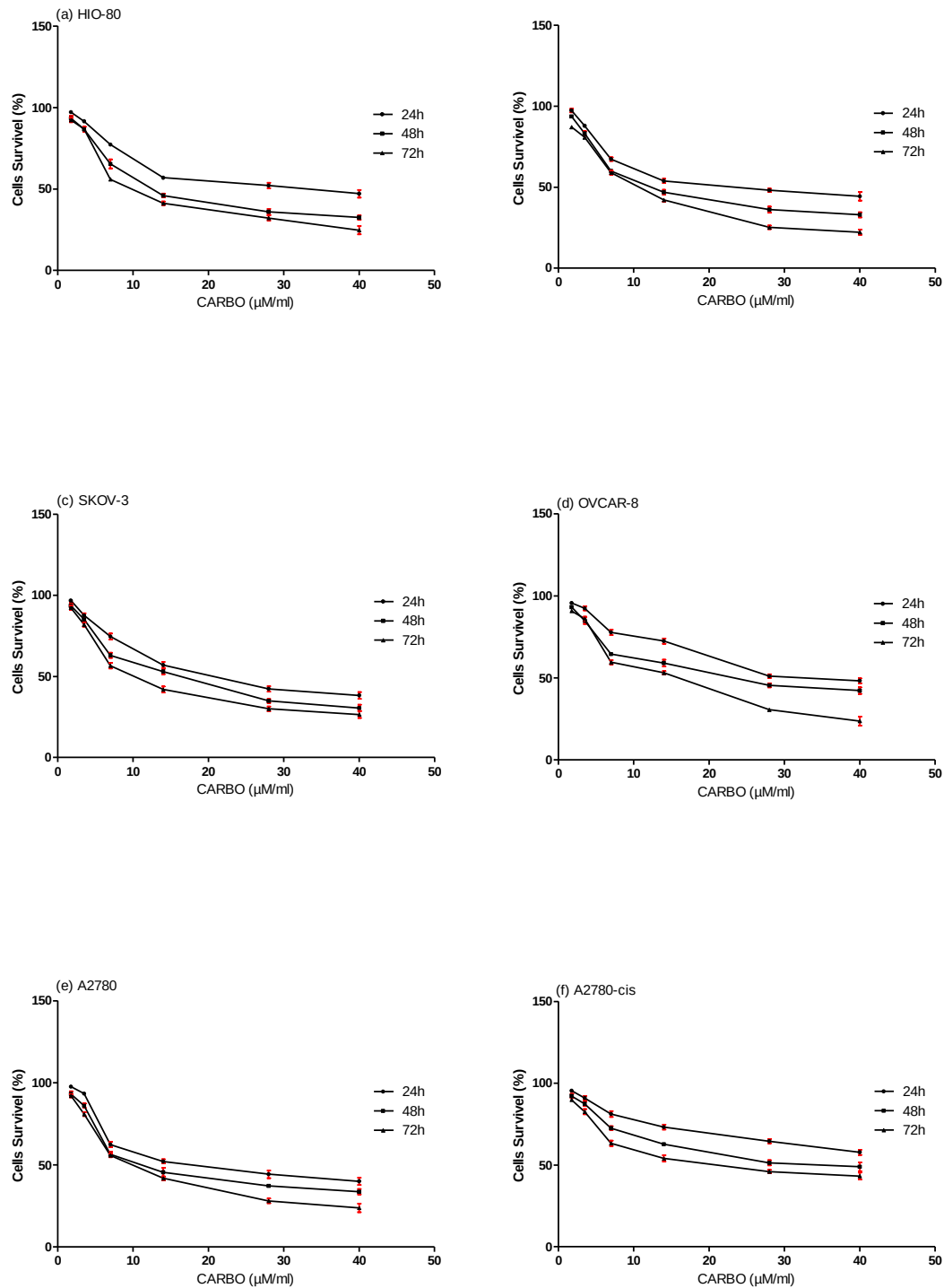


Figure 2.4: Dose optimisation of CARBO

Cells were treated with different doses of CARBO ranging from 1.75 – 40 $\mu\text{M/ml}$.

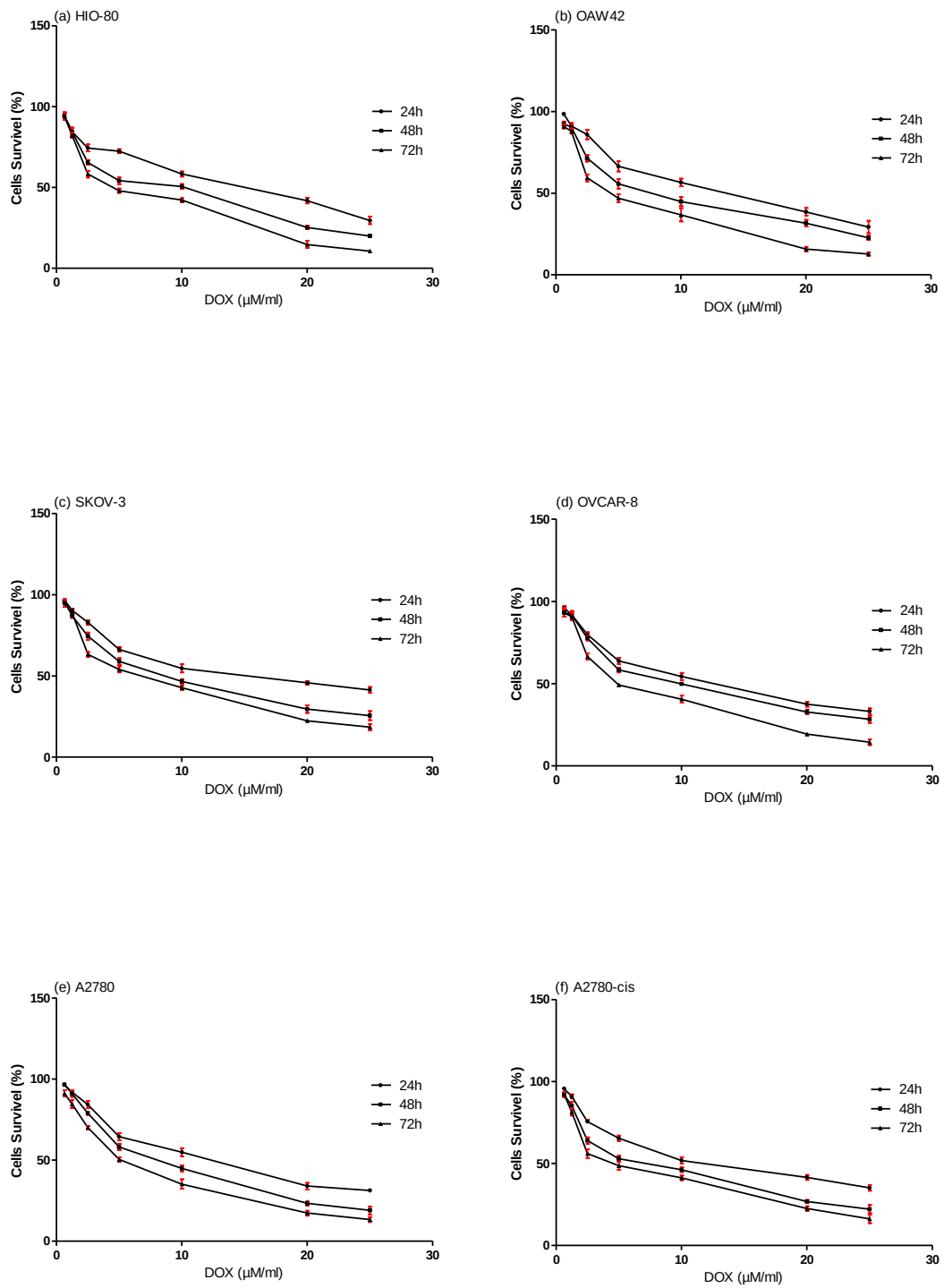


Figure 2.5: Dose optimisation of DOX

Cells were treated with different doses of DOX ranging from 0.65 – 25 $\mu\text{M/ml}$.

2.8 Nanodiamond size characterization

Methods to monitor ND characteristics such as core size and hydrodynamic diameter involve a range of established techniques as detailed in section 1.5.6. In this study the most appropriate methods included Dynamic Light Scattering (DLS) and Nanoparticle tracking analysis (NTA). In collaboration with Jennifer McIntyre and Hugh J. Byrne, (Dublin Institute of Technology (DIT)), DLS was used to measure the hydrodynamic diameter of individual nanomaterial dispersed in solution (NDs, ND/PTX and ND/CARBO). When this method was employed in this study to assess the functionalisation of PTX and CARBO, the size of bare NDs was 92 nm with a clear increase in size after functionalization with PTX and CARBO to 220 nm and 180 nm respectively (Table 2.9 and 2.10).

Table 2.9: Hydrodynamic size distribution of bare NDs and PTX loaded NDs

Mean and standard deviations (SD) of bare NDs and PTX loaded NDs was determined by DLS.

Size distribution	Bare ND	ND/PTX
Mean	95.2 nm	220 nm
SD	± 6.2	± 27.4

Table 2.10: Hydrodynamic size distribution of bare NDs and CARBO loaded NDs

Mean and standard deviations (SD) of bare NDs and CARBO loaded NDs was determined by DLS.

Size distribution	Bare ND	ND/CARBO
mean	95.2 nm	180 nm
SD	± 6.2	± 27.1

NTA was also used to measure the functionalisation with DOX and provided quantitative information about the hydrodynamic size of the NDs in aqueous solution. All bare NDs and NDs coupled with DOX (ND/DOX) were dispersed in ultrapure, 100 nm filtered DEPC water for NTA analysis. The hydrodynamic size of bare NDs was recorded to 92nm with a clear increase in size when functionalised with DOX to be 209nm (figure 2.6, Table 2.11).

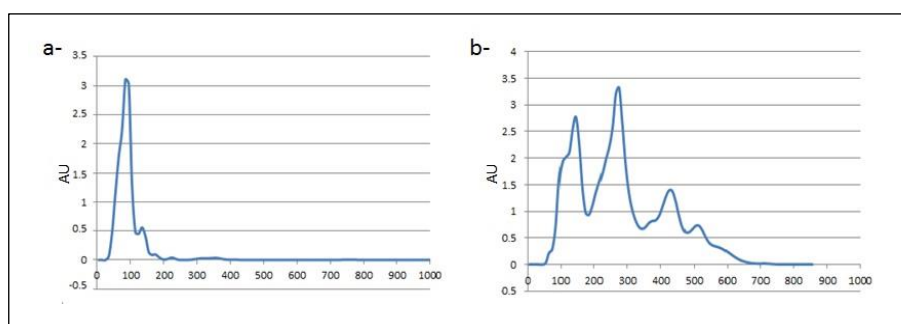


Figure 2.6: Nanodiamonds tracking analysis (bare) and after functionalisation with DOX
(a) Bare ND and (b) ND functionalized with drugs (i.e. DOX, PTX and CARBO).

Table 2.11: Hydrodynamic size distribution of bare NDs and DOX loaded NDs

Mean and standard deviations (SD) of bare NDs and DOX loaded NDs was determined by NTA.

Size distribution	Bare NDs	NDs/DOX
mean	95.2 nm	281.9 nm
SD	±51.2 nm	±135.0 nm

2.9 Uptake Visualisation

All ovarian cancer and normal cell lines were exposed to the ND/DOX conjugated with FITC labelled HE4 for 24h at 37°C, then cells were fixed with 3% PFA and stained with MMP tracker dye and DNA binding dye (Hoechst). Following this, cells were washed and scanned using the Cytell™ imaging system and uptake was analysed using IN Cell software (Figure 2.7).

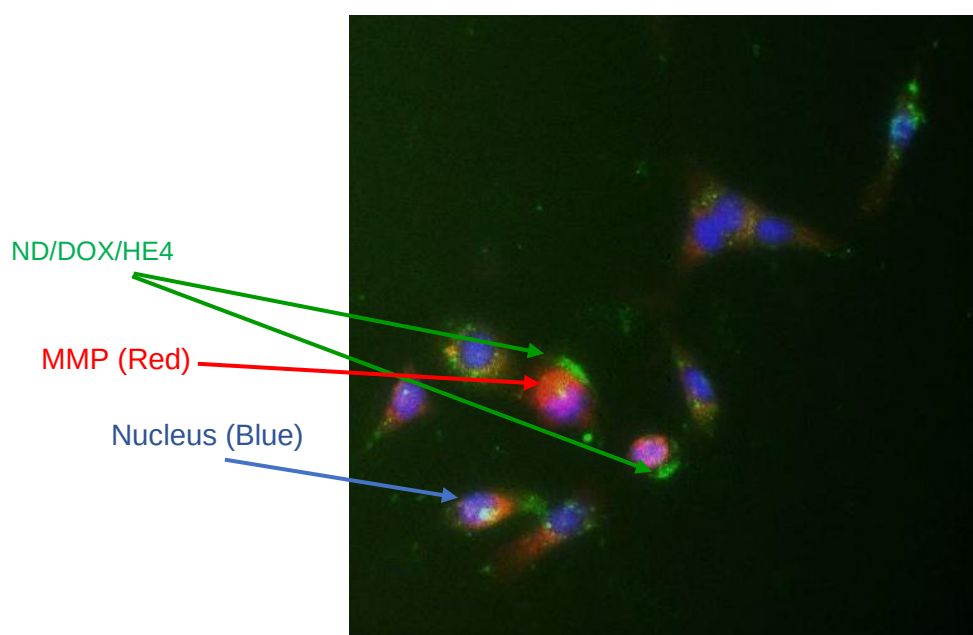


Figure 2.7: Cytoplasmic localisation of the nanocomplex

Representative image of SKOV-3 cells obtained by HCSA, the nanocomplexes were taken up by ovarian cancer cells marked with green arrows, nucleus labelled with Hoechst dye (blue colour) and mitochondria labelled with mitochondrial tracker (red colour).

2.10 The assessment of baseline HE4 and CA125 expression in the ovarian cell lines

To assess baseline expression of antibody targets (HE4 and CA125) in the cell lines chosen for this study, cells were cultured in T25 flasks with fresh RPMI media and incubated for 48h at 37°C and 5% CO₂. The protein was harvested from all cells and western blot was carried out for HE4 and CA125 as described previously (section 2.5). All cells expressed both proteins (HE4 and CA125) at various levels. CA125 was more highly expressed than HE4 in the majority of cell lines, except for SKOV3 where HE4 was more abundant (Figure 2.8).

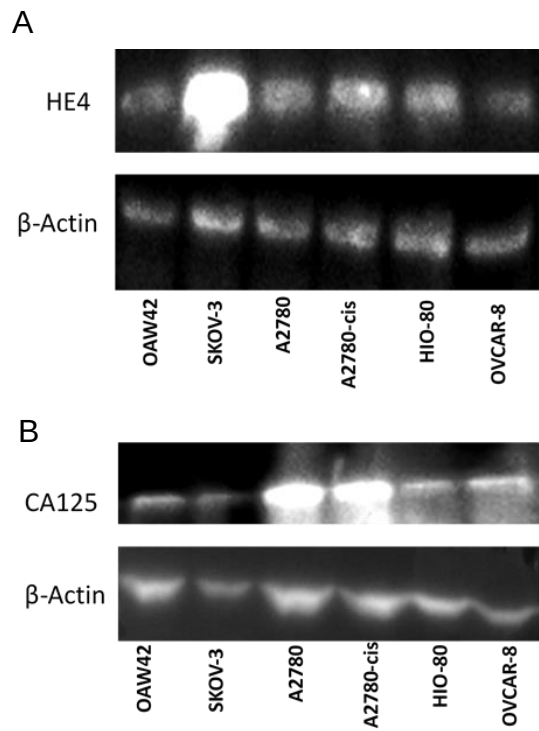


Figure 2.8: The expression level of HE4 and CA125 in ovarian cell lines

(A) Western blot result of HE4 for all cells, and (B) Western blot result of CA125 for all cells.

Chapter 3

Evaluation of response of ovarian cancer cells to nanocomplexes

3 Evaluation of response of ovarian cancer cells to nanocomplexes

3.1 Introduction

The traditional ovarian cancer treatment includes a combination of surgery and chemotherapy (GLANCE 2009). The prognosis for ovarian cancer following treatment is very poor in the majority of ovarian cancer patients, especially patients with stage III or IV disease on diagnosis. To improve the efficacy of chemotherapy, nanotechnology has been used in the treatment of recurrent ovarian cancer, in the form of Doxil (Polyethylene glycol PEGylated liposomal doxorubicin) (Misra, Acharya et al. 2010). As described in chapter 1 section 1.7, nanotechnology has been employed in cancer treatment to overcome some of the limitations seen with traditional therapy. Employing NPs loaded with drug molecules such as PTX, CARBO and DOX and conjugated with active targeting tools such as antibodies, siRNA, peptides and folic acid that specifically target ovarian cancer creates the potential for the development of novel nanocarriers to allocate valid alternatives to chemotherapeutics in the treatment of ovarian cancer. This will provide platforms to particularly reduce drug toxicity, enhance drug circulation, improve cellular drug uptake, and increase drug localization at target sites (Tuefferd, Couturier et al. 2007, Fleige, Quadir et al. 2012, Torchilin 2012, Engelberth, Hempel et al. 2014, Bazak, Houri et al. 2015, He, Poon et al. 2016, Lamberti, Scarano et al. 2016, Langdon and Sims 2016, Hamd-Ghadareh, Salimi et al. 2017, Hami, Rezayat et al. 2017, Pi, Zhang et al. 2017, Hoosen, Pradeep et al. 2018).

In general, nanotechnology in the medicine field may revolutionise the way that we detect and treat cancer in the human body in the future, with most nano-techniques making remarkable progress towards becoming realities. Most of these techniques have been used for diagnostic purposes to detect cancer at an early stage by attaching antibodies with fluorescent molecules. They then attach to cancer cells allowing their identification using imaging systems. In addition, NMs have taken a new direction to treat specifically cancer cells without causing any damage to the normal cells, minimising the side effects of treatment and increasing drug efficacy. This is achieved by using NPs complexed with drugs and coated with antibodies to specifically target and deliver the chemotherapy payload to tumour cells. This leads to increased drug

accumulation in cancer cells and induces higher cytotoxic effects by activation of cell kill pathways such as apoptosis and necrosis without affecting normal cells. Reduced side effects are also achieved by incorporating lower doses of chemotherapy into NPs (Subbiah, Veerapandian et al. 2010, Yallapu, Jaggi et al. 2010, Narvekar, Xue et al. 2014). In this thesis, we developed functionalized ND with specific anti-ovarian cancer agents and coated with specific antibodies to target and treat ovarian cancer cells. The aim of this chapter was to establish the response of ovarian cancer cells to these nanocomplexes.

3.2 Methods

Chemosensitive and chemoresistant ovarian cancer cell lines (OAW42, SKOV-3, OVCAR-8, A2780, and A2780-cis), and the immortalised human ovarian surface epithelial cell line (HIO-80) were exposed to drug-free media, bare ND (10 μ M/ml), three chemotherapy drugs (PTX 2.5nM/ml, CARBO 14 μ M/ml and DOX 10 μ M/ml) and three different concentrations of the following nanocomplexes:

1. ND loaded with PTX (1.25nM/ml, 2.5nM/ml, and 5nM/ml) and conjugated with HE4 (2.5 mg/ml), (ND/PTX/HE4).
2. ND loaded with PTX (1.25nM/ml, 2.5nM/ml, and 5nM/ml) and conjugated with CA125 (2.4mg/ml), (ND/PTX/CA125).
3. ND loaded with CARBO (7 μ M/ml, 14 μ M/ml, and 28 μ M/ml) and conjugated with HE4 (2.5 mg/ml) (ND/CARBO/HE4).
4. ND loaded with CARBO (7 μ M/ml, 14 μ M/ml, and 28 μ M/ml) and conjugated with CA125 (2.4 mg/ml), (ND/CARBO/CA125).
5. ND loaded with DOX (5 μ M/ml, 10 μ M/ml, and 20 μ M/ml) and conjugated with HE4 (2.5 mg/ml), (ND/DOX/HE4).
6. ND loaded with DOX (5 μ M/ml, 10 μ M/ml, and 20 μ M/ml) and conjugated with CA125 (2.4 mg/ml), (ND/DOX/CA125).

Cells were incubated for three different time intervals (24h, 48h, and 72h). Cells were fixed with PFA (3%) and stained with:

- DNA binding dye (Hoechst) to measure cell viability and cell cycle (blue).
- MMP tracker which measures the changes in MMP intensity and is an indicator of mitochondrial damage (red).

- CMP dye which measures CMP changes and is an indicator of cell membrane damage if the CMP dye has leaked to the nuclear region (green).
- Nocodazole (NC) was used as a positive control for cell cycle assessment.
- The cell cycle assessment was used as a further biological marker to confirm the cytotoxic effect seen by measuring CV, MMP and CMP changes after nanocomplexes treatment.

Cells were imaged using the HCSA imaging system (Cytell) (GE Healthcare, UK), and a representative image is shown in Figure 3.1. As can be seen in Figures 3.1A and B the untreated cells or cells exposed to bare NDs display a predominantly blue and green colour whereas the treated cells such as those seen in Figures 3.1C, D, E, F, G, H, I, J and K, display blue, green and red (the yellow colour seen is an interaction between the red and green colour and is an indicator of cell death). Viable cell count, MMP changes, and CMP changes were performed using IN cell software, and percentage of viable cells, MMP changes, and CMP changes versus time (24h, 48h, and 72h) were graphed using GraphPad Prism 5 for each treatment.

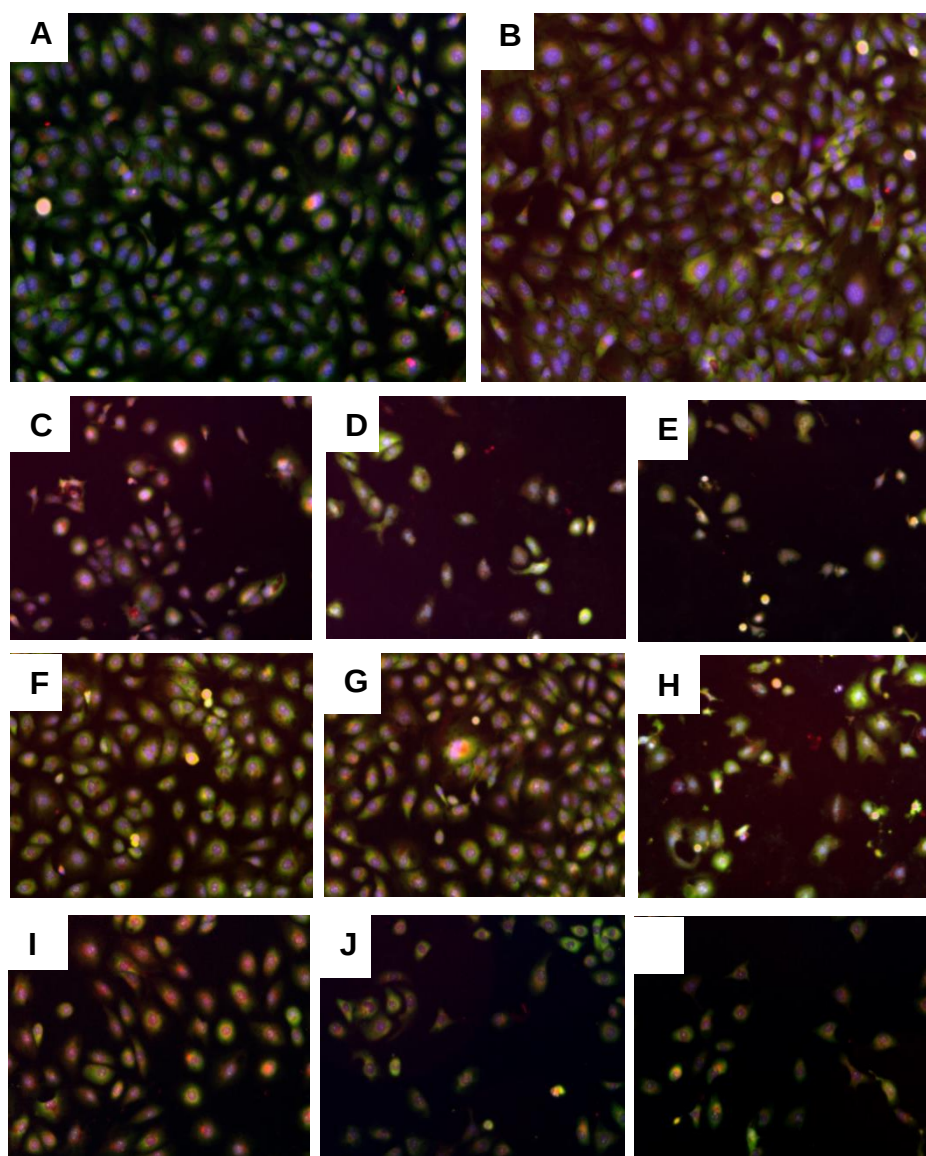


Figure 3.1: Representative images obtained using HCS imaging system

(A) untreated cells, (B) cells exposed to ND, (C) cells treated with PTX alone, (D) cells treated with ND/PTX/HE4, (E) cells treated with ND/PTX/CA125, (F) cells treated with CARBO alone, (G) cells treated with ND/CARBO/HE4, (H) cells treated with ND/CARBO/CA125, (I) cells treated with DOX alone, (J) cells treated with ND/DOX/HE4 and (K) cells treated with ND/DOX/CA125. Nucleus displayed in blue, cell membrane displayed in green and mitochondria displayed in red. The yellow colour is an interaction between CMP dye (green) and MMP tracker dye (red).

3.3 Statistical Analysis

Cell viability, MMP, CMP and total cell cycle proportions were normalised to control (untreated cells) and expressed as a percentage. Data are expressed as the mean $\pm$ standard error of the mean (S.E.M.) and were analysed using analysis of variance (2-way ANOVA) with *post hoc* analysis (Bonferroni or Dunnett; GraphPad Prism version 5). A 2-sample t-test was also used to compare differences between two cell lines. A value of $p < 0.05$ was considered statistically significant.

Prof Simon Wilson in Trinity College Dublin was consulted about the statistical approach taken in this study and he was in agreement with the statistical analysis performed.

3.4 Results

3.4.1 Cell viability assessment in response to ND/PTX/HE4 treatment

A significant cell viability reduction ($p < 0.001$) was seen after the cells were exposed to all nanocomplex concentrations at all time points, and the effect was cell, dose and time dependent (Figure 3.2).

SKOV-3 and OVCAR-8 cells showed the highest response to all concentrations of the nanocomplex compared to untreated cells. Decreases in viability ranging from 50.4% to 92.6% for SKOV-3 and 40.7% to 93.3% for OVCAR-8 were seen as time and concentration increased (Table 3.1). For other cell lines (OAW42, A2780, and A2780-cis), significant cell viability reductions ($p < 0.001$) were seen ranging between 22.6% to 53.7% in OAW42 cells, 27.5% to 88.4% in A2780 cells, and 28.7% to 88.8% A2780-cis cells (Figure 3.2 b, e, and f). A decrease was also seen when H10-80 cells were treated with the nanocomplex, but the percentage of cell viability reduction was not as high as the cancer cell lines (40.5% to 76.1%) (Figure 3.2 a).

The mid concentration (ND/PTX/HE4 2.5nM/ml) of the nanocomplex was more effective in reducing cell viability than the same dose of PTX alone (2.5nM/ml). Decreases in cell viability ranging from 27.8% to 89.8% across the ovarian cancer cell lines compared to 20.3% to 71.5% for the drug alone (Figure 3.2 b, c, d, e, and f). Interestingly, the lowest concentration (1.25nM/ml) of the nanocomplex used in this study was more effective in reducing cell viability than the clinically relevant dose of PTX (2.5nM/ml) alone in OAW42, SKOV-3 and OVCAR-8 cell lines at all time points (Figure 3.2 b, c and d).

A paired cisplatin sensitive and resistant ovarian cancer cell line (A2780 and A2780-cis) exhibited similar cytotoxic effects after exposure to all concentrations of the nanocomplex at three time points (Figure 3.2 e and f).

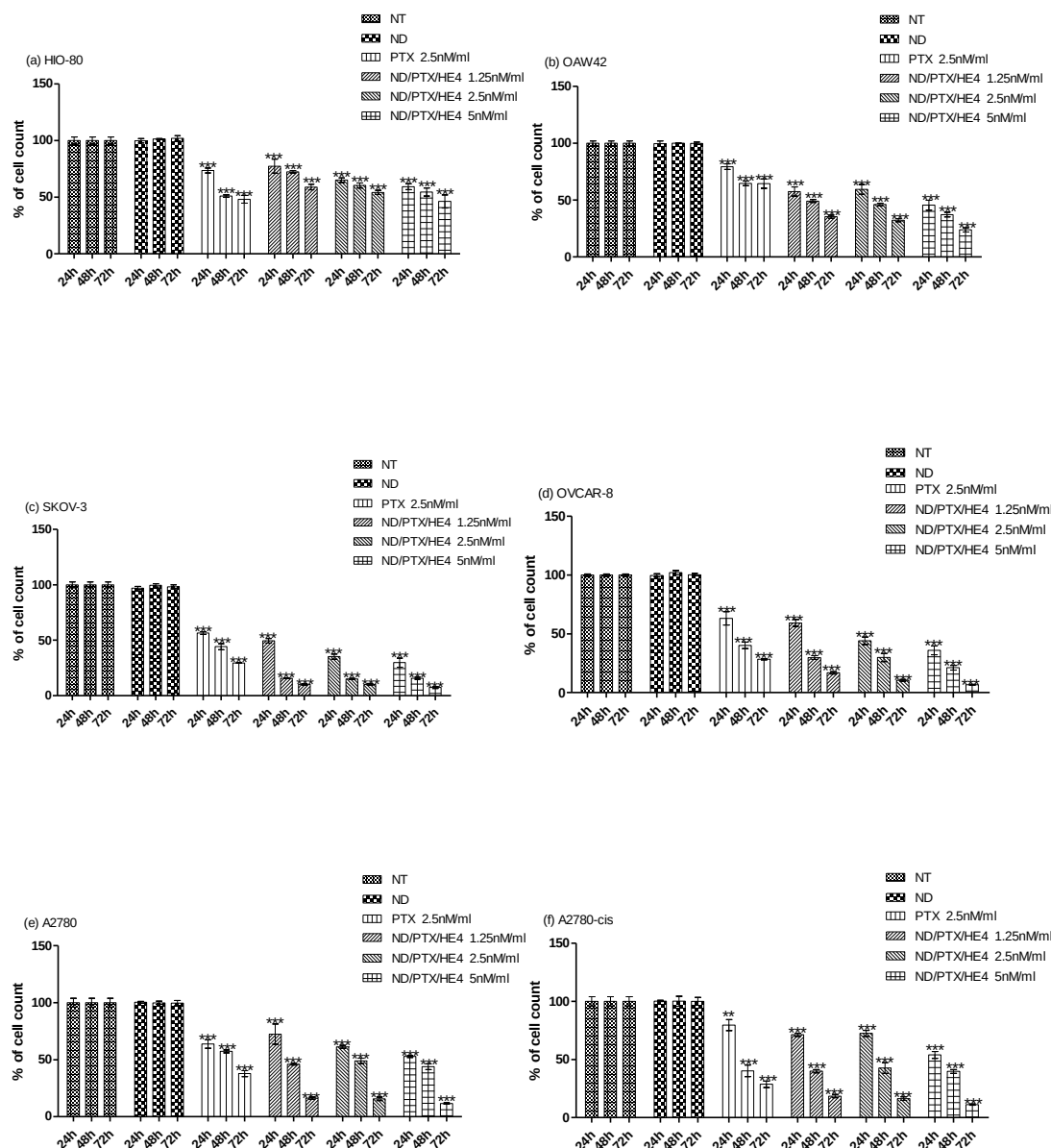


Figure 3.2: Cell viability assessment after ND/PTX/HE4 treatment

Cells were exposed to the ND/PTX/HE4 for three different time incubations and total cell count was measured using the Cytell™ imaging system and IN cell software, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) OVCAR8, (e) A2780 cells and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out. Statistical significance between treated and untreated cells reported as “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (PTX) paclitaxel.

Table 3.1: Percentages of cell viability reduction (cell kill) after ND/PTX/HE4 treatment. The percentage of cell viability reduction listed is in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	PTX 2.5nM/ml (%)	SEM	ND/PTX/HE4 1.25nM/ml (%)	SEM	ND/PTX/HE4 2.5nM/ml (%)	SEM	ND/PTX/HE4 5nM/ml (%)	SEM
HIO-80	24h	0.1 ± 3.3		0.2 ± 1.8		26.4 ± 2.2		42.3 ± 3.9		40.5 ± 4.2		54.4 ± 4.3	
	48h	0.0 ± 3.1		1.2 ± 0.4		48.9 ± 0.9		50.7 ± 1.3		53.8 ± 1.1		62.7 ± 2	
	72h	0.2 ± 2.9		1.9 ± 2.3		51.7 ± 3.4		64.5 ± 1.3		67.6 ± 1.2		76.1 ± 1.8	
OAW42	24h	0.3 ± 2.7		0.3 ± 2.4		20.6 ± 2.4		22.6 ± 6.1		34.9 ± 2.1		40.6 ± 2.6	
	48h	0.4 ± 1.9		0.2 ± 0.1		35.1 ± 2.1		27.5 ± 1		39.5 ± 2.3		45.4 ± 3.5	
	72h	0.0 ± 2.6		0.3 ± 1.1		35.5 ± 4		41.0 ± 2.5		45.7 ± 1.8		53.7 ± 5.8	
SKOV-3	24h	0.1 ± 3		2.9 ± 1.5		43.4 ± 1.1		50.4 ± 1.8		64.7 ± 2.2		70.1 ± 4.1	
	48h	0.1 ± 2.6		0.4 ± 1.4		55.7 ± 2.7		84.3 ± 0.1		85.0 ± 0.5		84.3 ± 0.8	
	72h	0.5 ± 2.2		1.7 ± 1.8		70.5 ± 0.3		89.8 ± 0.4		89.8 ± 0.9		92.6 ± 0.8	
OVCAR-8	24h	0.0 ± 1.1		0.2 ± 1.4		36.7 ± 5.6		40.7 ± 2.9		55.8 ± 3.1		64.0 ± 3.7	
	48h	0.1 ± 1		2.1 ± 1.8		59.7 ± 2.7		70.1 ± 1.6		70.1 ± 3.5		78.8 ± 1.9	
	72h	0.2 ± 1.2		0.3 ± 0.9		71.5 ± 0.5		83.1 ± 1		89.5 ± 0.8		93.3 ± 0.3	
A2780	24h	0.0 ± 3.8		0.3 ± 0.9		36.1 ± 3.6		27.5 ± 8.8		38.7 ± 1.2		47.2 ± 0.8	
	48h	0.3 ± 3.5		0.1 ± 1.4		42.7 ± 1.2		53.9 ± 0.7		51.0 ± 2.3		56.0 ± 2.5	
	72h	0.1 ± 2.9		0.2 ± 2.1		62.2 ± 2.6		83.6 ± 0.9		84.3 ± 1.6		88.4 ± 0.5	
A2780-cis	24h	0.1 ± 4		0.9 ± 0.5		20.3 ± 4.8		28.7 ± 1.2		27.4 ± 2.5		46.0 ± 3	
	48h	0.2 ± 3.2		0.8 ± 4.3		59.7 ± 4.9		59.9 ± 1.4		57.3 ± 4.6		60.1 ± 1.6	
	72h	0.6 ± 2.9		1.0 ± 3.4		71.3 ± 2.6		81.5 ± 1.4		83.6 ± 1.5		88.8 ± 0.6	

3.4.2 Triggering of mitochondrial membrane potential changes upon ND/PTX/HE4 treatment

A significant increase ($p < 0.001$) in MMP intensity was seen in all cells after all concentrations of ND/PTX/HE4 treatments, and the effect was dose, time and cell dependent (Figure 3.3) and this is displayed in colorimetric heat map in (Figure 3.3 g). SKOV-3 and OVCAR-8 cells showed the highest response to all concentrations of the nanocomplex compared to untreated cells. Increases in MMP changes ranging from 175.1% to 263% for SKOV-3 and 87.9% to 257% for OVCAR-8 were seen as time and concentration increased (Table 3.2). Significant MMP changes ($p < 0.001$) were seen in the other cell lines (OAW42, A2780, and A2780-cis) ranging between 14.5% to 52.4% in OAW42, 26.8% to 72.7% in A2780 and 19% to 60.2% in A2780-cis (Figure 3.3 b, e, and f). Similar MMP changes were also seen when the HIO-80 cells were treated with ND/PTX/HE4 with 31.1% to 77.1 increases compared to untreated cells (Figure 3.3 a).

Interestingly, the lowest concentration (1.25nM/ml) of the nanocomplex used in this study induced higher MMP changes than the clinically relevant dose of PTX (2.5nM/ml) alone in all cell lines at all time points (Figure 3.3).

A paired cisplatin sensitive and resistant ovarian cancer cell line (A2780 and A2780-cis) exhibited similar MMP changes after exposure to all concentrations of ND/PTX/HE4 at three time points (Figure 3.3 e and f).

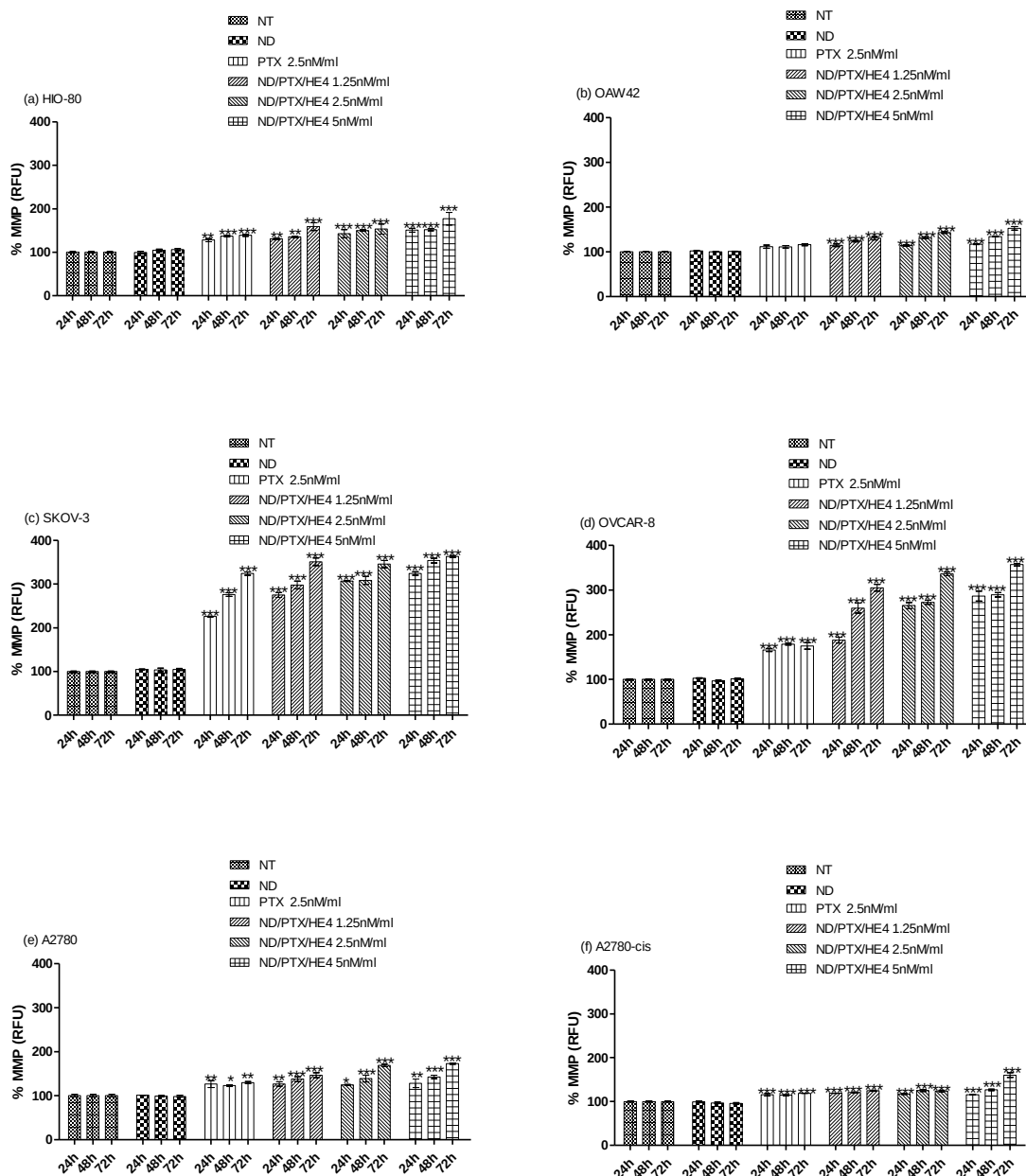


Figure 3.3: Mitochondrial Membrane Changes after ND/PTX/HE4 treatment

Cells were treated with ND/PTX/HE4 for three different time point and Cytell[™] imaging system and IN cell software were used to measure the mitochondrial changes (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) OVCAR8, (e) A2780 cells and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls, and statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (PTX) paclitaxel.

Table 3.2: Percentages of MMP increases after ND/PTX/HE4 treatment.

The percentage of MMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT	SEM	ND	SEM	PTX 2.5nM/ml	SEM	ND/PTX/HE4 1.25nM/ml	SEM	ND/PTX/HE4 2.5nM/ml	SEM	ND/PTX/HE4 5nM/ml	SEM
HIO-80	24h	0.1 ± 1.5		0.1 ± 2.7		28 ± 3.5		31.1 ± 1.9		42.8 ± 9.3		50.8 ± 4.4	
	48h	0.1 ± 1.5		1.5 ± 2.7		37.3 ± 1.1		35.4 ± 1.1		50.2 ± 1.5		52.1 ± 2.5	
	72h	0 ± 1.5		2.0 ± 2.4		39 ± 2.2		59 ± 8.9		53.3 ± 11.5		77.1 ± 14.4	
OAW42	24h	0.9 ± 0.3		2.0 ± 0.4		11.5 ± 3.3		14.5 ± 2.9		14.1 ± 1.8		16.8 ± 0.6	
	48h	1.1 ± 0.4		0.5 ± 0.3		11.2 ± 2.7		23.8 ± 1.7		31.4 ± 2.1		34.3 ± 0.6	
	72h	0 ± 0.1		0.9 ± 0.5		15.6 ± 2.1		30.3 ± 3.7		42.9 ± 1.9		52.4 ± 3.8	
SKOV-3	24h	0.1 ± 1.1		4.4 ± 1		126 ± 0.8		175.1 ± 5.7		206.8 ± 0.9		224.5 ± 3.9	
	48h	0 ± 1.2		3.8 ± 3.8		176.2 ± 4.2		197.9 ± 8.7		208.3 ± 8.9		253.7 ± 5.3	
	72h	0.1 ± 1		4.6 ± 2.6		224.2 ± 3.9		250.7 ± 8.6		245.7 ± 8.1		263 ± 1.9	
OVCAR-8	24h	0 ± 1.4		2.9 ± 0.8		65.9 ± 3.5		87.9 ± 6.5		165.5 ± 6.6		186.6 ± 11.1	
	48h	0.1 ± 1.4		0.8 ± 1.5		78.9 ± 1.7		159.9 ± 11		172.8 ± 4.5		189.7 ± 5	
	72h	0.2 ± 1.4		1.4 ± 1.4		75.1 ± 7.4		205.3 ± 7.4		236.4 ± 3.8		257 ± 2.4	
A2780	24h	1.3 ± 2.6		1.0 ± 0.2		26.3 ± 7.8		26.8 ± 4.9		24.5 ± 0.9		28.3 ± 9.7	
	48h	0.5 ± 2.6		0.9 ± 1.2		23.1 ± 1.4		37.6 ± 5.5		39 ± 7.6		42.6 ± 3.9	
	72h	0.3 ± 2.6		0.5 ± 2.1		29.9 ± 2.2		46.3 ± 5.6		86.8 ± 2.5		72.7 ± 1.2	
A2780-cis	24h	0 ± 1.4		0.5 ± 1.7		15.5 ± 2.5		19 ± 0.2		16.9 ± 0.9		15.6 ± 0.7	
	48h	0.2 ± 1.4		1.1 ± 2		14.5 ± 1		20.7 ± 0.6		25.2 ± 2.2		26.7 ± 1.8	
	72h	0.4 ± 1.4		0.9 ± 1.7		18.9 ± 0.3		24.3 ± 1.1		23.5 ± 2		60.2 ± 5.5	

3.4.3 Induction of cell membrane permeability upon treatment with ND/PTX/HE4

Significant CMP increases were seen ($p < 0.001$) after the cells were exposed to all nanocomplex concentrations at all time points in a cell, dose, and time dependent manner (Figure 3.4).

SKOV-3 and OVCAR-8 cells showed the highest CMP increases after treatment with all concentrations of the nanocomplex compared to untreated cells. Increases in CMP changes ranged from 7.8% to 39.2% for SKOV-3 and 14% to 39.8% for OVCAR-8 as time and concentration increased (Table 3.3). For other cell lines (OAW42, A2780, and A2780-cis), significant CMP increases ($p < 0.001$) ranged between 7.6% to 41% in OAW42 cells, 9.6% to 16% in A2780 cells, and 16.9% to 31.4% in A2780-cis cells (Figure 3.4 b, e, and f). Increases in CMP were also seen when H10-80 cells were treated with the nanocomplex, but the percentage of CMP increases was not as high as the cancer cell lines with changes ranging between 6% to 27% (Figure 3.4 a).

Interestingly, the lowest concentration (1.25nM/ml) of the nanocomplex used in this study was more effective in increasing CMP changes than the clinically relevant dose of PTX (2.5nM/ml) alone in OAW42, SKOV-3, A2780 and A2780-cis cell lines with increases in CMP changes ranging from 6% to 28% across those cell lines compared to 3.9 to 24% for the PTX alone (Table 3.3).

A paired cisplatin sensitive and resistant ovarian cancer cell line (A2780 and A2780-cis) exhibited similar CMP changes after exposure to all concentrations of the nanocomplex at the three time points (Figure 3.4 e and f).

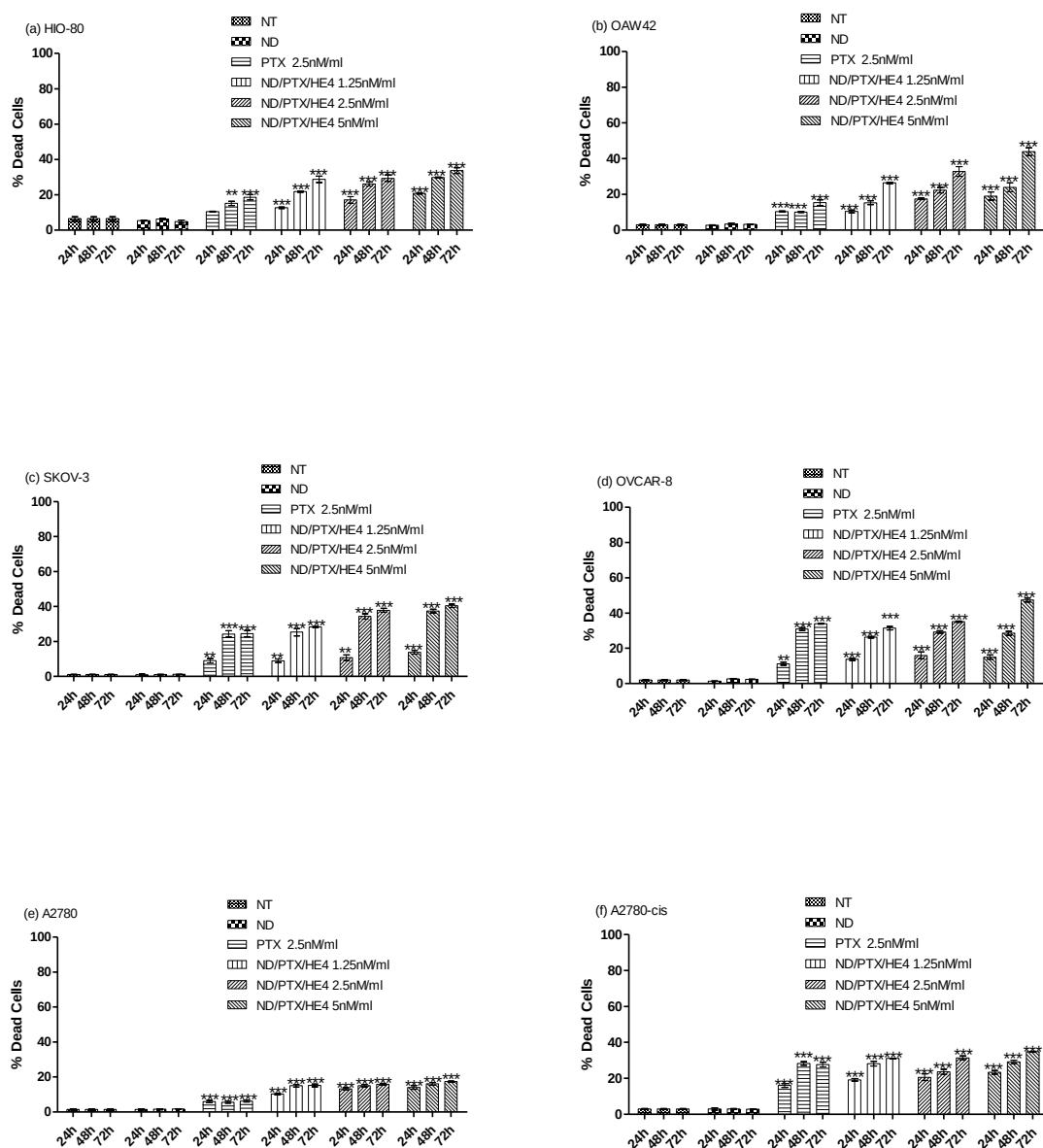


Figure 3.4: Cell membrane permeability measurement after ND/PTX/HE4 treatment

Cells were treated with ND/PTX/HE4 and incubated for 24h, 48h, and 72h and CMP was measured by the Cytell™ imaging system, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) OVCAR8, (e) A2780sen cells, and (f) A2780cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (PTX) paclitaxel.

Table 3.3: Percentages of CMP increases after ND/PTX/HE4 treatment.

The percentage of CMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%) SEM	ND (%) SEM	PTX 2.5nM/ml (%) SEM	ND/PTX/HE4 1.25nM/ml (%) SEM	ND/PTX/HE4 2.5nM/ml (%) SEM	ND/PTX/HE4 5nM/ml (%) SEM
HIO-80	24h	0 ± 1	0 ± 0.3	3.9 ± 0.3	6 ± 0.4	10.5 ± 1.9	14.2 ± 0.3
	48h	0 ± 1.1	0 ± 0.4	8.5 ± 1.2	15.2 ± 0.3	19.6 ± 1.1	23.2 ± 0.2
	72h	0 ± 1	0 ± 0.8	12 ± 1.5	22.2 ± 1.8	22.8 ± 1.8	27 ± 1.6
OAW42	24h	0 ± 0.5	0 ± 0.2	7.5 ± 0.3	7.6 ± 0.7	14.6 ± 0.4	16.1 ± 2.1
	48h	0 ± 0.5	0.5 ± 0.4	7.2 ± 0.2	12 ± 14.1	19.4 ± 1.5	21 ± 2.5
	72h	0 ± 0.5	0.2 ± 0.2	12.5 ± 1.5	23.5 ± 0.3	29.9 ± 2.7	41 ± 2.2
SKOV-3	24h	0 ± 0.1	0 ± 0.3	7.9 ± 1.2	7.8 ± 1	9.5 ± 1.5	12.6 ± 1
	48h	0 ± 0.1	0 ± 0.1	23.1 ± 1.8	24.1 ± 2.1	33.2 ± 1.5	36.2 ± 1
	72h	0 ± 0.2	4.6 ± 0.1	23.3 ± 1.8	27.2 ± 0.2	36.6 ± 1.1	39.2 ± 0.9
OVCAR-8	24h	0 ± 0.3	1.1 ± 0.1	12.1 ± 0.8	14 ± 0.5	16.9 ± 1.9	23.4 ± 1.2
	48h	0 ± 0.4	0.3 ± 0.2	12.2 ± 0.7	17 ± 0.4	23.5 ± 0.7	30.1 ± 1
	72h	0 ± 0.3	0 ± 0.1	18.5 ± 0.2	26.7 ± 1.1	27 ± 1.2	39.8 ± 1.1
A2780	24h	0 ± 0.4	0.1 ± 0.4	5 ± 0.6	9.6 ± 0.3	12.8 ± 0.6	13 ± 1
	48h	0 ± 0.5	0.2 ± 0.3	4.8 ± 0.5	14.6 ± 0.7	14.6 ± 0.6	15.7 ± 0.7
	72h	0 ± 0.4	0.3 ± 0.1	5.9 ± 0.6	14.7 ± 0.7	14.8 ± 0.5	16 ± 0.3
A2780-cis	24h	0 ± 0.2	0 ± 0.5	13 ± 1	16.9 ± 0.6	17.3 ± 1.9	20 ± 1
	48h	0 ± 0.3	0 ± 0.2	25.8 ± 0.9	25.9 ± 1.2	20.3 ± 1.4	26.9 ± 0.9
	72h	0 ± 0.2	0 ± 0.1	24.3 ± 1.2	28 ± 0.2	28.1 ± 1	31.4 ± 0.2

3.4.4 Cell Cycle analysis in response to ND/PTX/HE4 treatments

A significant increase in the number of cells in S phase was seen in OAW42, A2780, and A2780-cis cells after treatment with all concentrations of the nanocomplex at all time points compared to untreated cells (Figure 3.5 b, e and f). SKOV-3 and OVCAR-8 cells showed a significant increase in the number of cells in G2/M phase after all nanocomplex treatments at all time incubation points (Figure 3.5 c and d). An increase in S phase was also seen when HIO-80 cells were treated with the nanocomplex, (Figure 3.5 a), and the cell proportion in arrested (S) phase increased as time and concentration increased. However, the maximal cell proportion was in G2/M phase with 42% in OVCAR-8 and 26% in SKOV-3. For other cell lines the cell proportion in S phase was 25% in OAW42, 24% in A2780 and 18% in A2780-cis.

The mid concentration (ND/PTX/HE4 2.5nM/ml) of the nanocomplex was more effective in increasing the cell percentage in arrested (S and G2/M) phases than the same dose of PTX alone (2.5nM/ml) in OAW42, SKOV-3, OVCAR-8, A2780-cis and HIO-80 cells with increases in the cell proportions in the arrested phase ranging from 14% to 42% across those cell lines compared to 11% to 34% for the PTX alone at all time points (Figure 3.5 a, b, c, d and f).

The percentage of cells in S phase in cisplatin sensitive ovarian cancer cell (A2780) was higher than its resistance counterpart (A2780-cis) after treatment with all concentrations of the nanocomplex. Interestingly, the lowest concentration (1.25nM/ml) of the nanocomplex used in this study was more effective sending cells into arrest than the clinically relevant dose of PTX (2.5nM/ml) alone in OAW42, SKOV-3, OVCAR-8, A2780-cis and HIO-80 cells at all time points (Figure 3.5 a b, c, d, and f).

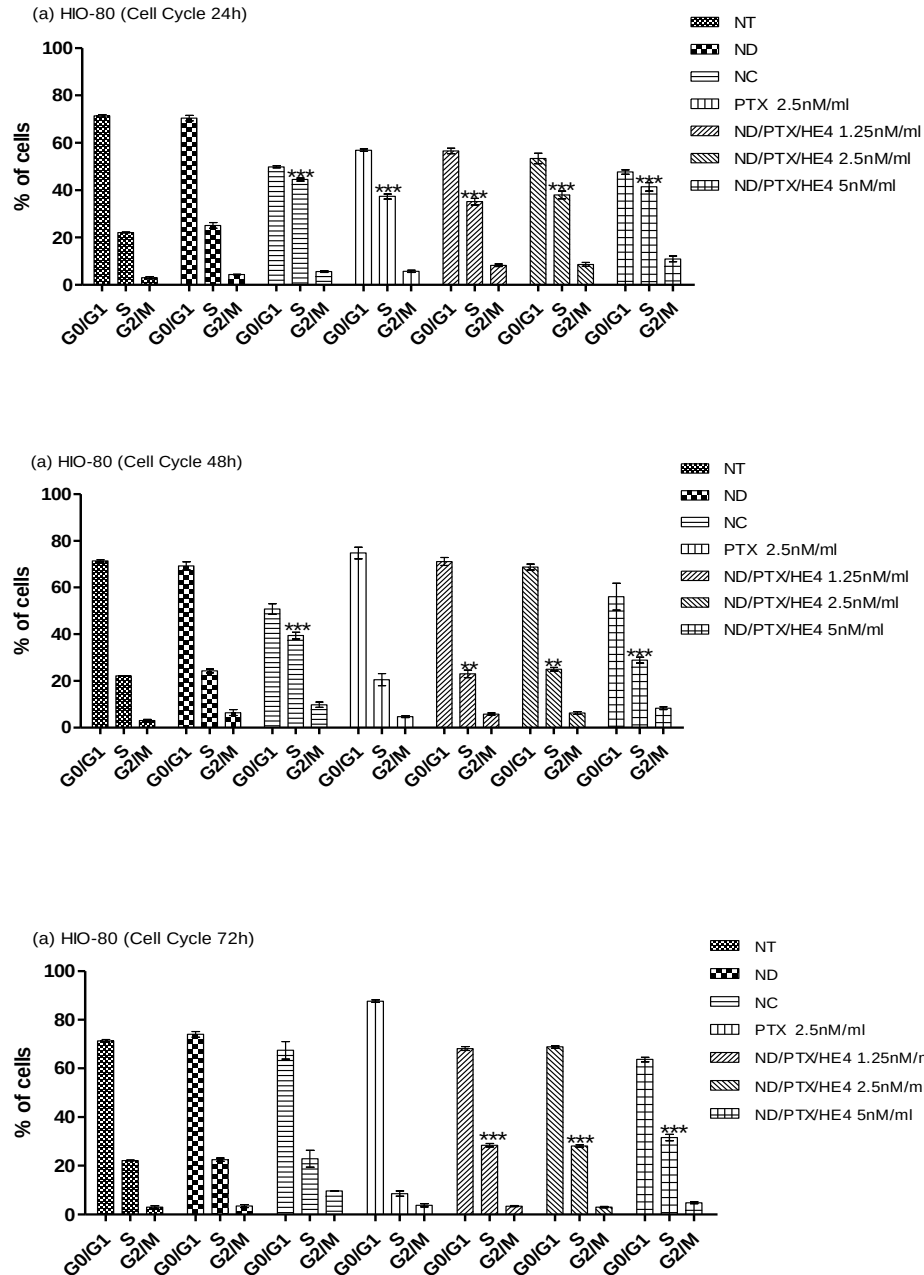


Figure 3.5a: Cell cycle for HIO-80 cells ND/PTX/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to control. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

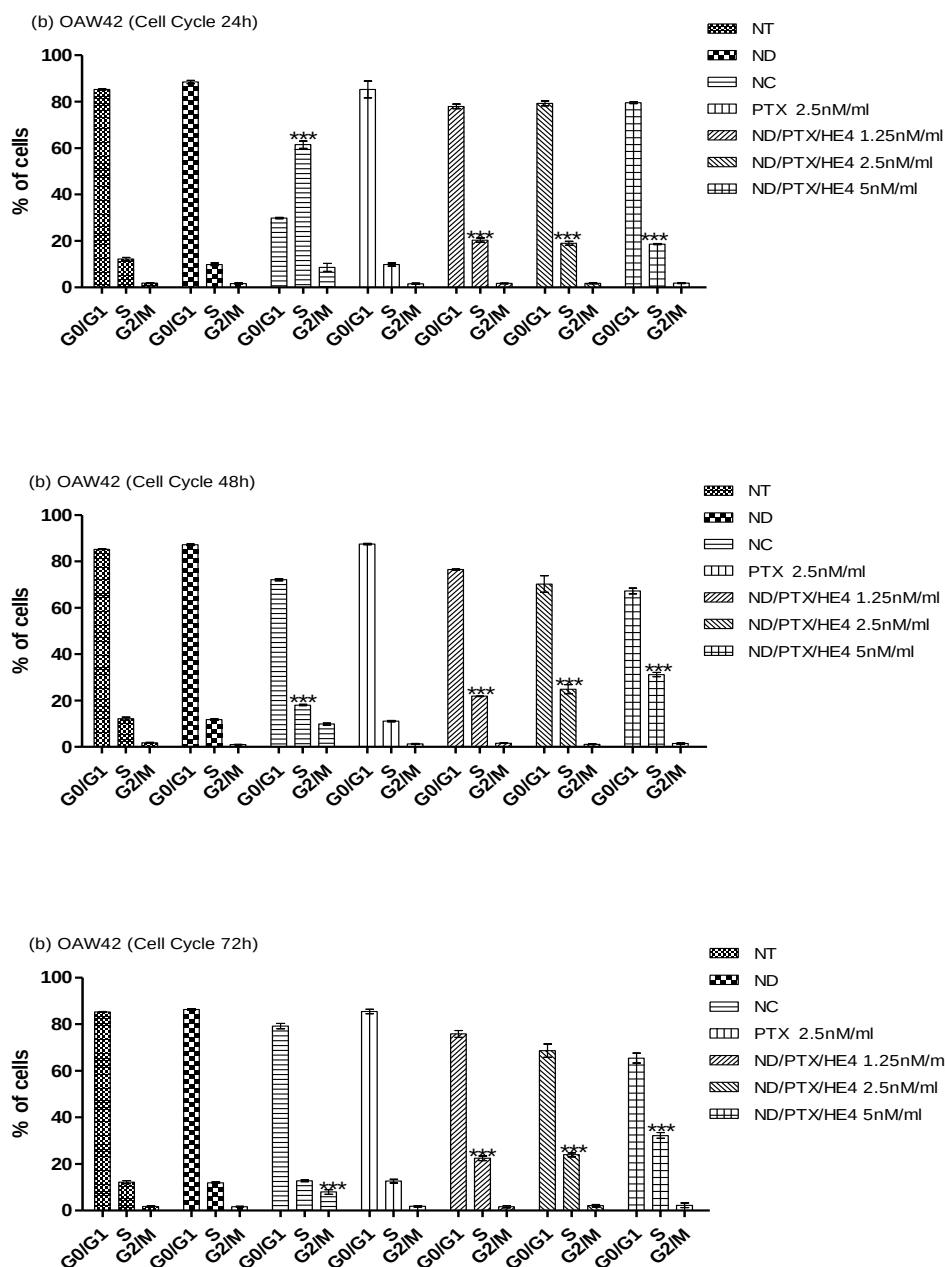


Figure 3.5b: Cell cycle for OAW42 cells ND/PTX/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “***” $p < 0.01$; “****” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

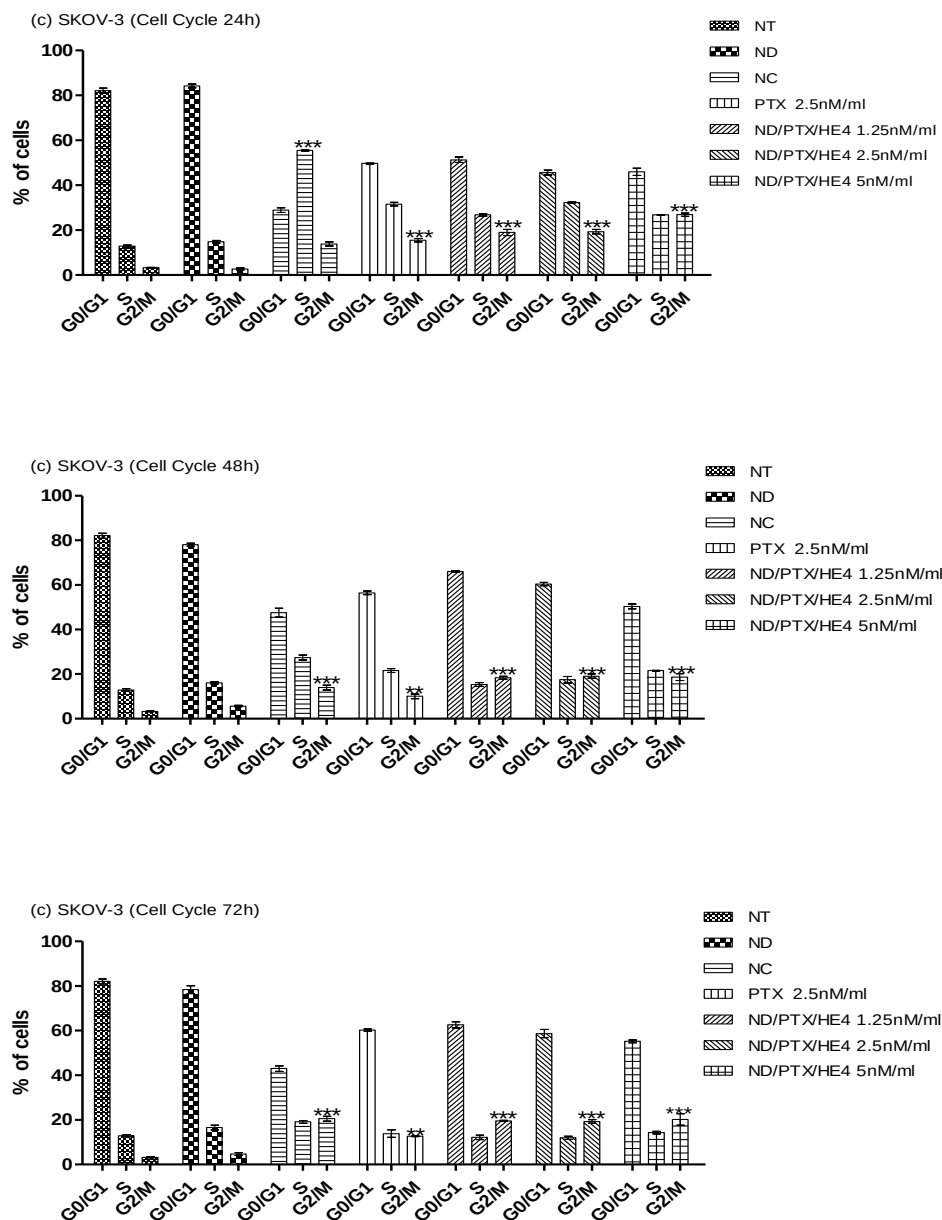


Figure 3.5c: Cell cycle for SKOV3 cells ND/PTX/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

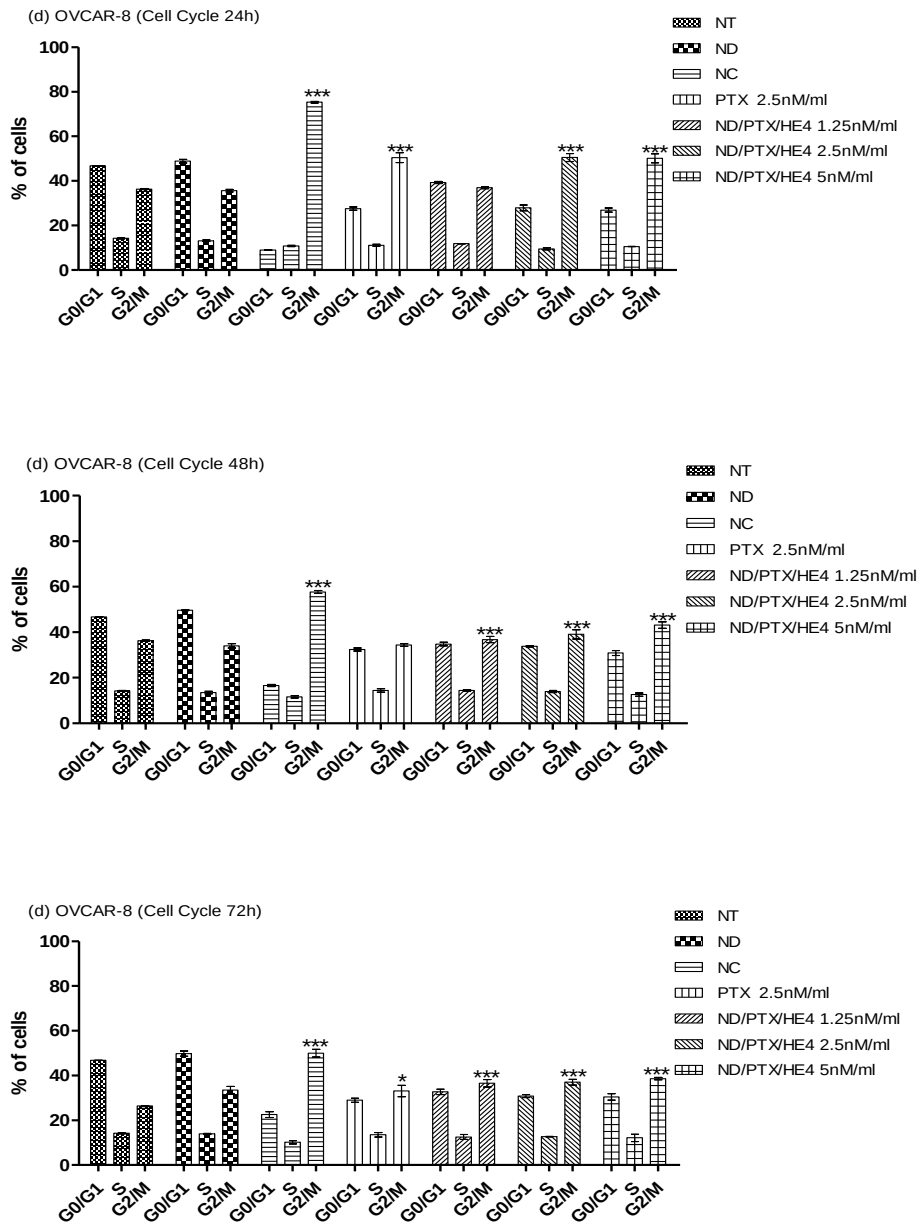


Figure 3.5d: Cell cycle for OVCAR-8 cells ND/PTX/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

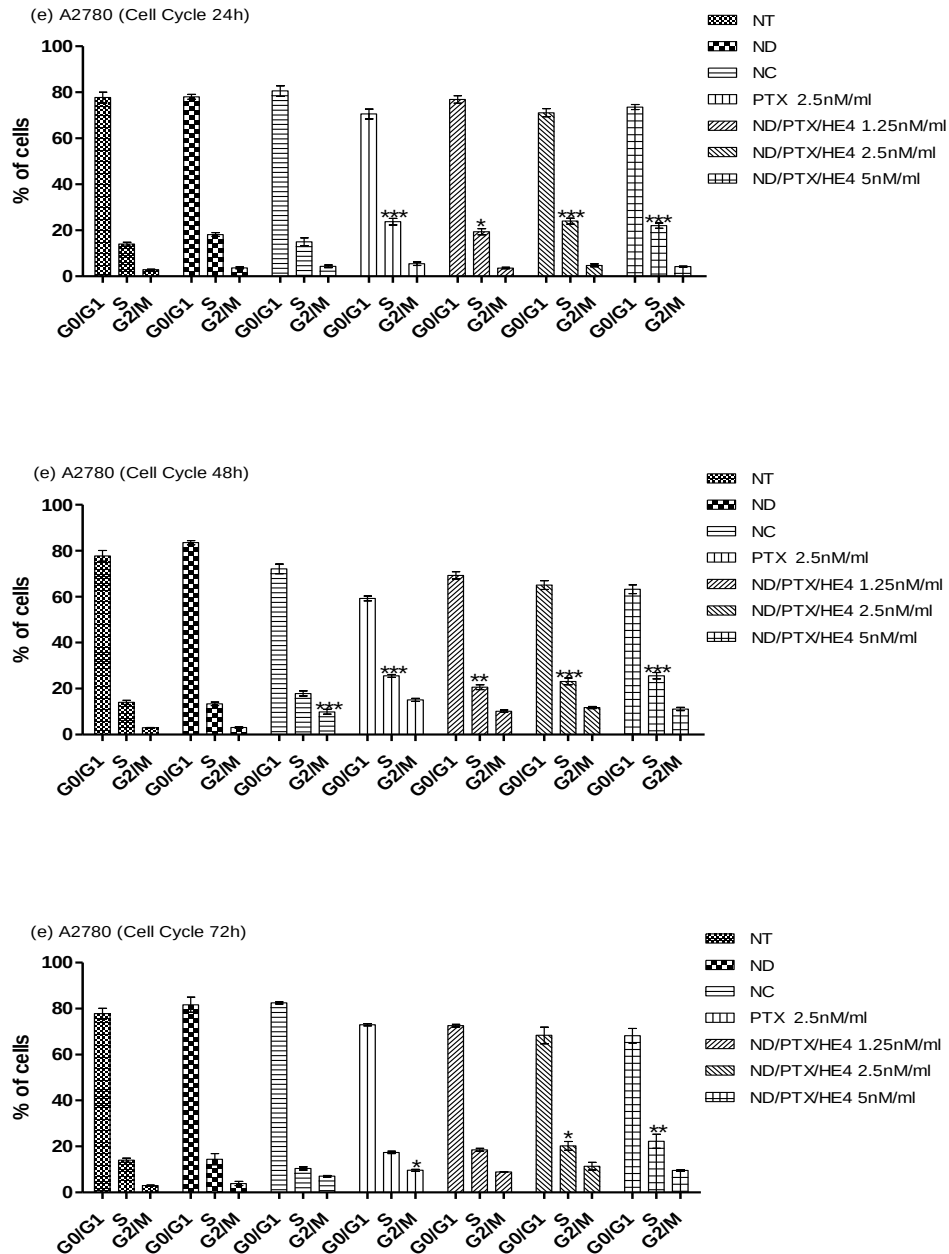


Figure 3.5e Cell cycle for A2780 cells ND/PTX/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

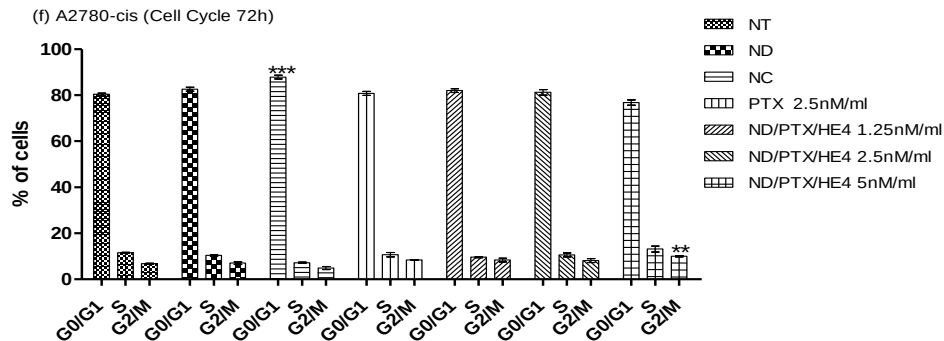
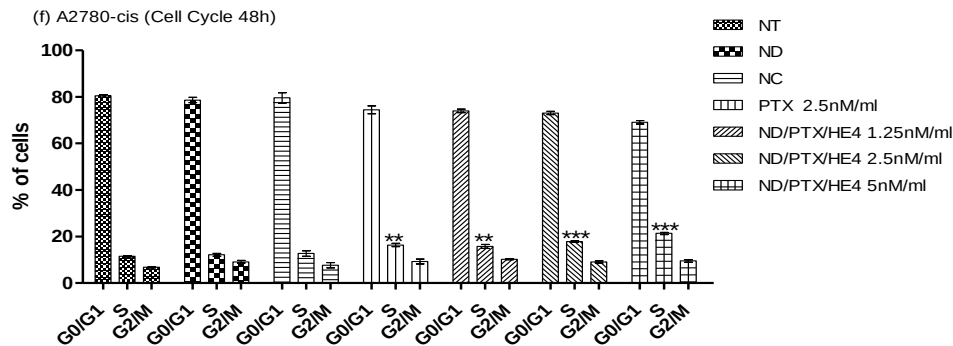
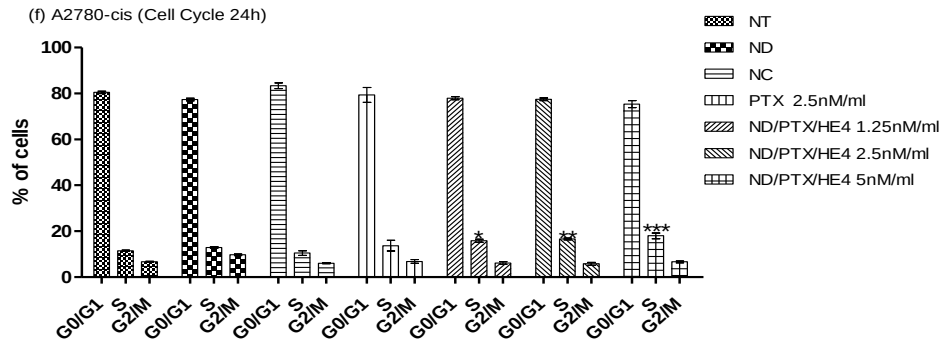


Figure 3.5 f Cell cycle for A2780-cis cells ND/PTX/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “***” $p < 0.01$; “****” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

3.4.5 Cell Viability assessment in response to ND/PTX/CA125 treatment

Significant cell viability reduction ($p < 0.001$) was seen for all cells after ND/PTX/CA125 exposure and the effect was cell, time, and dose dependent (Figure 3.6). The highest effect of the nanocomplex was seen in OVCAR-8 cells with cell viability reduction ranging between 63.1% to 91.8% as time and concentration increased compared to untreated cells (Table 3.4).

The cisplatin resistant ovarian cancer cell lines (A2780-cis) showed a higher cytotoxic effect ranging between 73.3% to 90.2% compared to its sensitive counterpart (A2780) with cytotoxic effects ranging from 57.45 to 90.6% after exposure to all concentrations of the nanocomplex at three time points (Figure 3.6 e and f).

Both SKOV-3 and OAW42 exhibited cell viability reduction ranging between 55.4% to 89.1% for SKOV-3 cells and 50.6% to 88.1% for OAW42 cells after exposure to all nanocomplex concentrations compared to the effect that induced after PTX alone with 50.4% to 74% for SKOV-3 cells and 49.5% to 80.4% for OAW42 cells at all time points (Figure 3.6 b and c).

The HIO-80 cell lines exhibited less cell viability reduction ranging from 21.1% to 81.4% after exposure to the nanocomplex at all time incubation points compared to untreated cells, and cell viability changes were not as high as the ovarian cancer cell lines (Figure 3.6 a).

Interestingly, the lower concentration (ND/PTX/CA125 1.25nM/ml) of the nanocomplex induced a higher cell viability reduction than the clinically relevant dose of (PTX 2.5nM/ml) across all ovarian cancer cells at all time intervals. Decreases in cell viability ranging from 21.1% to 86.7% were seen after all ovarian cancer cell lines were exposed to the lower concentration of the nanocomplex compared to the cell viability reduction after PTX exposure which ranged from 17.8% to 82% (Figure 3.6).

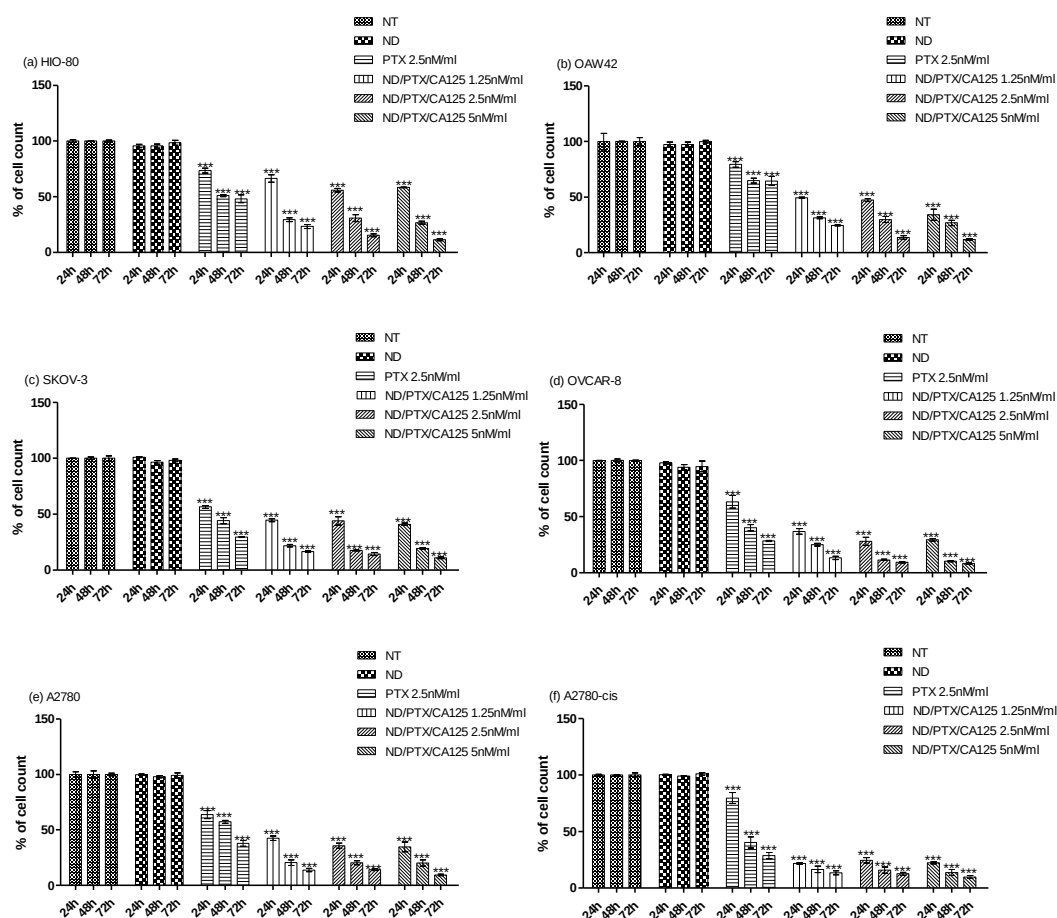


Figure 3.6: Cell viability assessment after ND/PTX/CA125 treatment

Cells were treated with ND/PTX/CA125 for three different time incubation and total cell count was measured using the Cytell™ imaging system and IN cell software, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) OVCAR8, (e) A2780 cells and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls. Statistically significant between treated and untreated cells reported by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (PTX) paclitaxel.

Table 3.4: Percentages of cell viability reduction (cell kill) after ND/PTX/CA125 treatment. The percentage of cell viability reduction listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	PTX (%)	SEM	ND/PTX/CA125 1.25nM/ml (%)	SEM	ND/PTX/CA125 2.5nM/ml (%)	SEM	ND/PTX/CA125 5nM/ml (%)	SEM
HIO-80	24h	0.0 ± 1		4.4 ± 0.8		17.8 ± 1.3		21.1 ± 0.7		34.3 ± 0.1		46.6 ± 2.8	
	48h	0.1 ± 0.3		1.0 ± 0.9		50.3 ± 2.4		49.5 ± 2.2		62.5 ± 1.5		72.2 ± 0.9	
	72h	0.1 ± 0.8		0.4 ± 1.4		66.4 ± 1.4		72.4 ± 0.3		75.0 ± 0.3		81.4 ± 0.6	
OAW42	24h	0.3 ± 7.4		2.5 ± 2.1		49.5 ± 1.1		50.6 ± 0.6		52.7 ± 1.3		65.7 ± 5	
	48h	0.4 ± 0.2		2.5 ± 2.1		69.2 ± 0.4		68.7 ± 0.7		70.1 ± 2.6		73.2 ± 2.4	
	72h	0.1 ± 3.5		0.1 ± 1.3		80.4 ± 0.4		75.4 ± 0.3		86.3 ± 1.6		88.1 ± 0.7	
SKOV-3	24h	0.2 ± 0.1		0.9 ± 0.4		50.4 ± 1.4		55.4 ± 1.3		55.9 ± 3.6		59.0 ± 1	
	48h	0.0 ± 1		3.6 ± 1.6		68.1 ± 1.8		78.1 ± 1		82.4 ± 0.4		80.6 ± 0.7	
	72h	0.3 ± 2.2		1.8 ± 1.1		74.0 ± 0.3		83.3 ± 0.4		85.7 ± 1.3		89.1 ± 0.7	
OVCAR-8	24h	0.2 ± 0.2		2.0 ± 0.7		61.9 ± 2.7		63.1 ± 2.4		71.9 ± 3.5		70.6 ± 1.1	
	48h	0.3 ± 1.3		5.9 ± 2.4		72.3 ± 0.2		74.9 ± 1.1		88.5 ± 0.5		89.7 ± 0.6	
	72h	0.0 ± 0.4		5.2 ± 4.7		85.2 ± 1.8		86.7 ± 1.4		90.7 ± 0.5		91.8 ± 0.7	
A2780	24h	0.0 ± 2.4		1.9 ± 1		36.1 ± 3.6		57.4 ± 2		64.2 ± 2.3		65.3 ± 4.4	
	48h	0.6 ± 3.1		1.9 ± 1		42.7 ± 1.3		79.4 ± 2.5		79.6 ± 1.8		79.8 ± 2.7	
	72h	0.1 ± 1.2		0.8 ± 2.1		62.2 ± 2.6		86.1 ± 1.5		85.8 ± 0.8		90.6 ± 0.6	
A2780-cis	24h	0.4 ± 0.7		0.2 ± 0.5		64.6 ± 4.4		73.3 ± 0.7		75.5 ± 2.3		77.7 ± 0.9	
	48h	0.1 ± 0.2		0.8 ± 0.2		77.1 ± 2.1		83.5 ± 2.9		84.1 ± 2.7		86.2 ± 2.6	
	72h	0.0 ± 1.9		1.0 ± 1.1		82.0 ± 0.8		86.5 ± 1.7		87.3 ± 1.2		90.2 ± 1.2	

3.4.6 Triggering of mitochondrial membrane potential changes upon treatment with ND/PTX/CA125

A significant increase ($p < 0.001$) in MMP intensity was seen in SKOV-3 and A2780-cis cells after exposure to all concentrations of ND/PTX/CA125, and the effect was dose, time and cell dependent (Figure 3.7 b, c, and d) and this is displayed in colorimetric heat map in (Figure 3.7 g).

OAW42, SKOV-3 and OVCAR-8 cells showed the highest response to all concentrations of the nanocomplex among studied cells compared to untreated cells. Increases in MMP changes ranging from 1.4% to 114.2% in OAW42, 56.8% to 121.4% for SKOV-3 and 12.4% to 107.8% for OVCAR-8 as time and concentration increased (Table 3.5). Similar MMP changes were also seen when the HIO-80 cells treated with ND/PTX/CA125, but the percentage of MMP changes were not as high as the cancer cell lines (Figure 3.7 a).

The cisplatin resistant ovarian cancer cell line (A2780-cis) exhibited higher MMP changes than the cisplatin sensitive (A2780) cells after exposure to all concentrations of ND/PTX/CA125 at three-time points (Figure 3.7 e and f).

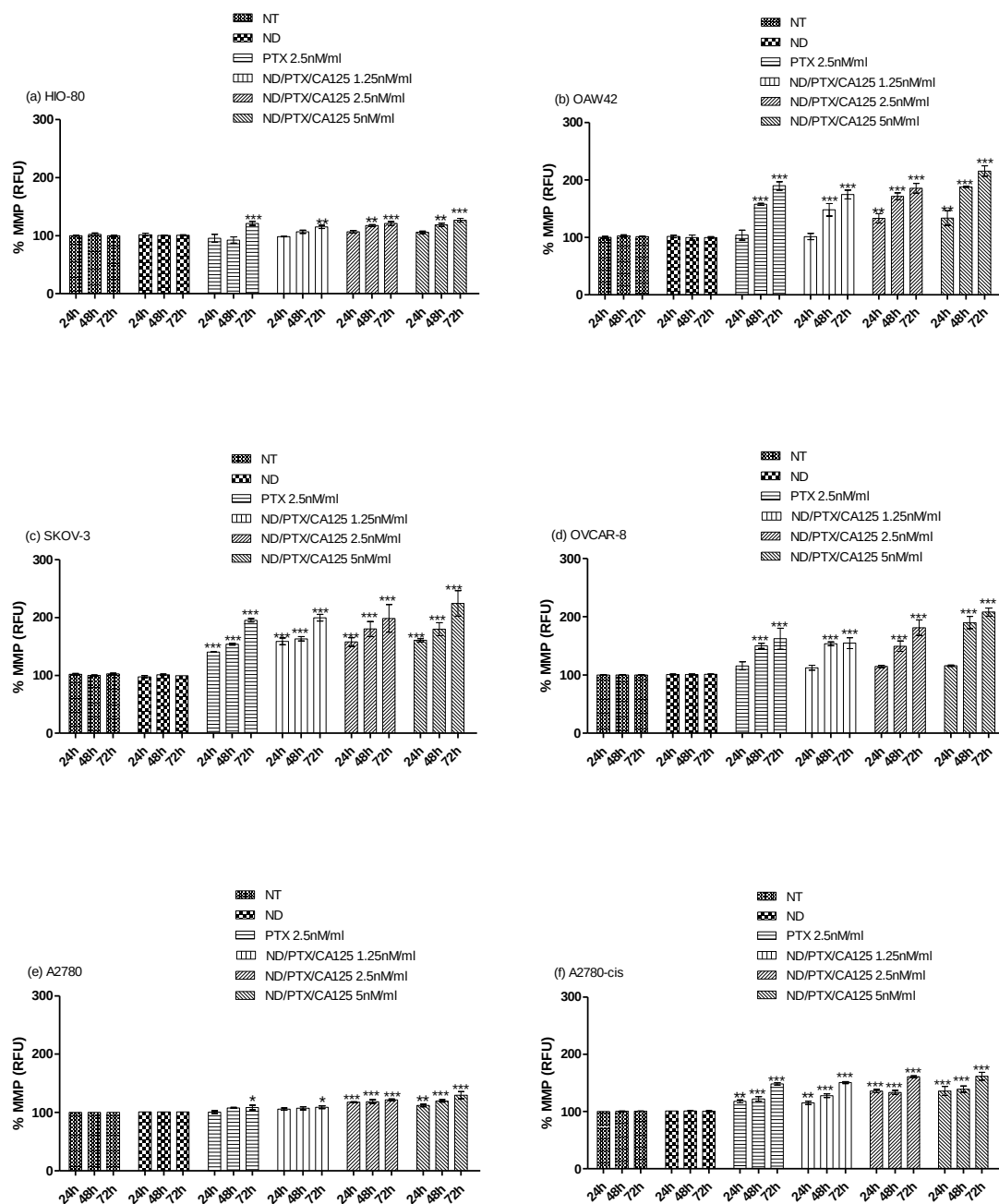


Figure 3.7: Mitochondrial membrane changes after ND/PTX/CA125 treatment

Cells were treated with ND/PTX/CA125 for three different time points and Cytell™ imaging system and IN cell software were used to measure the mitochondrial changes (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV3, (d) OVCAR8, (e) A2780 cells and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (PTX) paclitaxel.

Table 3.5: Percentages of MMP increases after ND/PTX/CA125 treatment.

The percentage of MMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	PTX 2.5nM/ml (%)	SEM	ND/PTX/CA125 (%)	SEM	ND/PTX/CA125 (%)	SEM	ND/PTX/CA125 (%)	SEM
HIO-80	24h	1.7 ± 0.8		1.6 ± 2.3		0.6 ± 6.7		0.6 ± 0.3		6.5 ± 2.1		5.8 ± 2.1	
	48h	1.3 ± 2.3		0.3 ± 0.3		0.0 ± 5.3		4.2 ± 2.9		14.8 ± 1.6		16.2 ± 2.9	
	72h	0.0 ± 0.9		0.6 ± 0.9		21.4 ± 3.2		15.2 ± 2.7		21.2 ± 3.3		26.7 ± 3.1	
OAW42	24h	0.7 ± 1.5		1.5 ± 2.2		4.0 ± 8.6		1.4 ± 5.5		33.2 ± 8.2		33.5 ± 12.7	
	48h	0.0 ± 2		0.9 ± 4.2		54.9 ± 1.8		45.4 ± 1.1		68.9 ± 5.9		85.3 ± 0.8	
	72h	0.4 ± 0.6		0.9 ± 1.2		88.3 ± 7.3		73.1 ± 7.7		84.1 ± 8.5		114.2 ± 9	
SKOV-3	24h	0.7 ± 1.6		0.4 ± 1.7		38.3 ± 0.4		56.8 ± 5.8		55.8 ± 7.2		58.8 ± 2.8	
	48h	0.1 ± 0.8		1.0 ± 1.3		53.9 ± 1.6		63.4 ± 3.8		80.4 ± 13		80.0 ± 11.1	
	72h	0.2 ± 1		0.6 ± 0.1		92.4 ± 2.9		96.9 ± 5.7		95.6 ± 24		121.4 ± 21.8	
OVCAR-8	24h	0.7 ± 0.3		0.7 ± 0.5		15.5 ± 7		12.4 ± 4.1		14.5 ± 2		15.8 ± 1.1	
	48h	0.1 ± 0.6		0.8 ± 0.6		49.9 ± 4.3		53.5 ± 3		49.3 ± 8.9		89.9 ± 10.6	
	72h	0.5 ± 0.4		1.0 ± 0.4		62.1 ± 18.1		54.4 ± 9.4		81 ± 13		107.8 ± 6.9	
A2780	24h	0.1 ± 0.1		0.7 ± 0.8		0.9 ± 2.4		5.8 ± 1.9		17.6 ± 0.7		11.8 ± 2.3	
	48h	0.0 ± 0.5		0.7 ± 0.4		7.8 ± 0.8		7 ± 2.6		18.7 ± 3.1		19.7 ± 2	
	72h	0.2 ± 0.2		0.8 ± 0.2		8.4 ± 4.1		8.9 ± 2.2		21.6 ± 1.6		29.7 ± 6.1	
A2780-cis	24h	0.1 ± 0.1		0.7 ± 0.1		17.8 ± 2.5		15.1 ± 2.7		35.6 ± 2.8		35.9 ± 7.8	
	48h	0.0 ± 0.6		0.6 ± 0.4		21.6 ± 3.8		27.2 ± 3.2		32.9 ± 3.4		38.7 ± 5.5	
	72h	0.4 ± 0.3		0.2 ± 0.8		47.6 ± 1		49.8 ± 1.9		60.2 ± 1.7		60.9 ± 6.7	

3.4.7 Cell Membrane Permeability measurement in response to ND/PTX/CA125 treatment

Significant CMP increases ($p < 0.001$) were seen after the cells were exposed to all nanocomplex concentrations at all time points, and the effect was cell, dose, and time dependent (Figure 3.8).

A2780 and OAW42 cells showed the highest CMP increases after treatment with all concentrations of the nanocomplex compared to untreated cells. Increases in CMP ranging from 19.5% to 88.1% for A2780 and 16% to 80.1% for OAW42 were seen as time and concentration increased (Table 3.6). For other cell lines (SKOV-3, OVCAR-8 and A2780-cis), significant CMP increases ($p < 0.001$) ranging between 13.5% to 38.3% in SKOV-3 cells, 12.6% to 48.6% in OVCAR-8 cells, and 25.9% to 50.9% A2780-cis cells (Figure 3.8 c, d, and f) were seen. Similar increases in CMP changes were also seen when H10-80 cells were treated with the nanocomplex ranging from 13.1% to 33.9% (Figure 3.8 a).

The mid concentration (ND/PTX/CA125 2.5nM/ml) of the nanocomplex was more effective in increasing CMP changes than the same dose of PTX alone (2.5nM/ml) in all cells with increases in CMP changes ranging from 16.3% to 81.3% across those cell lines compared to 8.2% to 57.3% for the PTX alone (Table 3.6).

A paired cisplatin sensitive and resistant ovarian cancer cell line (A2780 and A2780-cis) exhibited similar CMP changes after exposure to all concentrations of the nanocomplex at three time points (Figure 3.8 e and f).

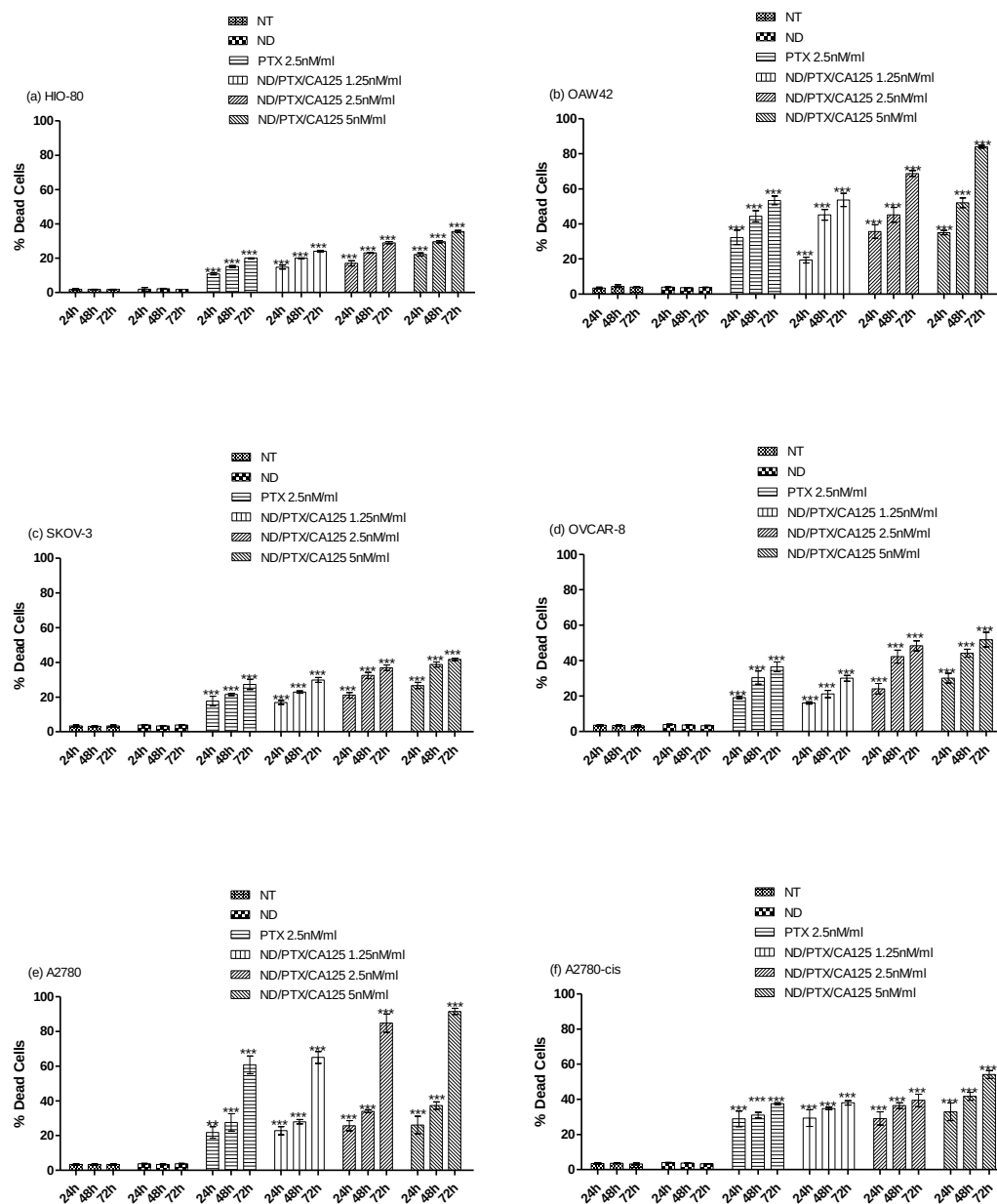


Figure 3.8: Cell membrane permeability measurement after ND/PTX/CA125 treatment

Cells were treated with ND/PTX/CA125 and incubated for 24h, 48h, and 72h and the CMP was measured by Cytell™ imaging system, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) OVCAR8, (e) A2780 cells, and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data, normalised to controls. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (PTX) paclitaxel.

Table 3.6: Percentages of CMP increases after ND/PTX/CA125 treatment.

The percentage of CMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	PTX 2.5nM/ml (%)	SEM	ND/PTX/CA125 1.25nM/ml (%)	SEM	ND/PTX/CA125 2.5nM/ml (%)	SEM	ND/PTX/CA125 5nM/ml (%)	SEM
HIO-80	24h	0 ± 0.4		0.2 ± 0.9		8.2 ± 0.6		13.1 ± 1.1		16.3 ± 1.4		20.5 ± 0.9	
	48h	0 ± 0.1		0 ± 0.2		13.5 ± 0.5		18.3 ± 0.1		21.4 ± 0.2		29.9 ± 0.7	
	72h	0 ± 0.1		0 ± 0.1		18.3 ± 0.1		22.4 ± 0.4		27.3 ± 0.7		33.9 ± 0.6	
OAW42	24h	0 ± 0.5		0.5 ± 0.1		28.9 ± 4.1		16 ± 1.6		32.2 ± 3.8		31.6 ± 1.2	
	48h	0 ± 0.7		0 ± 0.2		44 ± 3.1		40.8 ± 3		40.8 ± 4.2		47.6 ± 2.7	
	72h	0 ± 0.2		0 ± 0.1		49.5 ± 2.4		49.8 ± 3.7		64.9 ± 1.6		80.1 ± 0.8	
SKOV-3	24h	0 ± 0.5		0 ± 0.1		14.3 ± 2.8		13.5 ± 1.1		17.7 ± 1.6		23.3 ± 1.7	
	48h	0 ± 0.4		0 ± 0.2		18.4 ± 0.7		19.7 ± 0.6		29.2 ± 1.8		35.5 ± 1.4	
	72h	0 ± 0.5		0 ± 0.1		23.8 ± 2.9		26.5 ± 1.4		33.5 ± 1.6		38.3 ± 0.6	
OVCAR-8	24h	0 ± 0.5		0 ± 0.1		15.7 ± 0.5		12.6 ± 0.6		20.7 ± 3		26.7 ± 2.7	
	48h	0 ± 0.2		0 ± 0.2		27 ± 3.7		17.8 ± 2		38 ± 3.6		44 ± 2.1	
	72h	0 ± 0.3		0 ± 0.1		33.3 ± 2.7		26.8 ± 1.6		45.2 ± 2.8		48.6 ± 4	
A2780	24h	0 ± 0.5		0.3 ± 0.2		18.3 ± 3.3		19.5 ± 2.3		22.3 ± 3		22.7 ± 5	
	48h	0 ± 0.5		0 ± 0.3		24.3 ± 5		24.7 ± 1.3		30.7 ± 0.8		34 ± 2.1	
	72h	0 ± 0.6		0.3 ± 0.2		57.3 ± 5.1		61.6 ± 3.4		81.3 ± 5.2		88.1 ± 1.8	
A2780-cis	24h	0 ± 0.5		0.5 ± 0.1		25.5 ± 4.4		25.9 ± 4.7		25.7 ± 3.7		29.5 ± 5	
	48h	0 ± 0.2		0 ± 0.2		27.4 ± 1.6		31.1 ± 0.6		32.8 ± 1.6		38.2 ± 2.2	
	72h	0 ± 0.4		0.1 ± 0.1		34.3 ± 0.5		34.8 ± 1.2		36.2 ± 3.5		50.9 ± 2.2	

3.4.8 Cell cycle distribution in response to ND/PTX/CA125 treatment

The ND/PTX/CA125 induced significant increase in the number of cells in S phase ($p < 0.001$) in all ovarian cancer cell lines at all time points compared to untreated cells (Figure 3.9 b, c, d, e and f). An increase in S phase was also seen when H10-80 cells were treated with the nanocomplex at 24h and 48h, and in G0/G1 at 72h (Figure 3.9 a), among all cells studied, the highest cell cycle arrest proportion in S phase (53%) was seen in OAW42 cell. For other cells the cell proportions in S phase were 48% in OVCAR-8, 47% in SKOV-3 and A2780-cis and 45% in A2780.

A paired cisplatin sensitive and resistant ovarian cancer cell line (A2780 and A2780-cis) exhibited similar cell cycle arrest after exposure to all concentrations of the nanocomplex at the three time points (Figure 3.9 e and f).

The mid concentration (ND/PTX/CA125 2.5nM/ml) of the nanocomplex was more effective in increasing the cell percentage in arrested phase than the same dose of PTX alone (2.5nM/ml) in all cells with increases of cell proportions in arrested phase ranging from 28% to 51% across those cell lines compared to 20% to 42% for the PTX alone. Interestingly, the lowest concentration (1.25nM/ml) of this nanocomplex used in this study was more effective in sending cells into arrest than the clinically relevant dose of PTX (2.5nM/ml) alone in all cells at all time points (Figure 3.9).

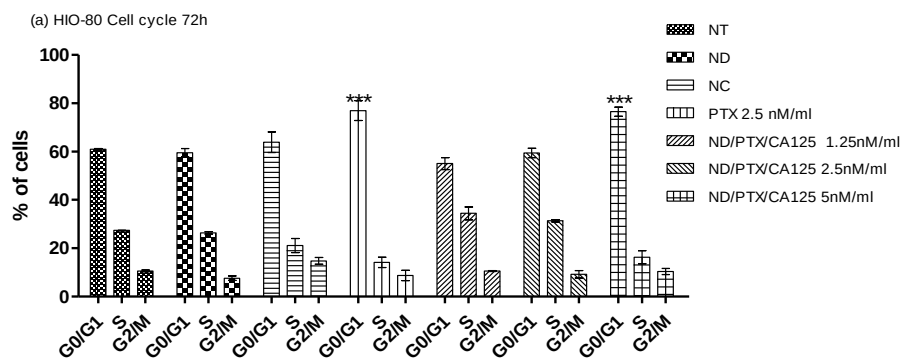
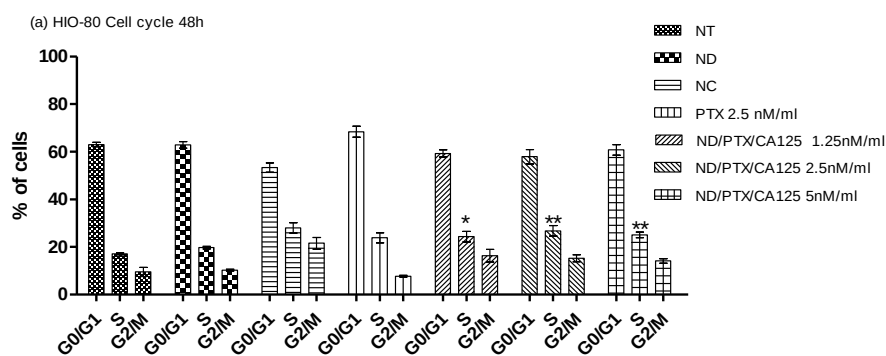
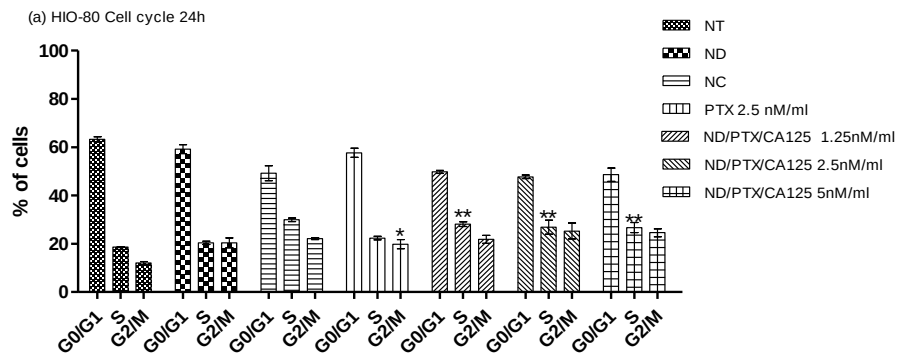


Figure 3.9 a: Cell cycle for HIO-80 cells after ND/PTX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

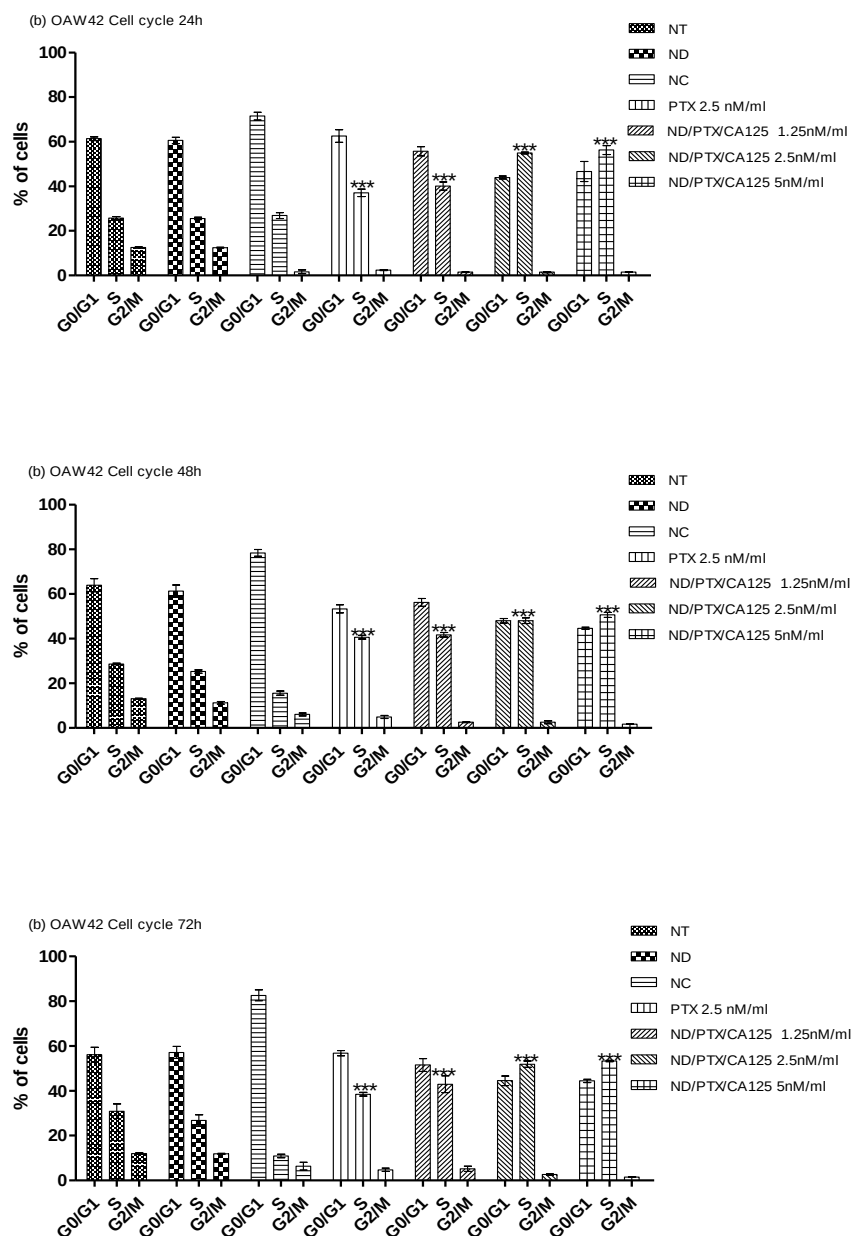


Figure 3.9 b: Cell cycle for OAW42 cells after ND/PTX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

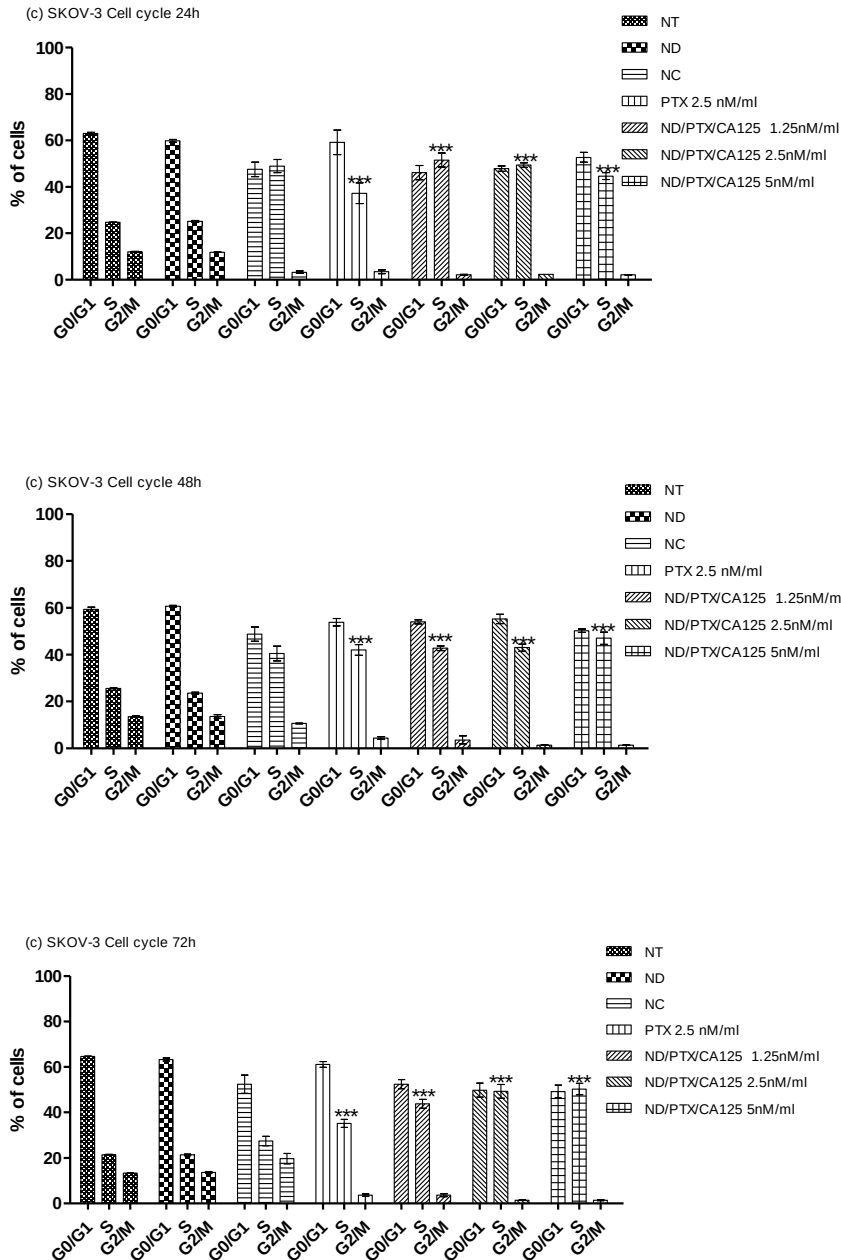


Figure 3.9 c: Cell cycle for SKOV-3 cells after ND/PTX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

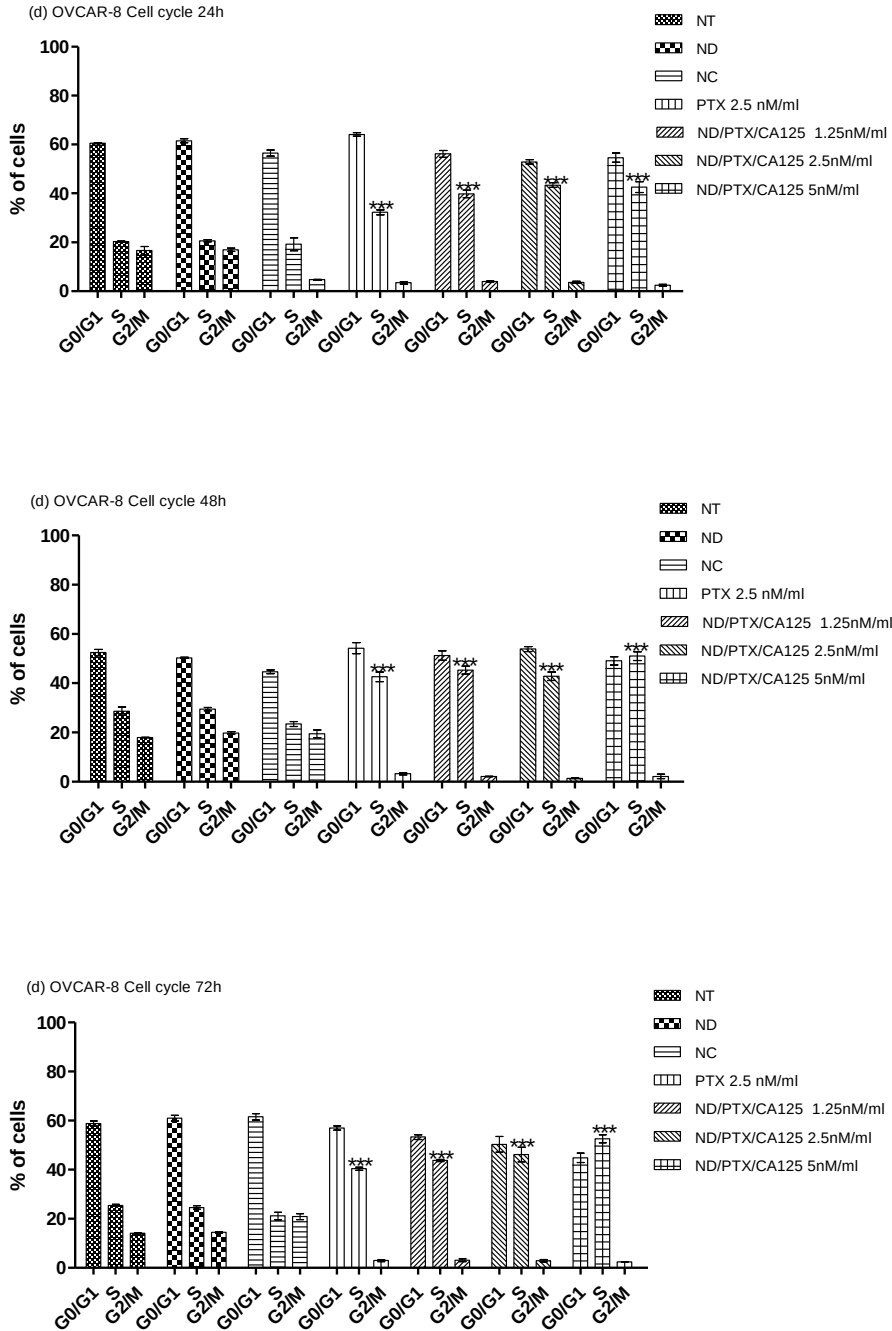


Figure 3.9 d: Cell cycle for OVCAR-8 cells after ND/PTX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

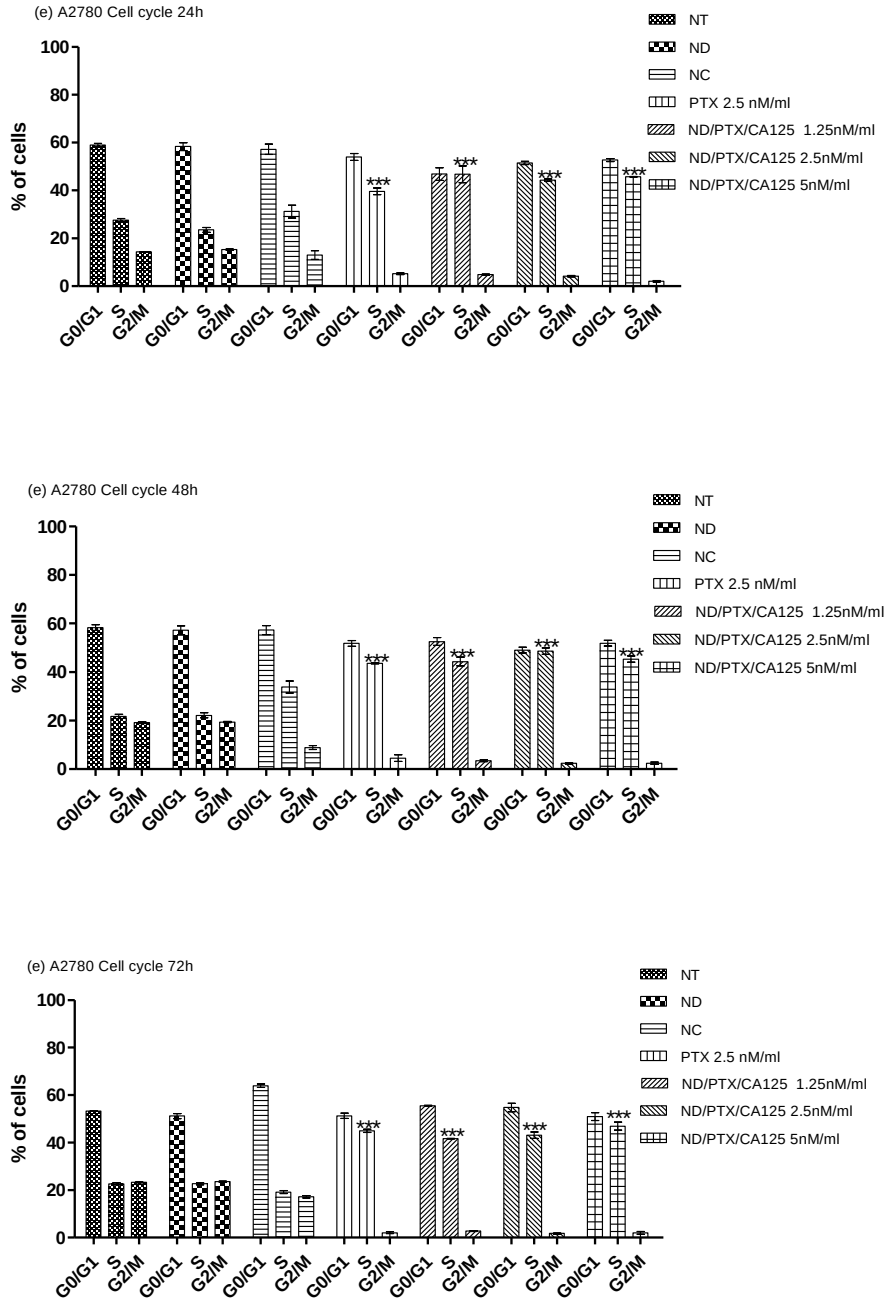


Figure 3.9 e: Cell cycle for A2780 cells after ND/PTX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

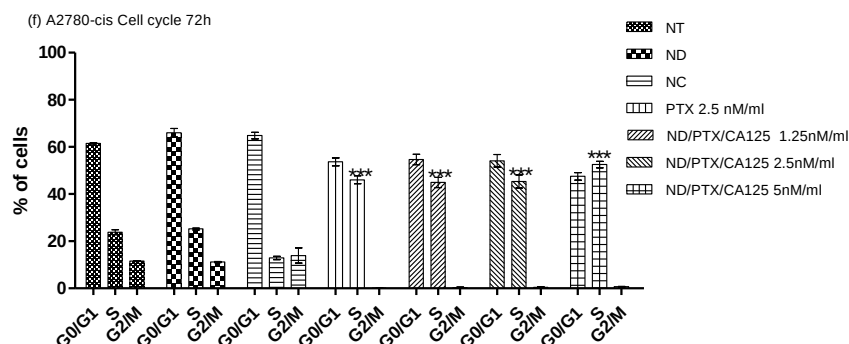
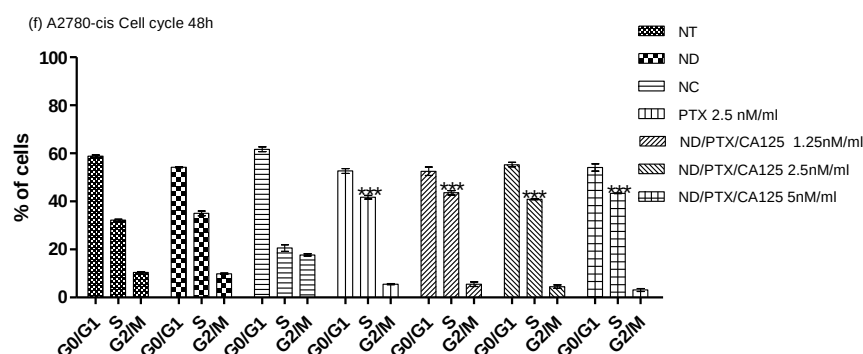
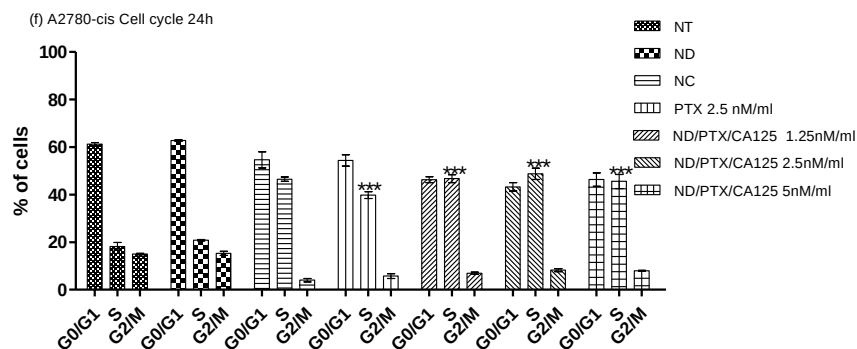


Figure 3.9 f: Cell cycle for A2780-cis cells after ND/PTX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/PTX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (PTX) paclitaxel.

3.4.9 Evaluation of cell viability in response to ND/CARBO/HE4 treatment

A significant cell viability reduction was seen ($p \leq 0.001$) after all cells were exposed to all nanocomplex concentrations compared to untreated cells, and the effect was time and concentration dependent (Figure 3.10).

The OAW42 and A2780 cells showed similar cell viability reduction after exposure to all nanocomplex concentrations with changes ranging from 33.8% to 73.7% compared to untreated cells across all time points (Table 3.7).

SKOV-3 and OVCAR-8 cells exhibited a significant cell viability reduction ($p \leq 0.001$) ranging from 25.3% to 65.4% for SKOV-3 cells and from 24.7% to 71.6% for OVCAR-8 after these cells were exposed to all concentration over all time points (Figure 3.10 b and c). HIO-80 cells showed significant cell viability reduction, and this effect was similar to the ovarian cancer cell lines. Decrease in cell viability ranked from 23.1% to 72% compared to untreated cells at all time intervals (Figure 3.10 a).

Cisplatin resistance ovarian cancer cells (A2780-cis) showed a significant cell viability reduction but not as high as other ovarian cancer cells (Figure 3.10 f). The lowest concentration (7 μ M/ml) of the nanocomplex used in this study was more effective in reducing cell viability than the clinically relevant dose of CARBO (14 μ M/ml) alone in all ovarian cancer cells at all time points (Figure 3.10). Decreases in cell viability ranging between 23.6% to 69.7% after exposure to 14 μ M/ml of the nanocomplex compared to the same dose of drug alone which showed changes ranging from 14.1% to 47.2% at all time points (Table 3.7).

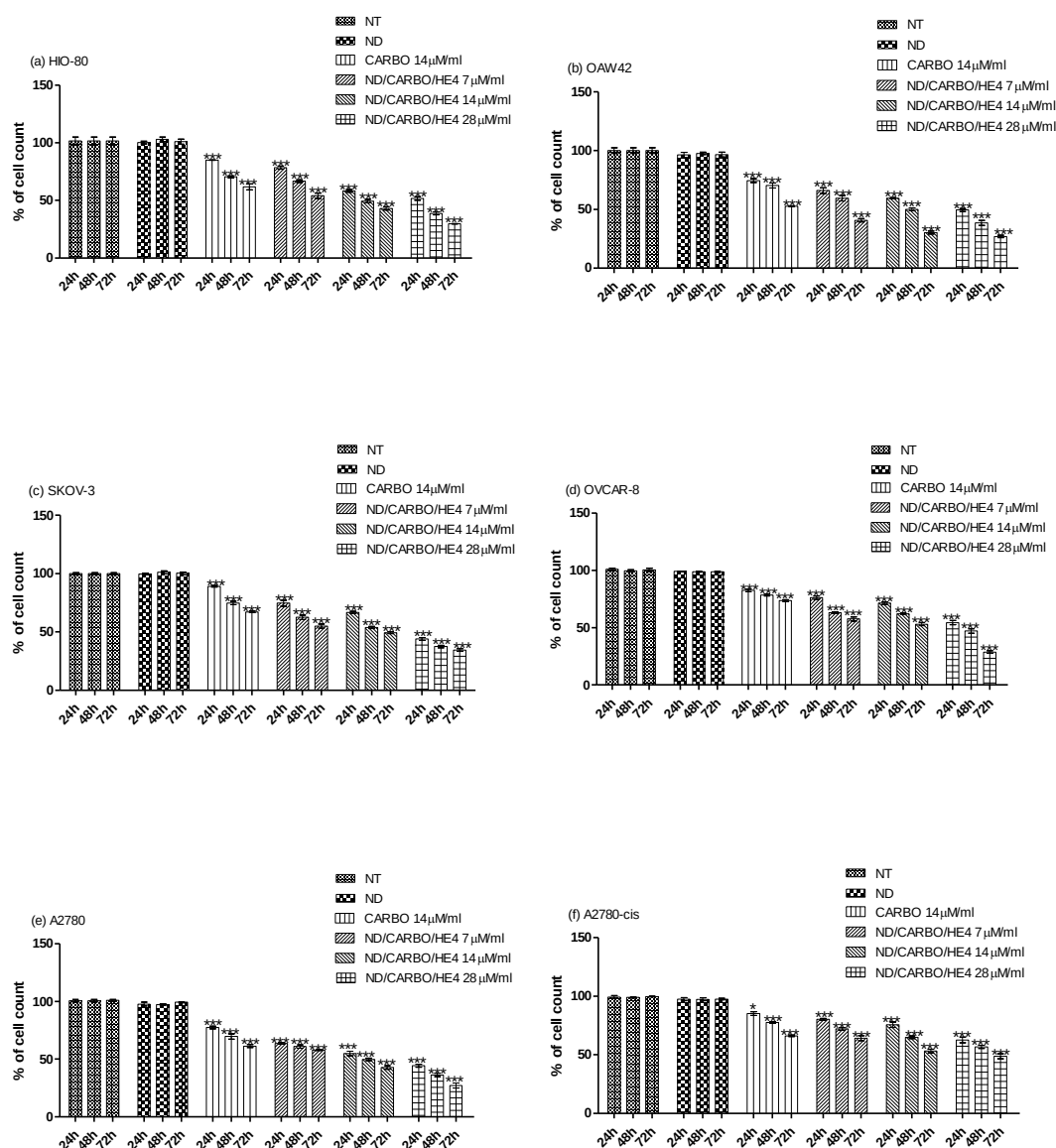


Figure 3.10: Cell viability assessment after ND/CARBO/HE4 treatment

Cells were treated with ND/CARBO/HE4 for three different time incubation and total cell count was measured using the Cytell™ imaging system and IN cell software, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) OVCAR8, (e) A2780 cells and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significant between treated and untreated cells reported by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (CARBO) carboplatin.

Table 3.7: Percentages of cell viability reduction (cell kill) after ND/CARBO/HE4 treatment. The percentage of cell viability reduction listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	CARBO 7µM/ml (%)	SEM	ND/CARBO/HE4 7µM/ml (%)	SEM	ND/CARBO/HE4 14µM/ml (%)	SEM	ND/CARBO/HE4 28µM/ml (%)	SEM
HIO-80	24h	4.7 ± 3.4		1.3 ± 1.2		16.5 ± 0.2		23.1 ± 1.4		43.3 ± 1.1		49.8 ± 1.5	
	48h	4.2 ± 3.2		1.3 ± 2		31.0 ± 0.7		34.8 ± 1.2		52.2 ± 1.4		62.9 ± 1.2	
	72h	4.0 ± 3.6		0.5 ± 2.1		39.8 ± 2.5		47.6 ± 2.2		58.6 ± 1.4		72.0 ± 0.2	
OAW42	24h	3.3 ± 2.4		3.5 ± 2.1		25.4 ± 1.6		33.8 ± 2.3		40.4 ± 0.6		50.4 ± 1.2	
	48h	3.1 ± 2.1		2.3 ± 1.1		29.6 ± 2.1		40.4 ± 2.3		49.9 ± 1.2		61.5 ± 2.1	
	72h	3.8 ± 2.2		3.2 ± 2		47.2 ± 0.3		59.2 ± 1.4		69.7 ± 1.3		73.0 ± 1	
SKOV-3	24h	0.9 ± 0.8		0.3 ± 0.8		10.9 ± 0.6		25.3 ± 2.5		33.2 ± 0.8		55.9 ± 1.1	
	48h	1.7 ± 0.8		1.1 ± 1.3		25.1 ± 1.4		37.4 ± 1.9		46.2 ± 0.9		62.4 ± 1	
	72h	1.4 ± 0.8		0.8 ± 1.1		32.7 ± 0.7		44.9 ± 1.7		50.5 ± 0.8		65.4 ± 1.1	
OVCAR-8	24h	4.7 ± 0.9		1.8 ± 0.2		18.4 ± 1		24.7 ± 1.4		29.5 ± 1		46.5 ± 1.9	
	48h	1.4 ± 0.8		0.8 ± 0.4		21.1 ± 0.7		36.5 ± 0.6		37.4 ± 0.6		52.5 ± 1.9	
	72h	2.4 ± 1.4		1.5 ± 0.6		26.6 ± 0.8		42.6 ± 1.7		47.2 ± 1.4		71.6 ± 1.6	
A2780	24h	1.2 ± 1.2		2.9 ± 1.6		23.2 ± 1.1		37.0 ± 0.6		45.8 ± 1.9		56.3 ± 1.2	
	48h	1.4 ± 1.2		3.3 ± 0.7		30.9 ± 2.2		39.7 ± 1.4		50.8 ± 1		64.3 ± 1.4	
	72h	1.5 ± 0.8		1.6 ± 0.5		39.7 ± 1.2		43.1 ± 0.4		58.2 ± 1.6		73.7 ± 2.2	
A2780-cis	24h	1.7 ± 1.1		1.8 ± 1.3		14.1 ± 1.6		18.9 ± 0.8		23.6 ± 2.2		36.6 ± 2.3	
	48h	0.0 ± 0.5		1.6 ± 1.2		21.3 ± 0.7		26.7 ± 1.1		34.0 ± 1.1		42.1 ± 1.3	
	72h	2.1 ± 0.2		2.1 ± 0.6		33.5 ± 0.6		35.4 ± 2.1		46.3 ± 1.5		50.9 ± 2.1	

3.4.10 MMP changes in response to ND/CARBO/HE4 treatment

A significant increase ($p < 0.001$) in MMP intensity was seen in all cells after all concentrations of ND/CARBO/HE4 treatments, and the effect was dose, time and cell dependent (Figure 3.11).

OAW42 and SKOV-3 cells showed the highest response to all concentrations of the nanocomplex compared to untreated cells with percentage of MMP changes ranging from 30.9% to 182.4% for OAW42 and 34% to 153.9% for SKOV-3 as time and concentration increased (Table 3.8). Significant MMP changes ($p < 0.001$) were also seen in the other cell lines (OVCAR-8, A2780, and A2780-cis) ranging between 37.1% to 139.5% in OVCAR-8, 10.3% to 110.2% in A2780 and 8.2% to 25.3% in A2780-cis (Figure 3.11 d, e, and f). The HIO-80 also showed similar MMP changes after treatment with ND/CARBO/HE4 with 16.4% to 105.1% increases compared to untreated cells (Figure 3.11 a).

Cisplatin sensitive (A2780) ovarian cancer cell line exhibited higher MMP changes than the cisplatin resistant cell line (A2780-cis) after exposure to all concentrations of ND/CARBO/HE4 at three time points (Figure 3.11 e and f).

The mid concentration (14 $\mu\text{M/ml}$) of the nanocomplex used in this study induced higher MMP changes than the clinically relevant dose of CARBO (14 $\mu\text{M/ml}$) alone in all cell lines at all time points (Figure 3.11).

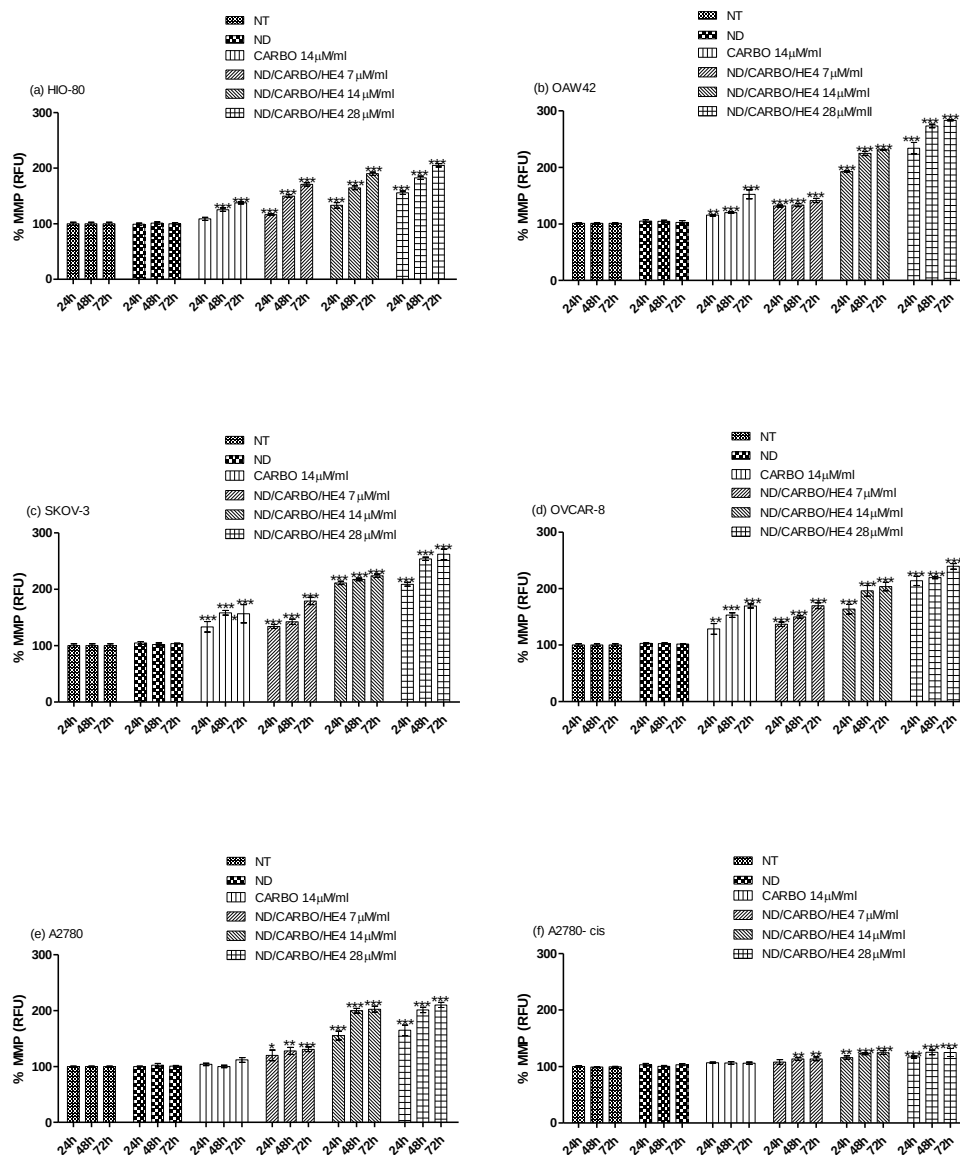


Figure 3.11: Mitochondrial membrane changes after ND/CARBO/HE4 treatment

Cells were treated with ND/CARBO/HE4 for three different time points and Cytell™ imaging system and IN cell software were used to measure the mitochondrial changes (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV3, (d) OVCAR8, (e) A2780 cells and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (CARBO) carboplatin.

Table 3.8: Percentages of MMP increases after ND/CARBO/HE4 treatment.

The percentage of MMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	CARBO 7µM/ml (%)	SEM	ND/CARBO/HE4 7µM/ml (%)	SEM	ND/CARBO/HE4 14µM/ml (%)	SEM	ND/CARBO/HE4 28µM/ml (%)	SEM
HIO-80	24h	0.1 ± 2.9		0.1 ± 2		8.9 ± 2.7		16.4 ± 1.4		33.1 ± 5		55.9 ± 3	
	48h	0.0 ± 3		1.2 ± 1.9		25.4 ± 3		49.8 ± 2.8		64.7 ± 3.4		82.7 ± 2.9	
	72h	0.2 ± 2		0.1 ± 1.7		37.5 ± 1.8		70.8 ± 2.9		90.1 ± 3		105.1 ± 3	
OAW42	24h	1.0 ± 1.2		4.1 ± 2.1		14.5 ± 1.5		30.9 ± 1.6		91.6 ± 1.1		133 ± 9.9	
	48h	0.5 ± 1.3		3.6 ± 2.2		19.1 ± 1.2		32.3 ± 2.7		123.7 ± 3.5		172.3 ± 2.8	
	72h	0.7 ± 1.2		1.9 ± 2.8		51.3 ± 7.9		40.4 ± 3.5		130.1 ± 0.7		182.4 ± 1	
SKOV-3	24h	0.0 ± 3		4.6 ± 2.4		33.3 ± 9.2		34 ± 4.5		111.1 ± 2.8		108.9 ± 3.4	
	48h	0.0 ± 2.5		2.0 ± 2.6		58.1 ± 4.1		42.2 ± 4.7		117.3 ± 2.5		153.9 ± 2.8	
	72h	0.0 ± 3		2.2 ± 0.6		58.1 ± 16.1		42.2 ± 6.3		117.3 ± 3		153.9 ± 9.3	
OVCAR-8	24h	0.1 ± 2.1		3.0 ± 0.7		28.7 ± 9.6		37.1 ± 3.9		63.9 ± 8.4		113.8 ± 8.1	
	48h	0.0 ± 2		2.6 ± 1.2		53.3 ± 4		50.1 ± 2.3		96.2 ± 9.2		119.5 ± 1.9	
	72h	0.2 ± 1.9		1.9 ± 0.3		69.2 ± 3.5		69.7 ± 5.1		103.7 ± 7.5		139.5 ± 4.9	
A2780	24h	1.8 ± 0.9		0.3 ± 0.7		6.3 ± 2.2		10.3 ± 9.6		41.7 ± 7.9		48.1 ± 9.3	
	48h	0.3 ± 1		2.3 ± 3		0.1 ± 2.2		27.7 ± 6.3		99.8 ± 4.3		101.2 ± 4.9	
	72h	0.5 ± 0.9		0.6 ± 1.3		11.7 ± 4		30.7 ± 4.1		102.4 ± 5.4		110.2 ± 4.5	
A2780-cis	24h	0.1 ± 1.5		3.4 ± 2.1		6.7 ± 1.3		8.2 ± 4.1		15.9 ± 2.9		16.7 ± 1.9	
	48h	0.0 ± 0.9		1.7 ± 1.1		7.3 ± 2.5		14.3 ± 2.6		23.9 ± 1.6		26.2 ± 4.1	
	72h	0.0 ± 0.7		3.5 ± 1.4		6.6 ± 2		14.1 ± 3.1		25.2 ± 3.3		25.3 ± 7.1	

3.4.11 Cell membrane permeability assessment in response to ND/CARBO/HE4 treatment

The ND/CARBO/HE4 induced a significant increase in CMP ($p < 0.001$) after the cells were exposed to all nanocomplex concentrations at all time points and the effect was cell, dose, and time dependent (Figure 3.12) and this is displayed in colorimetric heat map in (Figure 3.12 g).

OAW42 cells showed the highest CMP increases after treatment with all concentrations of the nanocomplex compared to untreated cells, ranging from 7.4% to 19.7% as time and concentration increased (Table 3.9). For other cell lines (SKOV-3, OVCAR-8, A2780, and A2780-cis), significant CMP increases ($p < 0.001$) ranging between 3.8% to 13.8% in SKOV-3 cells, 3.6% to 11.3% in OVCAR-8 cells, 6.8% to 13.5% in A2780 cells, and 3.2% to 5.8% A2780-cis cells (Figure 3.12 b, c, e, and f). Similar increases were also seen in H10-80 cells after treatment with the nanocomplex with 5.8% to 21.1% increases observed (Figure 3.12 a).

The mid concentration (ND/CARBO/HE4 14 $\mu\text{M}/\text{ml}$) of the nanocomplex was more effective in increasing CMP changes than the same dose of CARBO alone (14 $\mu\text{M}/\text{ml}$) in all cells with increases in CMP ranging from 3.7% to 15.8% across those cell lines compared to 2.1% to 12.5% for the CARBO alone (Table 3.9). Interestingly, the lowest concentration (7 $\mu\text{M}/\text{ml}$) of the nanocomplex used in this study was more effective in increasing CMP than the clinically relevant dose of CARBO (14 $\mu\text{M}/\text{ml}$) alone in OVCAR-8, A2780 and A2780-cis cell lines at all time points (Figure 3.12 d, e, and f).

Cisplatin sensitive ovarian cancer cell line (A2780) exhibited higher CMP changes than its cisplatin resistant counterpart (A2780-cis) after exposure to all concentrations of the nanocomplex at three time points (Figure 3.12 e and f).

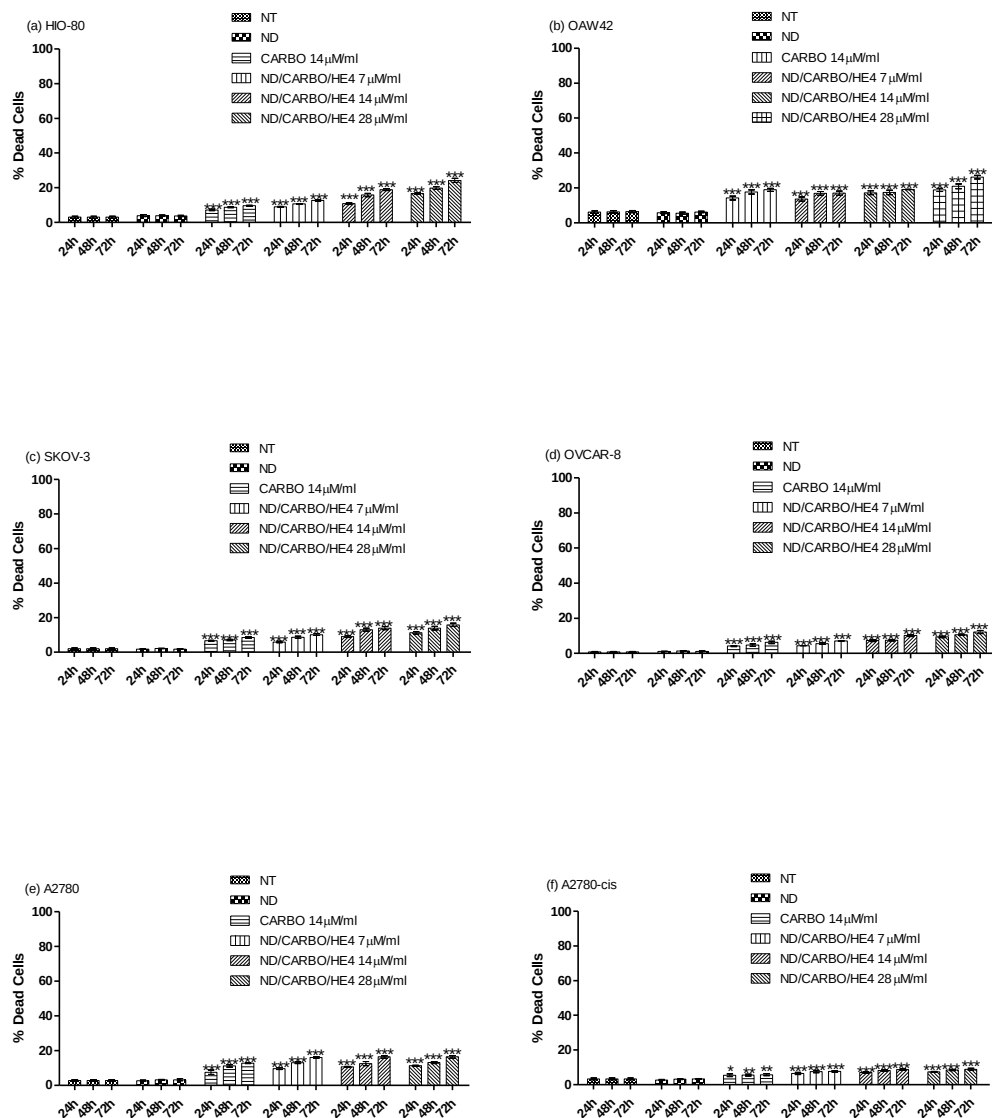


Figure 3.12: Cell membrane permeability measurement after ND/CARBO/HE4 treatment

Cells were treated with ND/CARBO/HE4 and incubated for 24h, 48h, and 72h and the CMP was measured by Cytell TM imaging system, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) OVCAR8, (e) A2780 cells, and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for p < 0.05; “**” p < 0.01; “***” for p < 0.001. (NT) untreated controls, (ND) bare nanodiamonds, (CARBO) carboplatin.

Table 3.9: Percentages of CMP increases after ND/CARBO/HE4 treatment.

The percentage of CMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%) SEM	ND (%) SEM	CARBO 7μM/ml (%) SEM	ND/CARBO/HE4 7μM/ml (%) SEM	ND/CARBO/HE4 14μM/ml (%) SEM	ND/CARBO/HE4 28μM/ml (%) SEM
HIO-80	24h	0 ± 0.5	0.8 ± 0.5	4.1 ± 0.4	5.8 ± 0.2	7.7 ± 0.4	13.5 ± 0.4
	48h	0 ± 0.6	0.8 ± 0.3	5.7 ± 0.3	7.6 ± 0.1	12.7 ± 1	16.7 ± 0.7
	72h	0 ± 0.5	0.6 ± 0.4	6.5 ± 0.3	9.6 ± 0.4	15.8 ± 0.5	21.1 ± 1.2
OAW42	24h	0 ± 0.6	0.1 ± 0.5	8.1 ± 1.1	7.4 ± 1.2	11 ± 1.1	12.6 ± 0.9
	48h	0 ± 0.6	0 ± 0.6	11.3 ± 1.2	10.6 ± 1	11.1 ± 1.3	14.6 ± 1.3
	72h	0 ± 0.7	0.1 ± 0.5	12.5 ± 0.8	10.5 ± 1.2	12.7 ± 0.1	19.7 ± 1
SKOV-3	24h	0 ± 0.3	0 ± 0.2	4.5 ± 0.3	3.8 ± 0.5	7 ± 0.5	9.1 ± 0.7
	48h	0 ± 0.2	0.1 ± 0.1	5 ± 0.4	6.6 ± 0.6	10.9 ± 0.8	11.8 ± 1
	72h	0 ± 0.4	0 ± 0.2	6.4 ± 0.4	8.1 ± 0.5	11.9 ± 0.9	13.8 ± 0.9
OVCAR-8	24h	0 ± 0.2	0.2 ± 0.1	3.3 ± 0.3	3.6 ± 0.1	6.4 ± 0.6	8.5 ± 0.4
	48h	0 ± 0.3	0.5 ± 0.2	3.8 ± 0.6	4.7 ± 0.3	6.6 ± 0.3	9.8 ± 0.3
	72h	0 ± 0.1	0.3 ± 0.1	5.4 ± 0.6	6.2 ± 0.1	9.3 ± 0.5	11.3 ± 0.9
A2780	24h	0 ± 0.5	0 ± 0.4	4.7 ± 1.2	6.8 ± 0.5	7.8 ± 0.2	8.5 ± 0.3
	48h	0 ± 0.5	0.2 ± 0.2	8.3 ± 0.6	10.2 ± 0.5	9.6 ± 1.1	10.2 ± 0.5
	72h	0 ± 0.4	0.3 ± 0.6	9.8 ± 0.2	13.2 ± 0.3	13.5 ± 0.6	13.5 ± 0.7
A2780-cis	24h	0 ± 0.5	0 ± 0.3	2.1 ± 0.7	3.2 ± 0.6	3.7 ± 0.4	4.1 ± 0.2
	48h	0 ± 0.5	0 ± 0.2	2.2 ± 0.5	4.2 ± 0.7	4.9 ± 0.5	5.1 ± 0.4
	72h	0 ± 0.5	0 ± 0.3	2.4 ± 0.6	4.4 ± 0.4	5.4 ± 0.4	5.6 ± 0.5

3.4.12 Cell Cycle distribution in response to ND/CARBO/HE4 treatment

A significant increase ($p < 0.001$) in the number of cells in G2/M phase was seen in all cells after treatment with all concentrations of the nanocomplex at all time points compared to untreated cells (Figure 3.13 b, c, d, e and f), and the cell proportion in arrested phase (G2/M) increased as time and concentration increased (Figure 3.13 a). While CARBO alone induced cell cycle arrest in S phase in the SKOV-3, OVCAR-8, A2780 and A2780-cis cell lines, it did not induce significant changes at any cell cycle stage in OAW42 or HIO-80.

The highest percentage of cells in arrested phase was 48 % and 37% in A2780 and OVCAR-8 cells respectively. For other cells the cell proportions arrested at G2/M phase were 26% in SKOV-3, 22% in OAW42, 29% in A2780-cis and 33% in HIO-80 cells.

The mid concentration (ND/CARBO/HE4 14 $\mu\text{M/ml}$) of the nanocomplex was more effective in increasing the cell percentage in arrested phase than the same dose of CARBO alone (14 $\mu\text{M/ml}$) in all cells with increases in cell proportions in arrested phase ranging from 20% to 47% across those cell lines compared to 9% to 26% for the CARBO alone. Interestingly, the lowest concentration (ND/CARBO/HE4 7 $\mu\text{M/ml}$) of the nanocomplex used in this study was more effective in sending cells into arrest than the clinically relevant dose of CARBO (14 $\mu\text{M/ml}$) alone in all cell lines at all time points (Figure 3.13 a b, c, d, e and f).

Cisplatin sensitive ovarian cancer cell line (A2780) exhibited higher cell cycle arrest in G2/M phase (48%) compared to the cisplatin resistant ovarian cancer cell line (A2780-cis) with 29% in G2/M phase.

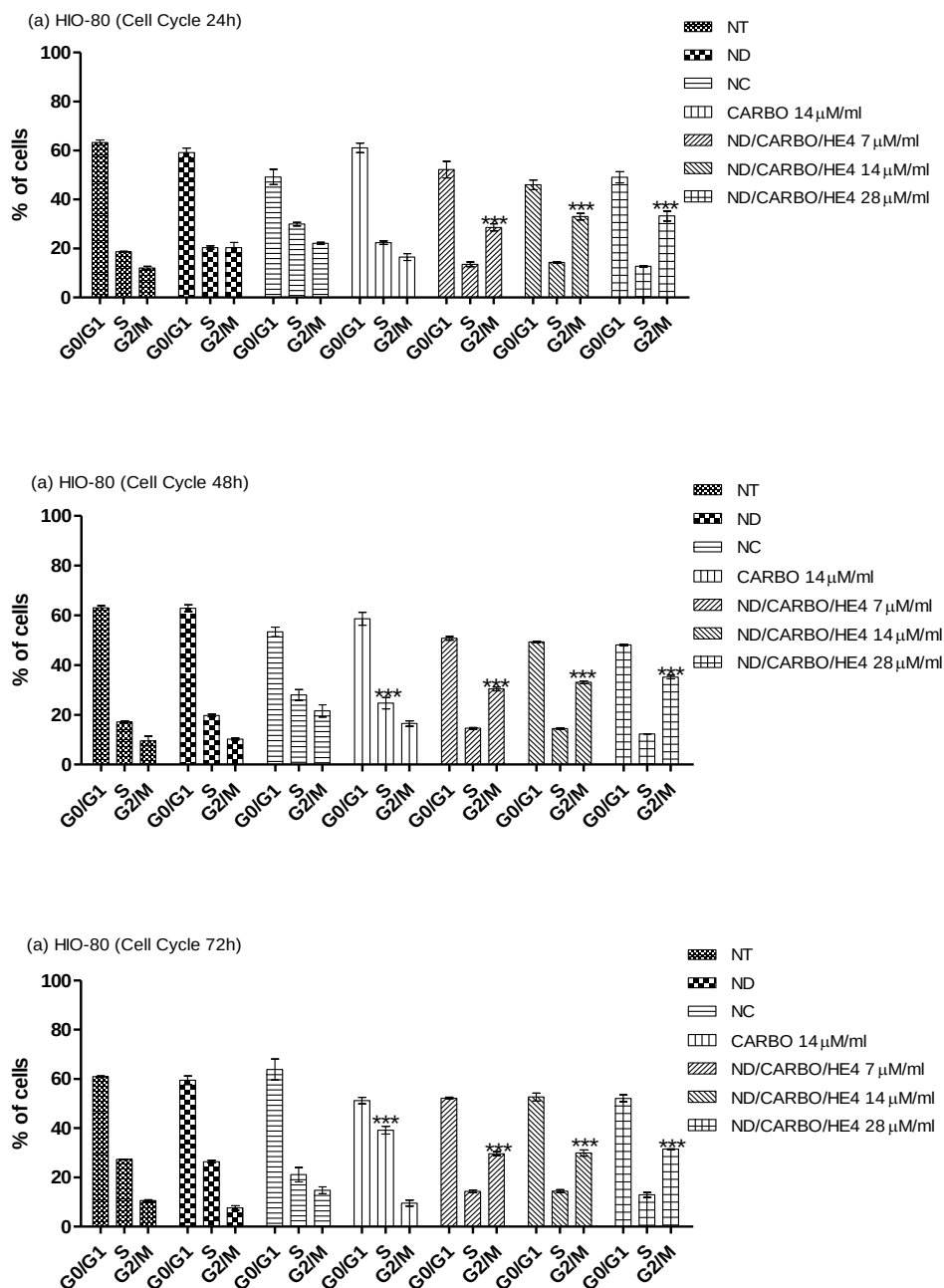


Figure 3.13 a: Cell cycle for HIO-80 cells after ND/CARBO/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

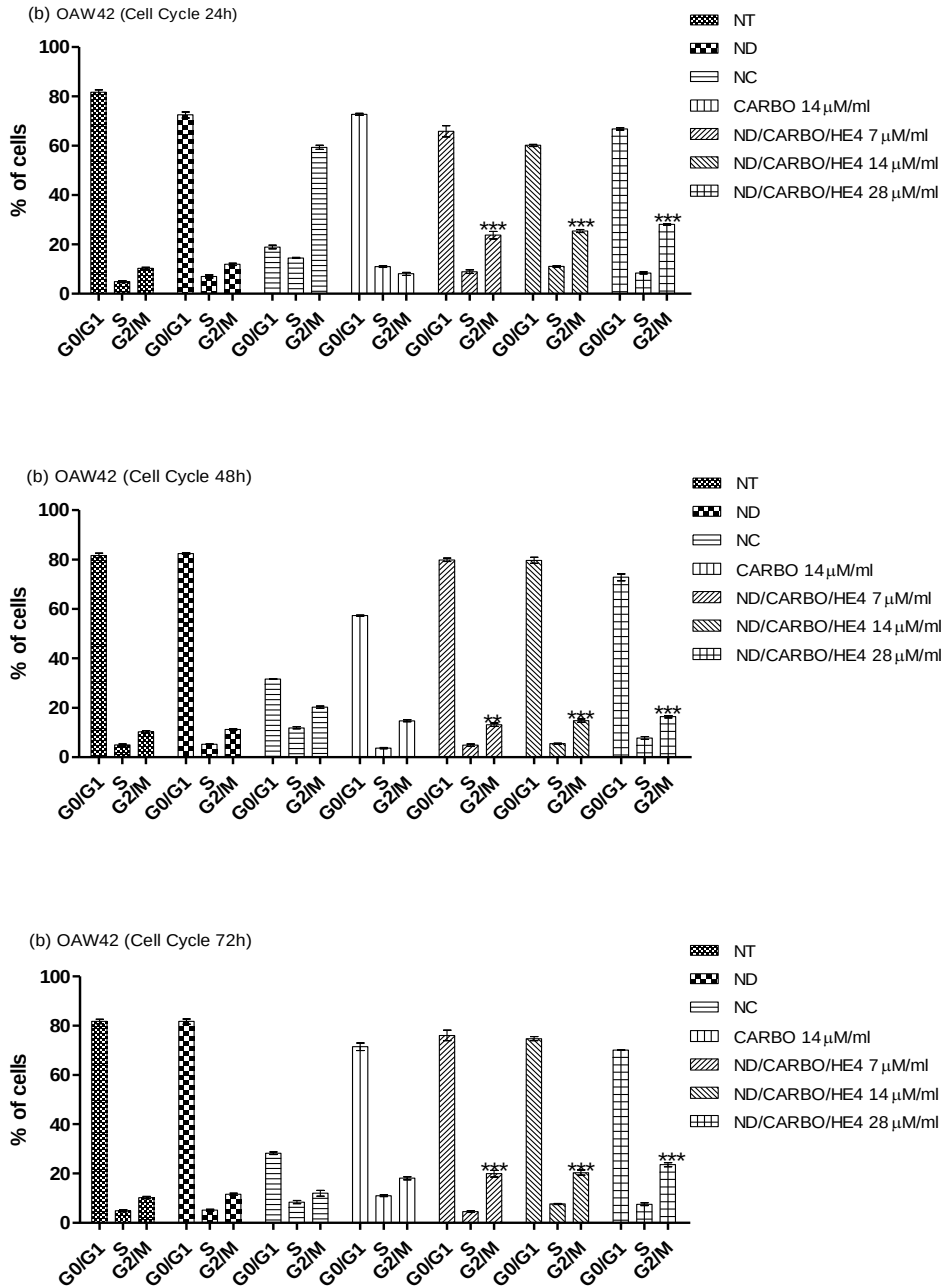


Figure 3.13 b: Cell cycle for OAW42 cells after ND/CARBO/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

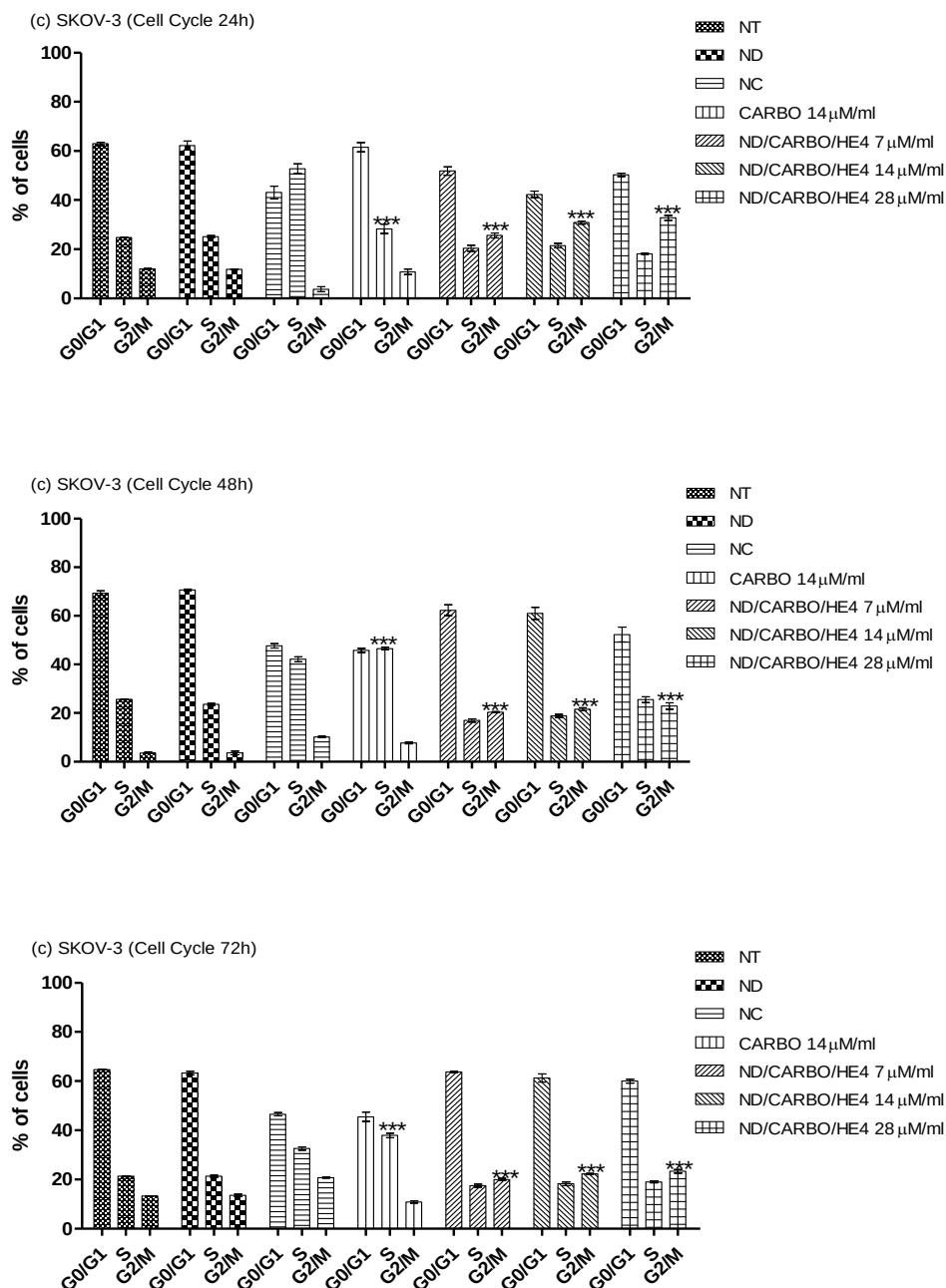


Figure 3.13 c: Cell cycle for SKOV-3 cells after ND/CARBO/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “***” $p < 0.01$; “****” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

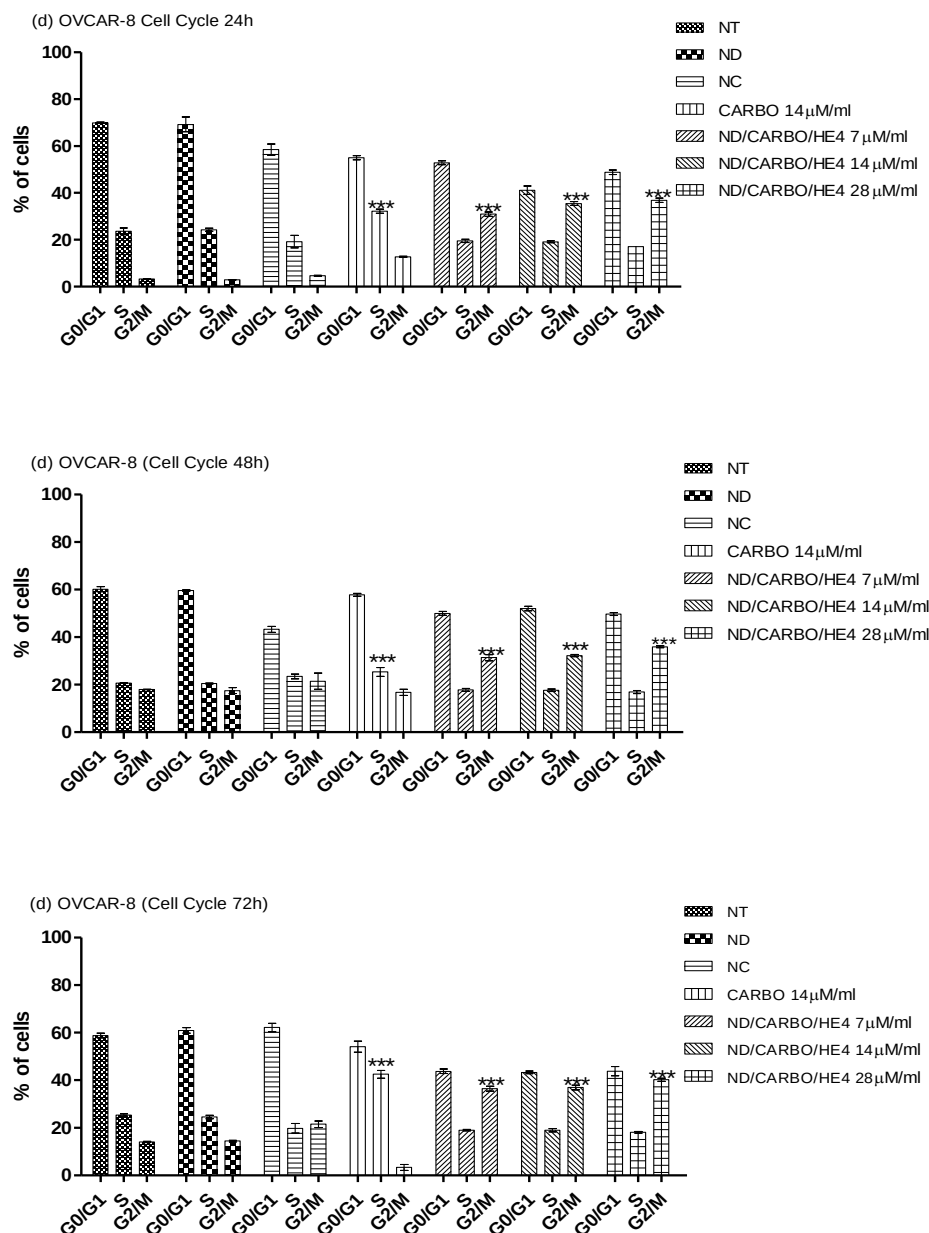


Figure 3.13 d: Cell cycle for OVCAR-8 cells after ND/CARBO/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

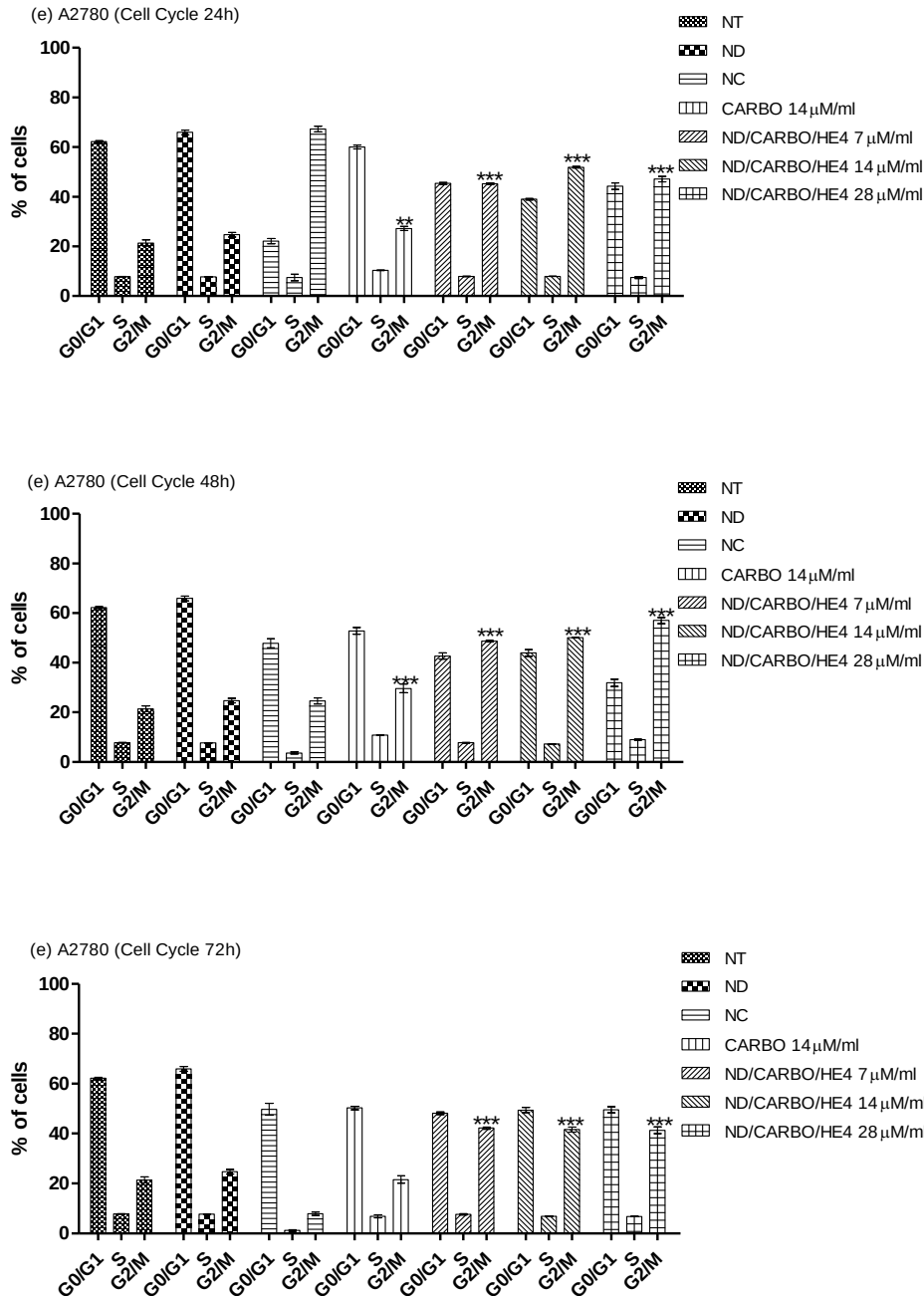


Figure 3.13 e: Cell cycle for A2780 cells after ND/CARBO/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

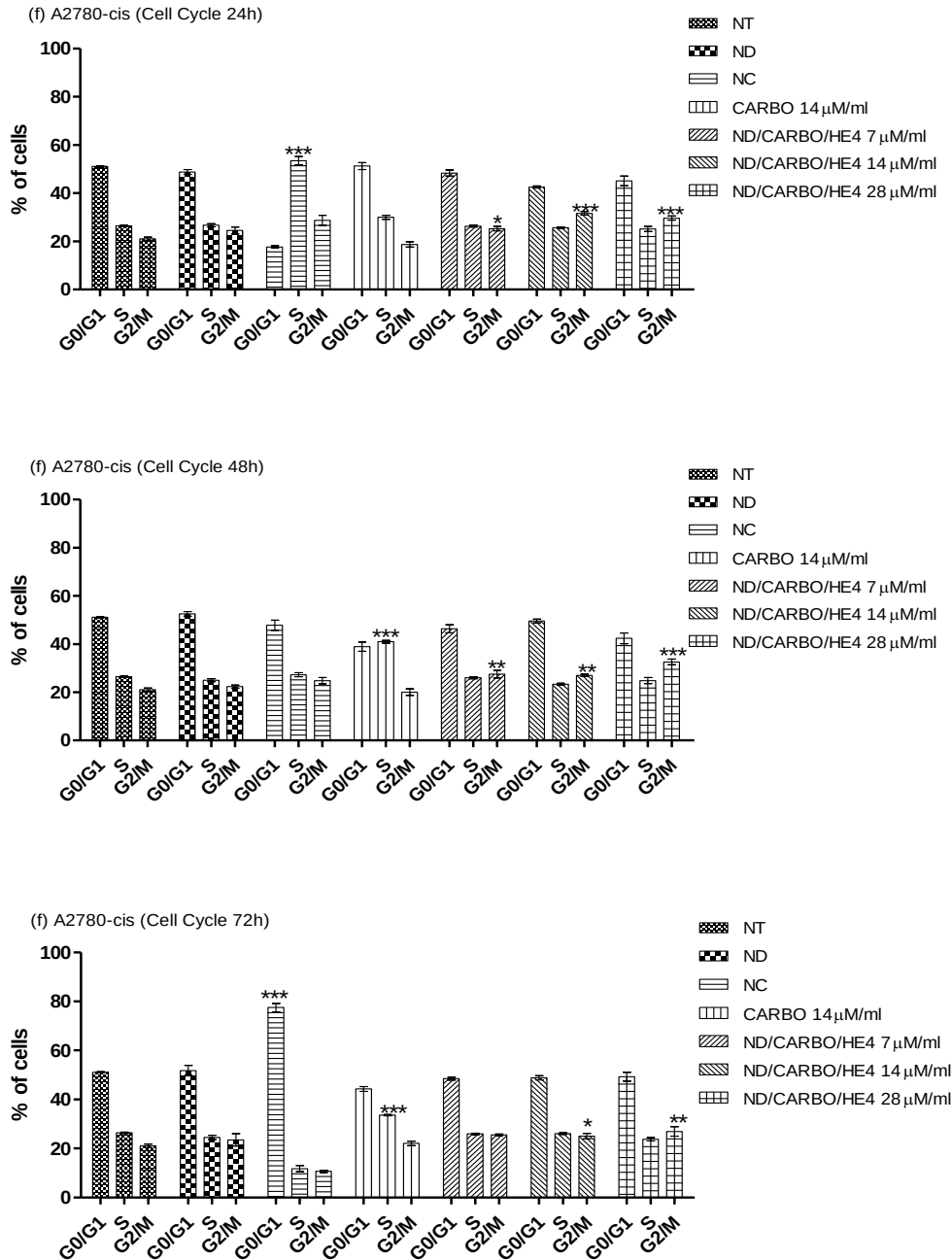


Figure 3.13 f: Cell cycle for A2780-cis cells after ND/CARBO/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “***” $p < 0.01$; “****” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

3.4.13 Cell viability assessment in response to ND/CARBO/CA125 treatment

All cells studied showed significant cell viability reduction ($p \leq 0.001$) after exposure to all nanocomplex concentrations at all time points compared to untreated cells (Figure 3.14). OVCAR-8 and A2780 showed more cell viability reduction than other cell lines with decreases ranging between 15.1% to 74.6% for OVCAR-8 cells and 17.9 to 75.5% for A2780 compared to untreated cells (Figure 3.14 d and e).

All other ovarian cancer cells (OAW42, SKOV-3 and A2780-cis) and HIO-80 showed similar cell viability reduction ranging between 8.2% to 69.9% after these cells were exposed to all concentrations of the nanocomplex compared to untreated cells (Table 3.10).

The mid concentration (ND/CARBO/CA125 14 μ M/ml) of the nanocomplex was more effective than the similar dose of drug alone (CARBO 14 μ M/ml) in OAW42, OVCAR-8, and A2780. Decrease in cell viability ranging from 22.2% to 68.9% were seen compared to drug alone with cell viability reduction ranging between 11.1% to 63.3% (Table 3.10). The lowest concentration (7 μ M/ml) of the nanocomplex induced more cell viability reduction in the A2780 cell line than the clinically relevant dose of CARBO across all time points (Figure 3.14 e).

Cisplatin sensitive ovarian cancer cell line (A2780) showed more cell viability reduction than the resistance ovarian cancer cell line (A2780-cis). Decreases in cell viability ranging from 17.9% to 75.5% for A2780 cells were seen compared to 8.2% to 66.6% for A2780-cis (Figure 3.14 e and f).

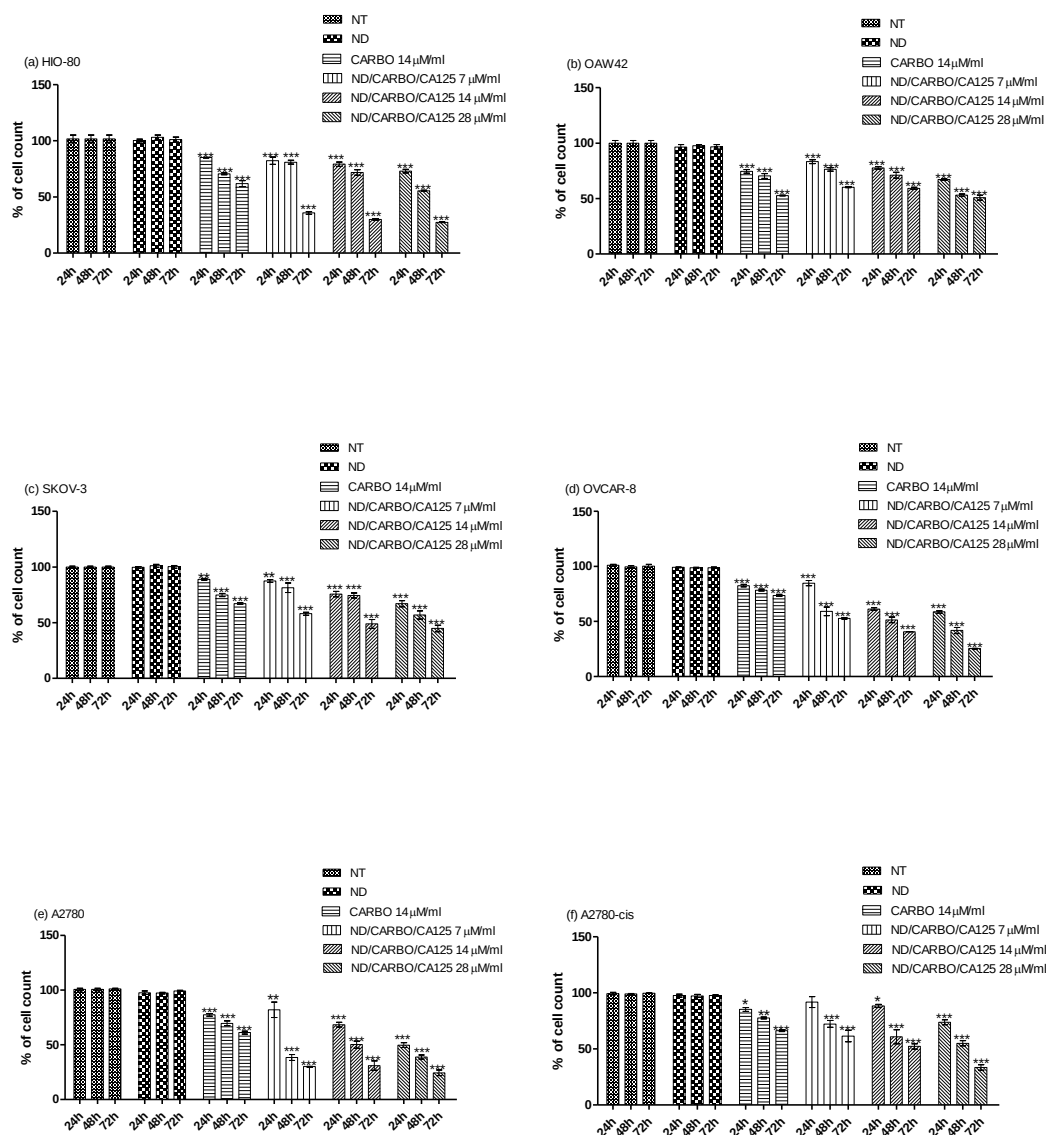


Figure 3.14: Cell viability assessment after ND/CARBO/CA125 treatment

Cells were treated with ND/CARBO/CA125 for three different time incubations and total cell count was measured using Cytell™ imaging system and IN cell software, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) OVCAR8, (e) A2780 cells and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significant between treated and untreated cells reported by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (CARBO) carboplatin.

Table 3.10: Percentages of cell viability reduction (cell kill) after ND/CARBO/CA125 treatment. The percentage of cell viability reduction listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	CARBO 7 μ M/ml (%)	SEM	ND/ CARBO /CA125 7 μ M/ml (%)	SEM	ND/ CARBO /CA125 14 μ M/ml (%)	SEM	ND/ CARBO /CA125 28 μ M/ml (%)	SEM
HIO-80	24h	3.3 $\pm$ 1		4.2 $\pm$ 0.5		1.0 $\pm$ 3		17.6 $\pm$ 3.2		20.8 $\pm$ 1.8		27.0 $\pm$ 1.6	
	48h	1.3 $\pm$ 0.2		3.0 $\pm$ 1.3		29.3 $\pm$ 3.9		19.0 $\pm$ 1.8		28.1 $\pm$ 2.5		44.5 $\pm$ 0.5	
	72h	0.9 $\pm$ 0.8		0.6 $\pm$ 0.3		46.8 $\pm$ 0.3		64.2 $\pm$ 1.1		69.9 $\pm$ 0.6		72.5 $\pm$ 0.3	
OAW42	24h	1.3 $\pm$ 7.4		1.5 $\pm$ 2.7		11.1 $\pm$ 0.9		16.6 $\pm$ 1.7		22.2 $\pm$ 1.1		32.7 $\pm$ 0.7	
	48h	1.7 $\pm$ 0.2		2.5 $\pm$ 1.7		15.9 $\pm$ 3.8		23.5 $\pm$ 1.5		28.8 $\pm$ 2.6		46.9 $\pm$ 1.1	
	72h	0.3 $\pm$ 3.5		0.4 $\pm$ 3.4		36.3 $\pm$ 2		32.9 $\pm$ 3		40.8 $\pm$ 0.9		49.1 $\pm$ 2.1	
SKOV-3	24h	4.7 $\pm$ 0.1		1.5 $\pm$ 0.3		4.2 $\pm$ 1.5		12.5 $\pm$ 1.1		24.3 $\pm$ 2.3		32.9 $\pm$ 2.7	
	48h	0.0 $\pm$ 1		3.3 $\pm$ 2.6		39.2 $\pm$ 3.6		18.6 $\pm$ 4.2		25.5 $\pm$ 2.2		43.0 $\pm$ 3.6	
	72h	1.6 $\pm$ 2.2		2.5 $\pm$ 1		51.1 $\pm$ 1.5		41.9 $\pm$ 1.3		51.2 $\pm$ 4.1		55.2 $\pm$ 2.9	
OVCAR-8	24h	0.3 $\pm$ 0.2		2.2 $\pm$ 0.4		12.9 $\pm$ 0.8		15.1 $\pm$ 2.3		38.6 $\pm$ 1		41.3 $\pm$ 1.1	
	48h	3.3 $\pm$ 1.3		0.7 $\pm$ 1.3		24.8 $\pm$ 3.1		40.8 $\pm$ 3.9		48.6 $\pm$ 2.5		58.2 $\pm$ 2.8	
	72h	0.9 $\pm$ 0.4		0.6 $\pm$ 0.4		39.5 $\pm$ 1.7		47.1 $\pm$ 0.5		59.3 $\pm$ 0.8		74.6 $\pm$ 0.3	
A2780	24h	4.3 $\pm$ 2.4		3.0 $\pm$ 1.9		11.3 $\pm$ 5.9		17.9 $\pm$ 7.1		31.5 $\pm$ 2.1		50.2 $\pm$ 2.1	
	48h	3.3 $\pm$ 3.1		2.5 $\pm$ 1.3		43.7 $\pm$ 3.2		61.5 $\pm$ 2.7		49.8 $\pm$ 3.1		61.1 $\pm$ 1.8	
	72h	0.3 $\pm$ 1.2		0.1 $\pm$ 1.2		63.3 $\pm$ 4.6		69.9 $\pm$ 0.4		68.9 $\pm$ 4.1		75.5 $\pm$ 2.6	
A2780-cis	24h	3.3 $\pm$ 0.7		1.5 $\pm$ 1.9		13.3 $\pm$ 1.9		8.2 $\pm$ 4.8		11.7 $\pm$ 1.4		26.2 $\pm$ 2.3	
	48h	0.0 $\pm$ 0.2		0.4 $\pm$ 0.1		13.8 $\pm$ 4		27.7 $\pm$ 3		39.2 $\pm$ 6.2		45.0 $\pm$ 2.3	
	72h	0.4 $\pm$ 1.9		1.8 $\pm$ 1		26.4 $\pm$ 3.5		38.5 $\pm$ 5.1		47.8 $\pm$ 2.3		66.6 $\pm$ 2.4	

3.4.14 MMP changes in response to ND/CARBO/CA125 treatment

Significant MMP changes ($p \leq 0.001$) were seen after all cells were treated with the higher concentration of the nanocomplex at 72h, but no significant changes in MMP intensity were seen at the lower concentration of ND/CARBO/HE4 (Figure 3.15). OAW42 cells showed the highest MMP changes with 62.7% increase after the higher concentration treatment at 72h compared with untreated cells (Figure 3.15 b).

All other cells (SKOV-3, OVCAR-8, A2780, and A2780-cis) exhibited MMP changes ranging between 1% to 48.7% after all nanocomplex concentrations, and the effect was time, cell and dose dependent (Table 3.11). Similar MMP changes were also seen when the HIO-80 cells treated with ND/CARBO/CA125 compared to untreated cells (Figure 3.15 a).

Cisplatin resistant ovarian cancer cell line (A2780-cis) exhibited higher MMP changes than the cisplatin sensitive ovarian cancer cells after exposure to all concentrations of ND/CARBO/CA125 at 48h and 72h (Figure 3.15 e and f).

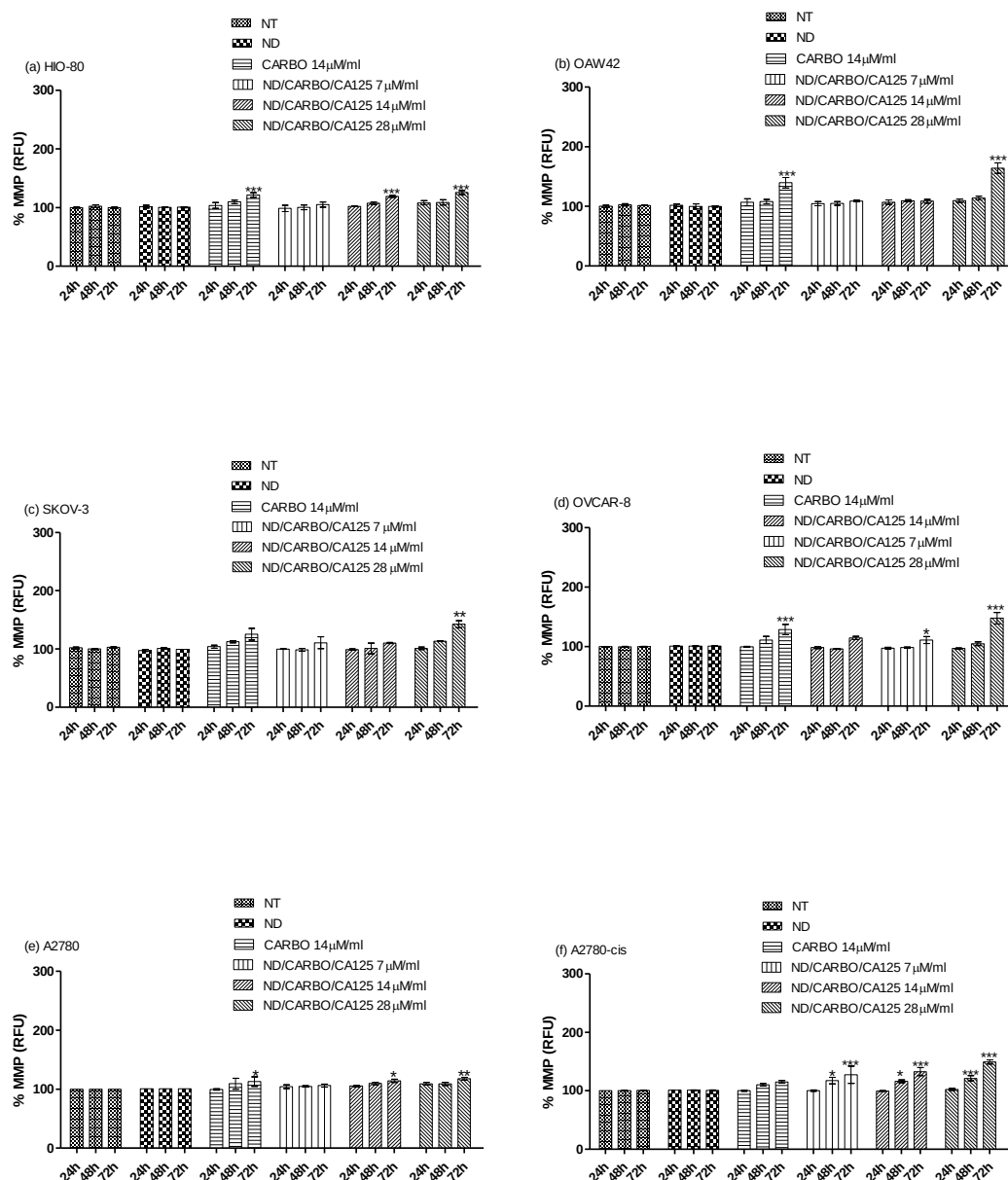


Figure 3.15: Mitochondrial membrane changes after ND/CARBO/CA125 treatment

Cells were treated with ND/CARBO/CA125 for three different time points and Cytell TM imaging system and IN cell software were used to measure the mitochondrial changes (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV3, (d) OVARC8, (e) A2780 cells and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “***” $p < 0.01$; “****” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (CARBO) carboplatin.

Table 3.11: Percentages of MMP increases after ND/CARBO/CA125 treatment.

The percentage of MMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	CARBO 7µM/ml (%)	SEM	ND/ CARBO /CA125 7µM/ml (%)	SEM	ND/ CARBO /CA125 14µM/ml (%)	SEM	ND/ CARBO /CA125 28µM/ml (%)	SEM
HIO-80	24h	0.1 ± 0.8		1.6 ± 2.3		3.7 ± 5		0.1 ± 5.1		2.1 ± 0.6		8.1 ± 3.5	
	48h	0 ± 2.3		0.2 ± 0.3		7.3 ± 2.9		0.1 ± 4		5.0 ± 2.1		6.3 ± 4.5	
	72h	0 ± 0.9		0.6 ± 0.9		21.6 ± 3.9		5.4 ± 4.2		19.1 ± 1.6		25.9 ± 3.3	
OAW42	24h	0 ± 1.5		1.5 ± 2.2		6.8 ± 5.7		4.6 ± 3.4		6.9 ± 3.7		9.5 ± 2.9	
	48h	0 ± 2		0 ± 4.2		5.0 ± 3.6		2.1 ± 3		6.7 ± 1.6		11.2 ± 3	
	72h	0 ± 0.6		0 ± 1.2		38.1 ± 8.5		7.4 ± 0.8		7.0 ± 3.1		62.7 ± 8.9	
SKOV-3	24h	0.1 ± 1.6		0 ± 1.7		9.6 ± 13.7		0.0 ± 4.5		0.0 ± 2.5		0.0 ± 5.6	
	48h	0 ± 0.8		1 ± 1.3		12.5 ± 1.9		0.0 ± 2.3		1.0 ± 9		13.5 ± 0.6	
	72h	0.1 ± 1		0 ± 0.1		22.5 ± 9.8		14.3 ± 21.9		17.3 ± 20.9		39.8 ± 6	
OVCAR-8	24h	0.2 ± 0.3		0.7 ± 0.5		0.0 ± 4		0.0 ± 7.2		0.0 ± 7		0.0 ± 0.6	
	48h	0.7 ± 0.6		0.8 ± 0.6		11.3 ± 6.2		0.0 ± 0.7		0.0 ± 2.9		2.8 ± 5	
	72h	0.3 ± 0.4		1 ± 0.4		28.7 ± 8.4		11.1 ± 5.8		14.5 ± 2.7		47.7 ± 9.6	
A2780	24h	0 ± 0.1		0.7 ± 0.1		0.0 ± 1.1		4.2 ± 3.1		5.3 ± 1.5		9.2 ± 2.1	
	48h	0 ± 0.1		0.8 ± 0.2		9.3 ± 9.2		4.7 ± 1.1		9.7 ± 2		8.7 ± 2.7	
	72h	0 ± 0.1		0 ± 0.1		13 ± 8		6.0 ± 2.5		14.1 ± 2.6		17.6 ± 2.3	
A2780-cis	24h	0 ± 0.1		0.7 ± 0.1		0.0 ± 1.2		0.0 ± 2.2		0.0 ± 2.1		2.2 ± 1.7	
	48h	0 ± 0.6		0.6 ± 0.4		9.9 ± 1.9		17.0 ± 5.6		15.8 ± 2.5		20.8 ± 4.3	
	72h	0 ± 0.3		0 ± 0.8		14.5 ± 2.3		26.6 ± 14.7		32.1 ± 7.1		48.7 ± 3.5	

3.4.15 Cell membrane permeability assessment after ND/CARBO/CA125 treatments

ND/CARBO/CA125 induced a significant increase in CMP ($p < 0.001$) after the cells were exposed to all nanocomplex concentrations at all time points and the effect was cell, dose, and time dependent (Figure 3.16).

SKOV-3 and A2780 cells showed the highest increase in CMP after treatment with all concentrations of the nanocomplex compared to untreated cells, with increases ranging from 13.2% to 38.7% for SKOV-3 and 11.9% to 51.6% for A2780 as time and concentration increased (Table 3.12). For other cell lines (OAW42, OVCAR-8 and A2780-cis), significant CMP increases ($p < 0.001$) ranging between 11% to 24.8% in OAW42 cells, 2% to 15.4% in OVCAR-8 cells, and 3.6% to 30.9% in A2780-cis cells (Figure 3.16 b, d, and f) were seen. Similar increases in CMP were also seen in H10-80 cells after treatment with the nanocomplex with percentages of CMP changes ranging from 3.8% to 40.5% (Figure 3.16 a).

The mid concentration (ND/CARBO/CA125 14 $\mu\text{M}/\text{ml}$) of the nanocomplex was more effective in increasing CMP than the same dose of CARBO alone (14 $\mu\text{M}/\text{ml}$) in SKOV-3, OVCAR-8, A2780 and A2780-cis cell lines with increases ranging from 4.2% to 44.5% across those cell lines compared to 2.4% to 29.9% for the CARBO alone (Table 3.12). Interestingly, the lowest concentration (7 $\mu\text{M}/\text{ml}$) of the nanocomplex used in this study was more effective in increasing CMP than the clinically relevant dose of CARBO (14 $\mu\text{M}/\text{ml}$) alone in the A2780 cell line at all time points (Figure 3.16 e).

Cisplatin sensitive ovarian cancer cell line (A2780) exhibited higher CMP changes than cisplatin resistant to ovarian cancer cell line (A2780-cis) after exposure to all concentrations of the nanocomplex at three time points (Figure 3.16 e and f).

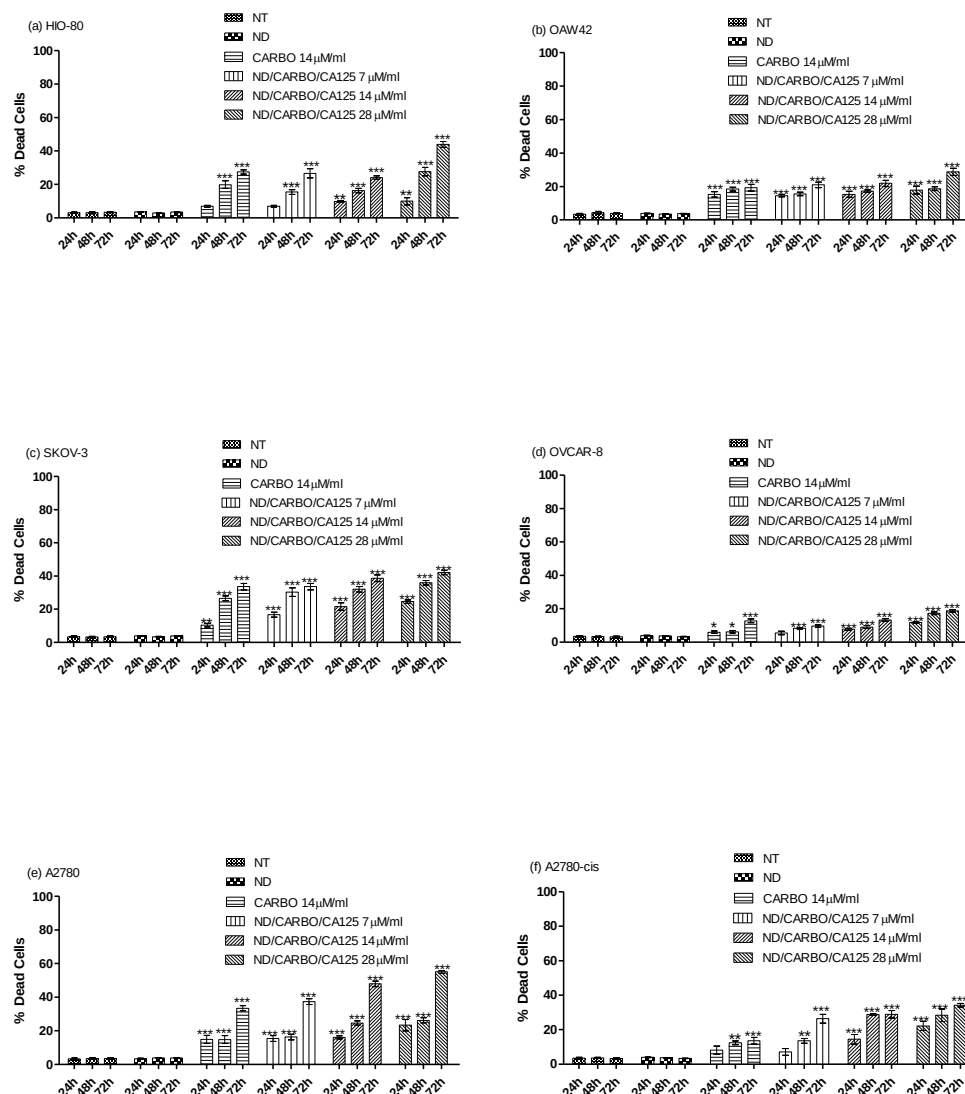


Figure 3.16: Cell membrane permeability measurement after ND/CARBO/CA125 treatment

Cells were treated with ND/CARBO/CA125 and incubated for 24h, 48h, and 72h and the CMP was measured by Cytell TM imaging system, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) OVCAR8, (e) A2780 cells, and (f) A2780-cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (CARBO) carboplatin.

Table 3.12: Percentages of CMP increases after ND/CARBO/CA125 treatment.

The percentage of CMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	CARBO 7µM/ml (%)	SEM	ND/ CARBO /CA125 7µM/ml (%)	SEM	ND/ CARBO /CA125 14µM/ml (%)	SEM	ND/ CARBO /CA125 28µM/ml (%)	SEM
HIO-80	24h	0 ± 0.4		0.5 ± 0.2		3.8 ± 0.5		3.8 ± 0.5		6.7 ± 0.5		6.8 ± 2.1	
	48h	0 ± 0.5		0.0 ± 0.1		16.7 ± 2.3		12.3 ± 1.4		13.1 ± 1.3		24.5 ± 2.5	
	72h	0 ± 0.2		0.1 ± 0.3		24.0 ± 1.2		23.1 ± 2.7		20.7 ± 1		40.5 ± 1.7	
OAW42	24h	0 ± 0.5		0.4 ± 0.1		11.8 ± 1.5		11.0 ± 0.7		11.9 ± 1.7		14.4 ± 2.3	
	48h	0 ± 0.7		0.0 ± 0.2		14.0 ± 1.1		11.1 ± 1		12.8 ± 0.6		14.2 ± 1.1	
	72h	0 ± 0.2		0.0 ± 0.1		15.3 ± 1.9		17.0 ± 1.5		17.9 ± 1.8		24.8 ± 2.1	
SKOV-3	24h	0 ± 0.5		0.4 ± 0.1		6.9 ± 1		13.2 ± 1.4		18.1 ± 2.2		21.1 ± 1	
	48h	0 ± 0.4		0.1 ± 0.2		23.3 ± 1.5		27.1 ± 2.5		28.7 ± 1.6		32.8 ± 1.3	
	72h	0 ± 0.5		0.4 ± 0.2		30.1 ± 1.9		30.1 ± 1.9		35.1 ± 2		38.7 ± 1.3	
OVCAR-8	24h	0 ± 0.5		0.4 ± 0.1		2.4 ± 0.7		2.0 ± 1.1		4.2 ± 0.5		8.3 ± 0.4	
	48h	0 ± 0.2		0.3 ± 0.2		2.8 ± 0.6		4.8 ± 0.3		5.6 ± 0.9		13.9 ± 0.8	
	72h	0 ± 0.2		0.0 ± 0.1		9.5 ± 1		6.4 ± 0.7		10 ± 0.7		15.4 ± 0.6	
A2780	24h	0 ± 0.5		0.0 ± 0.3		11.4 ± 2.4		11.9 ± 1.7		12.6 ± 0.9		19.9 ± 3.3	
	48h	0 ± 0.5		0.2 ± 0.2		11.2 ± 2.3		12.8 ± 1.6		21.1 ± 1.2		22.7 ± 1.4	
	72h	0 ± 0.6		0.3 ± 0.2		29.9 ± 1.5		33.9 ± 1.7		44.5 ± 1.6		51.6 ± 0.8	
A2780-cis	24h	0 ± 0.5		0.4 ± 0.1		4.6 ± 2.4		3.6 ± 1.8		10.9 ± 2.7		18.7 ± 2.6	
	48h	0 ± 0.3		0.0 ± 0.2		8.7 ± 1		9.9 ± 1.3		25.1 ± 1.5		24.7 ± 3.5	
	72h	0 ± 0.4		0.1 ± 0.2		10.4 ± 1.9		23.1 ± 2.5		25.6 ± 2.1		30.9 ± 1	

3.4.16 Cell cycle changes in response to ND/CARBO/CA125 treatments

A significant increase ($p < 0.001$) in the number of cells in S phase in all cell lines was seen after treatment with all concentrations of the nanocomplex at all time points compared to untreated cells (Figure 3.17 b, c, d, e and f), and the cell proportions in arrested (S) phase increased as time and concentration increased. Although HIO-80 showed increase in S phase (Figure 3.17 a), this was not as high as was seen in ovarian cancer cells.

The highest percentage of cells showing cell cycle arrest at S phase was 42% and 40% in OAW42 and SKOV-3 cell lines respectively. For other cells the cell proportions in S phase were 39% in OVCAR-8, 26% in A2780, 37% in A2780-cis and 22% in G2/M in HIO-80 cells.

Interestingly, the lowest concentration (ND/CARBO/CA125 7 $\mu\text{M}/\text{ml}$) of the nanocomplex used in this study was more effective in sending cells into arrest than the clinically relevant dose of CARBO (14 $\mu\text{M}/\text{ml}$) alone in all cells at all time points (Figure 3.17 a b, c, d, e and f).

Cisplatin resistant ovarian cancer cell (A2780-cis) exhibited higher cell cycle arrest in S phase (37%) compared to the cisplatin sensitive ovarian cancer cell (A2780) with 26% in S phase.

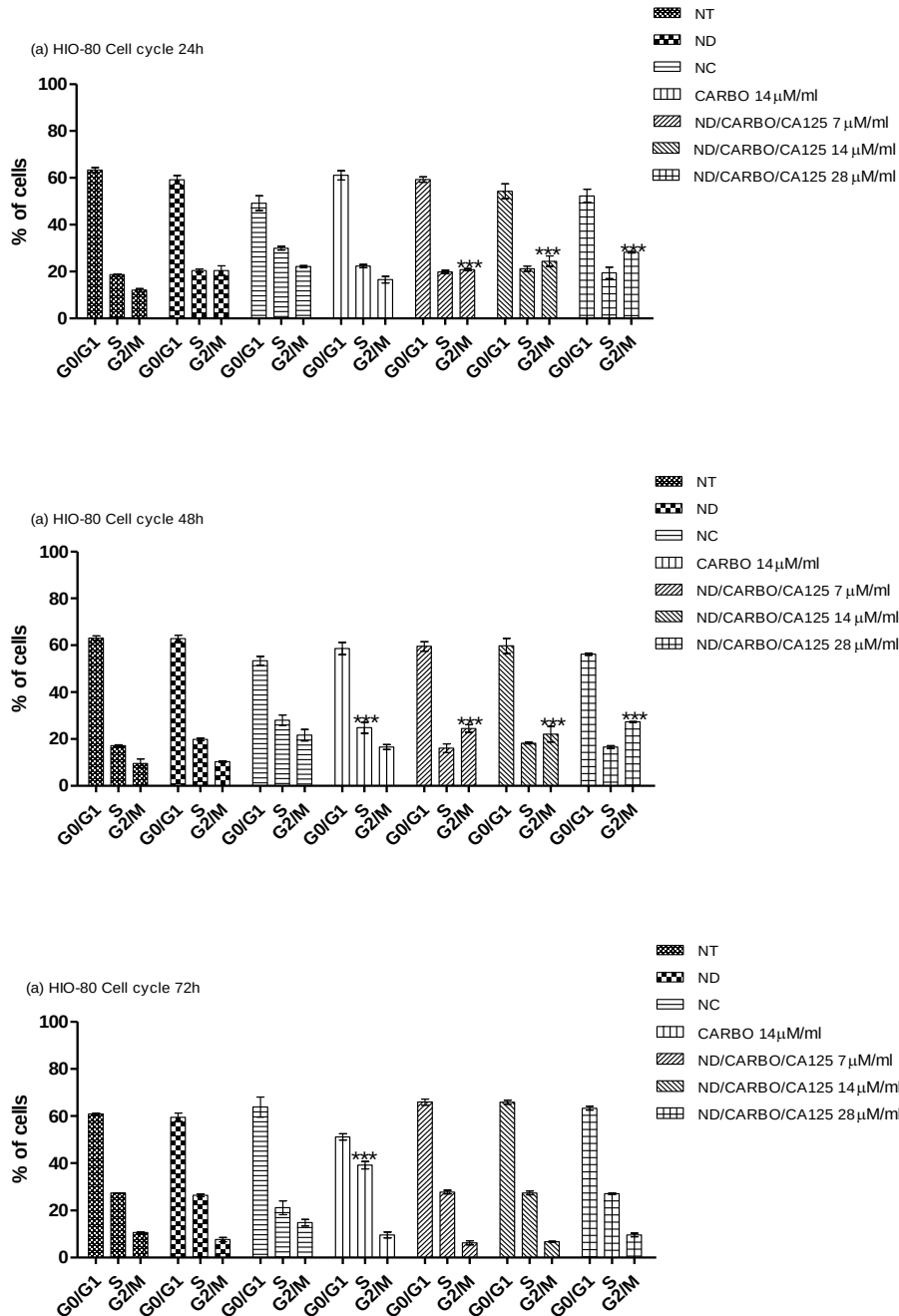


Figure 3.17 a: Cell cycle for HIO-80 cells after ND/CARBO/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

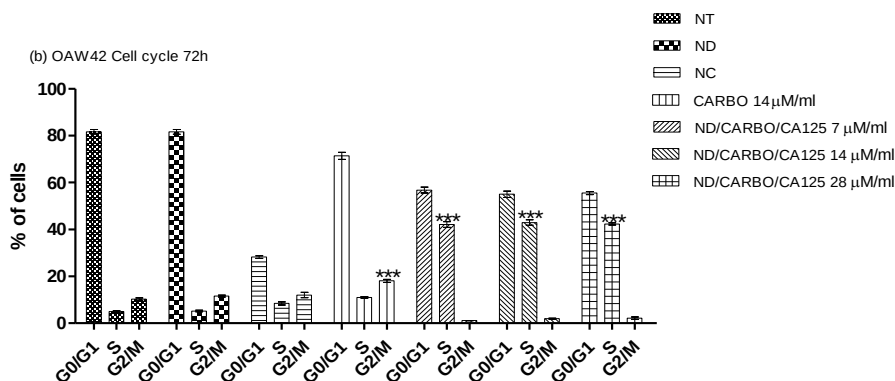
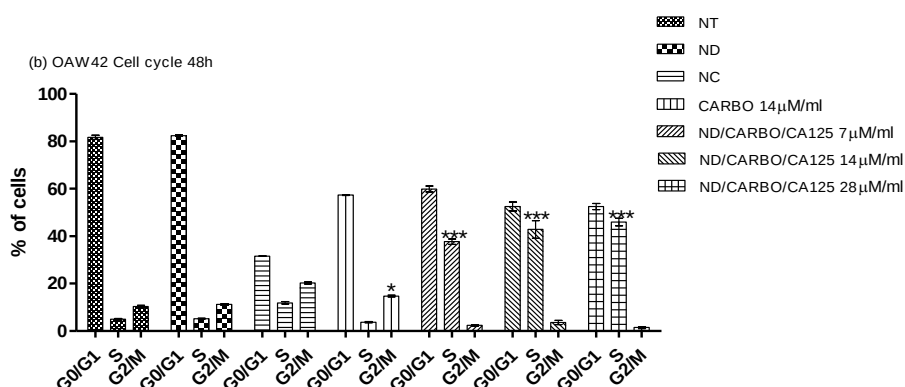
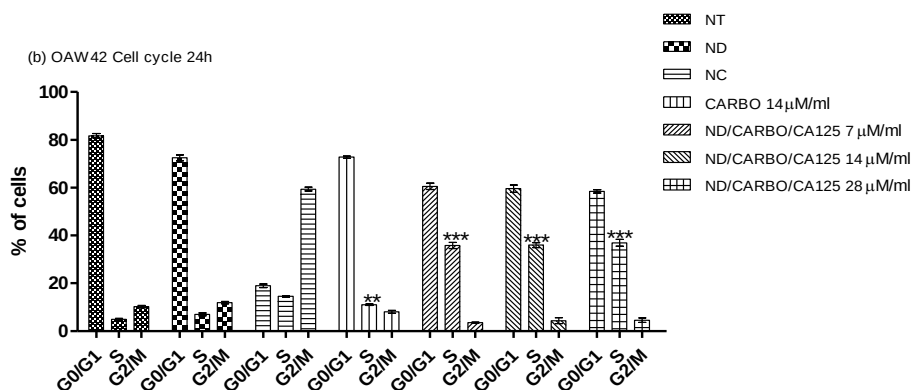


Figure 3.17 b: Cell cycle for OAW42 cells after ND/CARBO/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

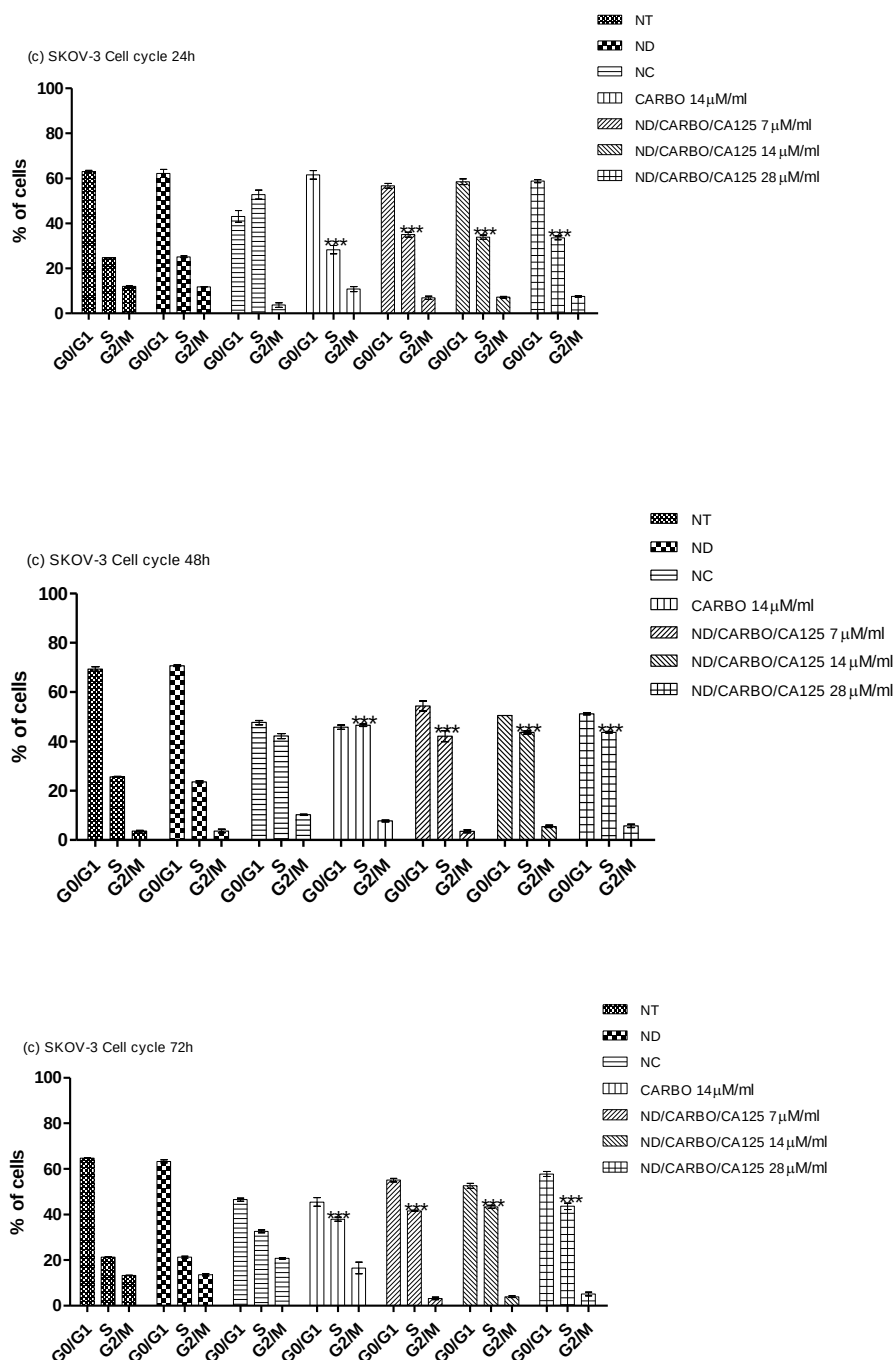


Figure 3.17 c: Cell cycle for SKOV-3 cells after ND/CARBO/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

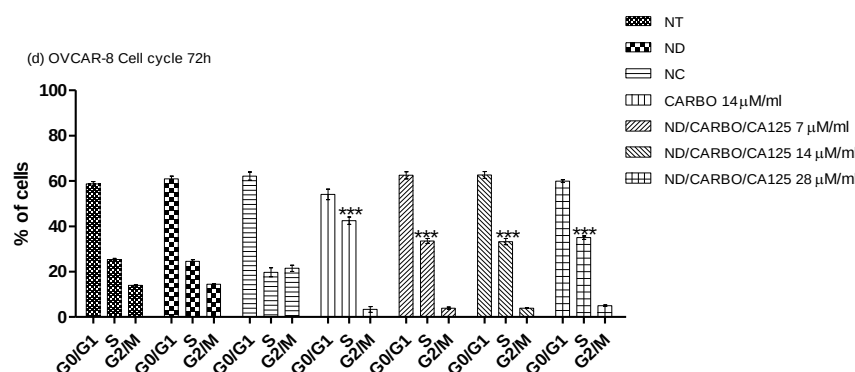
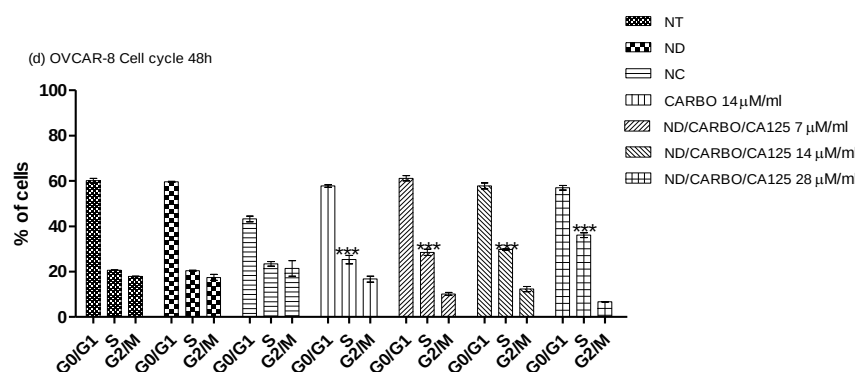
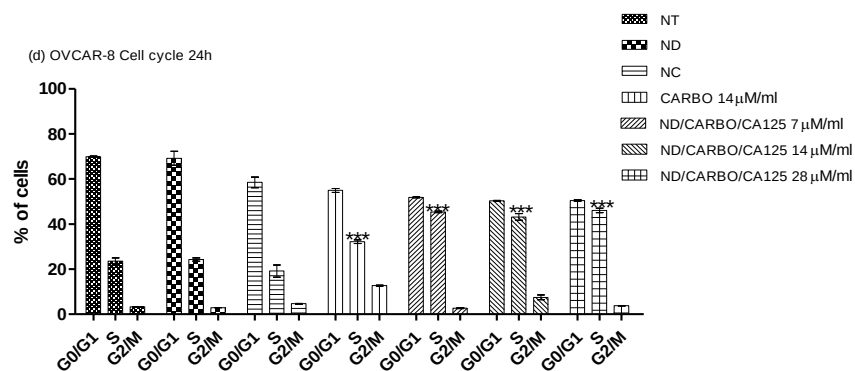


Figure 3.17 d: Cell cycle for OVCAR-8 cells after ND/CARBO/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

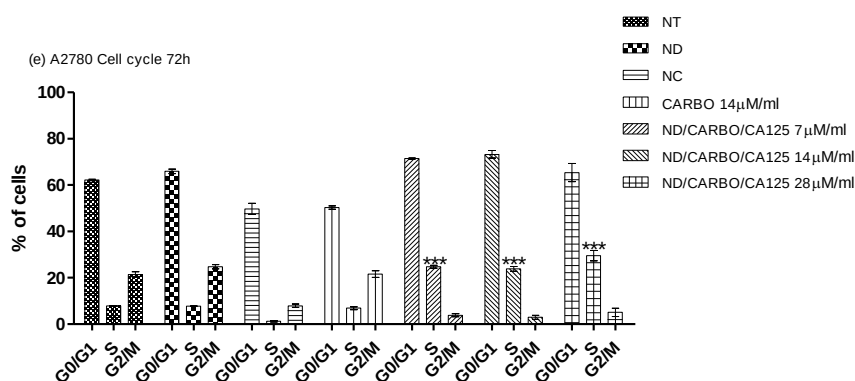
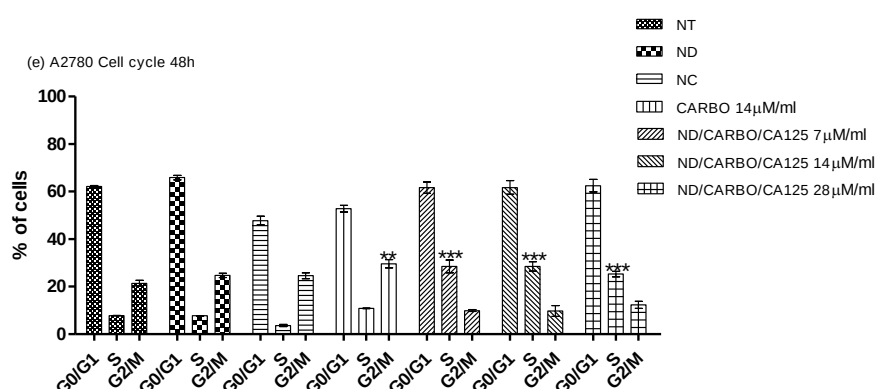
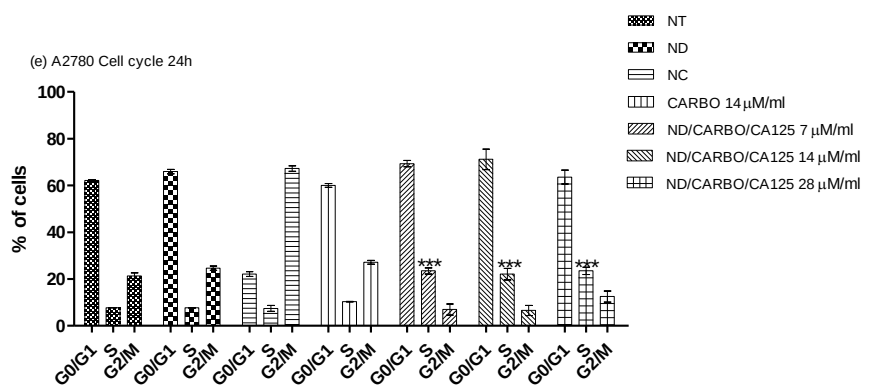


Figure 3.17 e: Cell cycle for A2780 cells after ND/CARBO/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

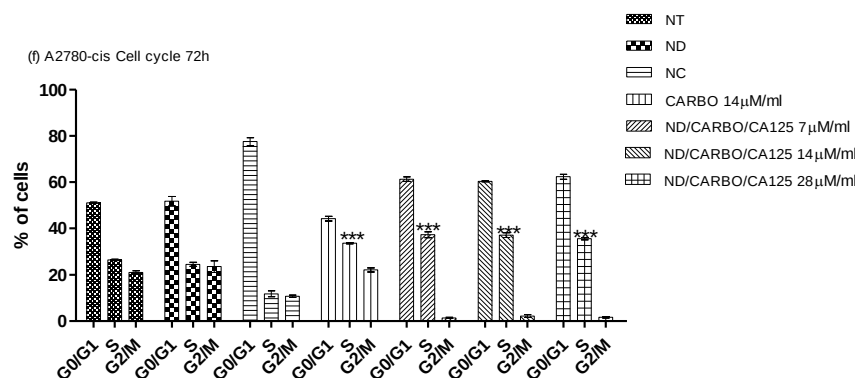
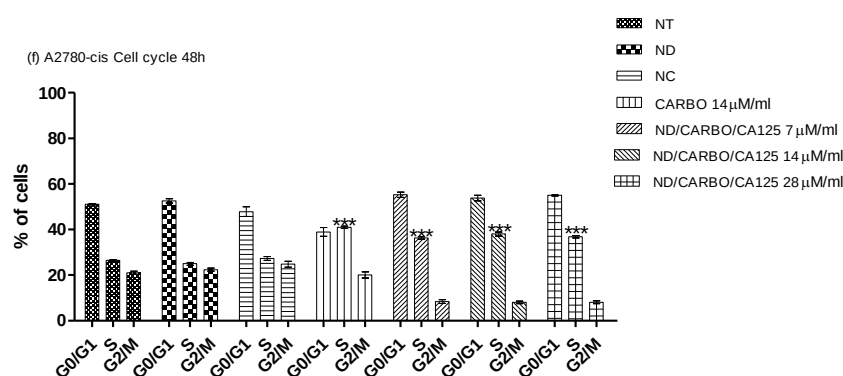
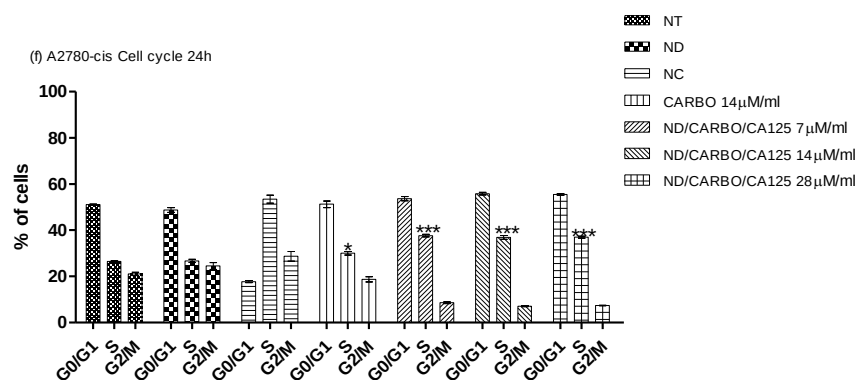


Figure 3.17 f: Cell cycle for A2780-cis cells after ND/CARBO/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/CARBO/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (CARBO) carboplatin.

3.4.17 Cell viability assessment in response to ND/DOX/HE4 treatment

A significant reduction ($p \leq 0.001$) in cell viability was seen in all ovarian cancer cell lines and HIO-80 cell line compared to untreated cells (Figure 3.18). The cisplatin sensitive ovarian cancer cell line (A2780) showed the highest response to all nanocomplex concentrations with cell viability reduction from 39.9% to 98.9% compared to untreated cells (Table 3.13). This was 21.8% higher than its resistant counterpart (Figure 3.18 e and f).

A similar cell viability reduction was seen in the other ovarian cancer cell lines after exposure to all nanocomplex concentrations at all time points. Decreases in cell viability ranged from 22.4% to 76.3% for OAW42, 30.9% to 78.2% for SKOV-3, 34.7% to 81.8% for OVCAR-8, and 31.3% to 77.1% for A2780-cis compared to their untreated cells at all time points (Figure 3.18 b, c, d, and f). The HIO-80 cells showed significant cell viability reduction but not as high as the ovarian cancer cell lines (Figure 3.18 a).

The mid concentration (ND/DOX/HE4 10 μ M/ml) of the nanocomplex was more effective in reducing cell viability than the same dose of DOX 10 μ M/ml alone at all time points. Decrease in cell viability ranging between 29.7% to 97.4% across all ovarian cancer cell lines compared to 20.7% to 64.8% for the drug alone (Table 3.13) were seen.

The SKOV-3, OVCAR-8 and A2780-cis showed more cell viability reduction after exposure to the lowest concentration (5 μ M/ml) of the nanocomplex than the clinically relevant dose of DOX (10 μ M/ml) alone at all time points (Figure 3.18 c, d, and f).

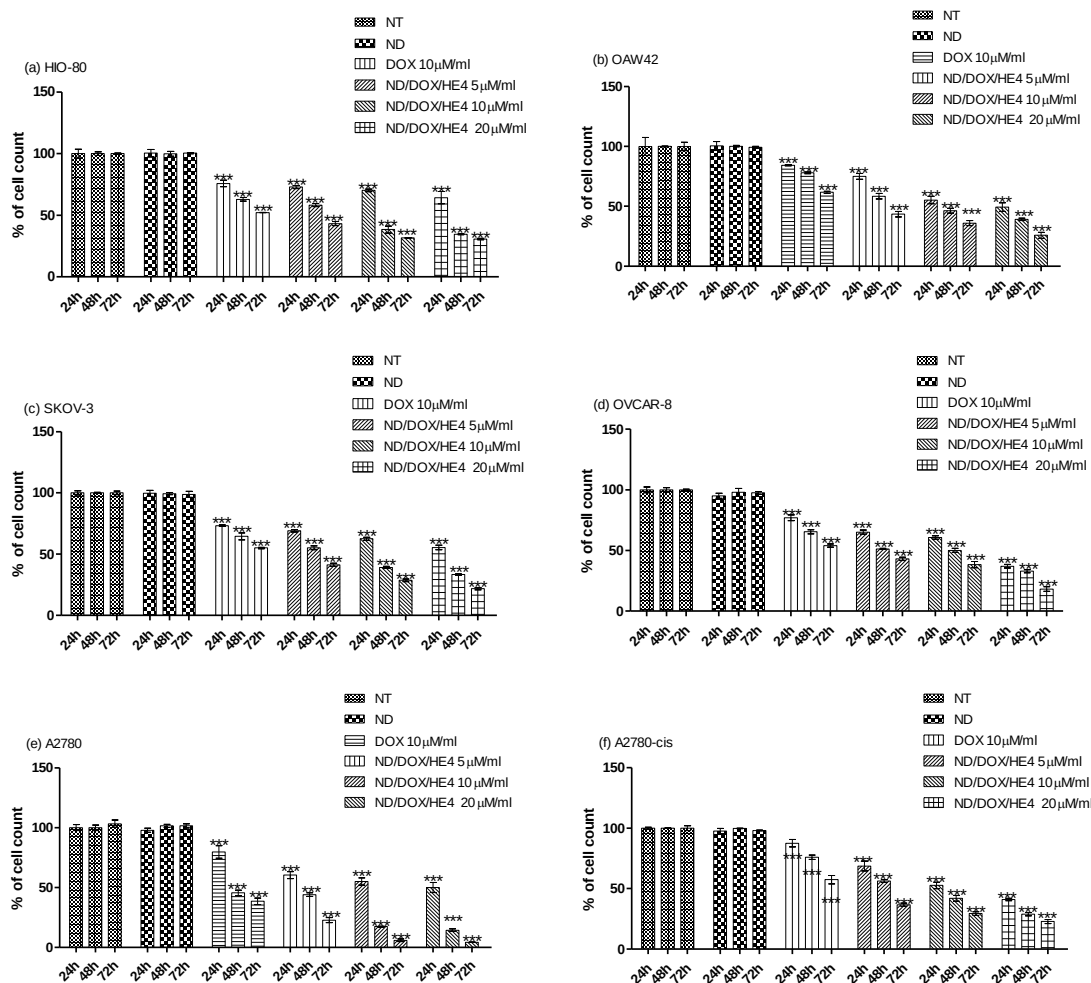


Figure 3.18: Cell viability assessment after treatment with ND/DOX/HE4

Cells were treated by ND/DOX/HE4 for three different time incubations and total cell count was measured using IN cell 1000 software, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) A2780 cells and (e) A2780 cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistically significant between treated and untreated cells reported by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (DOX) doxorubicin.

Table 3.13: Percentages of cell viability reduction (cell kill) after ND/DOX/HE4 treatment. The percentage of cell viability reduction listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	DOX 10µM/ml (%)	SEM	ND/DOX /HE4 5µM/ml (%)	SEM	ND/DOX /HE4 10µM/ml (%)	SEM	ND/DOX/HE4 20µM/ml (%)	SEM
HIO-80	24h	0.2 ± 3.5		0.3 ± 2.9		24.1 ± 2.5		27.0 ± 1.2		29.7 ± 1.1		35.6 ± 4.9	
	48h	0.0 ± 1.4		0.1 ± 1.9		37.0 ± 1.4		41.7 ± 1.3		61.6 ± 2.8		76.7 ± 1.1	
	72h	0.6 ± 0.4		0.4 ± 0.5		47.7 ± 0.1		57.4 ± 2		78.3 ± 0.1		89.2 ± 0.4	
OAW42	24h	0.0 ± 0.5		1.2 ± 0.5		24.7 ± 3.7		22.4 ± 3		47.6 ± 3.1		53.5 ± 3.6	
	48h	0.8 ± 0.4		0.6 ± 0.9		48.4 ± 1.9		40.0 ± 0.8		56.0 ± 2.1		63.3 ± 1	
	72h	0.3 ± 0.7		0.4 ± 0.9		56.4 ± 2.5		52.0 ± 1.2		59.4 ± 1.4		76.3 ± 2.4	
SKOV-3	24h	0.1 ± 1.7		0.3 ± 2.4		26.8 ± 0.6		30.9 ± 0.9		37.4 ± 1.3		44.5 ± 1.8	
	48h	0.4 ± 0.5		0.6 ± 0.9		35.4 ± 2.7		44.7 ± 1.6		60.8 ± 0.7		66.7 ± 0.4	
	72h	0.3 ± 1.5		1.2 ± 2.3		44.9 ± 0.5		58.7 ± 1.3		71.3 ± 1		78.2 ± 0.8	
OVCAR-8	24h	0.1 ± 2.3		4.8 ± 2		23.0 ± 2.3		34.7 ± 1.6		39.2 ± 1.4		62.9 ± 1.5	
	48h	0.5 ± 1.8		2.0 ± 3.2		34.5 ± 1.6		48.9 ± 0.3		49.8 ± 1.6		67.0 ± 1.2	
	72h	0.3 ± 0.5		2.4 ± 1		45.8 ± 1.2		56.9 ± 1.3		61.5 ± 2.5		81.8 ± 1.9	
A2780	24h	0.4 ± 2.5		2.1 ± 1.6		20.3 ± 5.2		39.7 ± 2.9		45.0 ± 2.9		50.0 ± 4.2	
	48h	0.0 ± 2.3		0.6 ± 1.3		54.4 ± 2.2		52.5 ± 4.5		82.6 ± 0.4		80.6 ± 1.8	
	72h	1.4 ± 3.1		1.8 ± 1.6		64.8 ± 2.6		80.5 ± 2.1		97.4 ± 1.1		98.9 ± 0.9	
A2780-cis	24h	0.1 ± 5.5		3.9 ± 1.5		28.1 ± 0.4		31.3 ± 3.9		47.3 ± 2.6		59.0 ± 1	
	48h	0.3 ± 2.6		2.4 ± 1.9		40.1 ± 2.5		43.7 ± 1.2		58.0 ± 2.3		71.2 ± 1.4	
	72h	0.5 ± 1.7		1.0 ± 2.8		62.8 ± 0.5		63.2 ± 1.2		70.6 ± 1.3		77.1 ± 1.7	

3.4.18 Triggering of mitochondrial membrane potential changes upon ND/DOX/HE4 treatment

Significant increases ($p \leq 0.001$) in MMP were seen in OAW42, SKOV-3, OVCAR-8, and A2780-cis ovarian cancer cell lines and HIO-80 cell line after all concentrations of ND/DOX/HE4 at 48h and 72h compared to untreated cells (Figure 3.19 a, b, c, e and f). The A2780 cell line showed no significant MMP changes at all nanocomplex concentrations except with the highest concentration of the nanocomplex at 72h with a 19.1% increase compared to untreated cells (Figure 3.19 e).

SKOV-3 cells showed the highest MMP increases ranging between 13.8% to 65.4% compared to untreated cells. Similar MMP changes were seen in the other ovarian cancer cell lines and HIO-80 cells after exposure to all nanocomplex concentrations at all time points. Increases in MMP ranged from 2.5% to 49.7% (Table 3.14).

The cisplatin resistant ovarian cancer cell lines (A2780-cis) exhibited higher MMP increases than the cisplatin sensitive ovarian cancer cell lines (A2780) (Figure 3.19 e and f).

The mid concentration (10 $\mu\text{M}/\text{ml}$) induced higher MMP changes in OAW42, SKOV-3 and OVCAR-8 than the clinically relevant dose of DOX alone (Figure 3.19 b, c, and d).

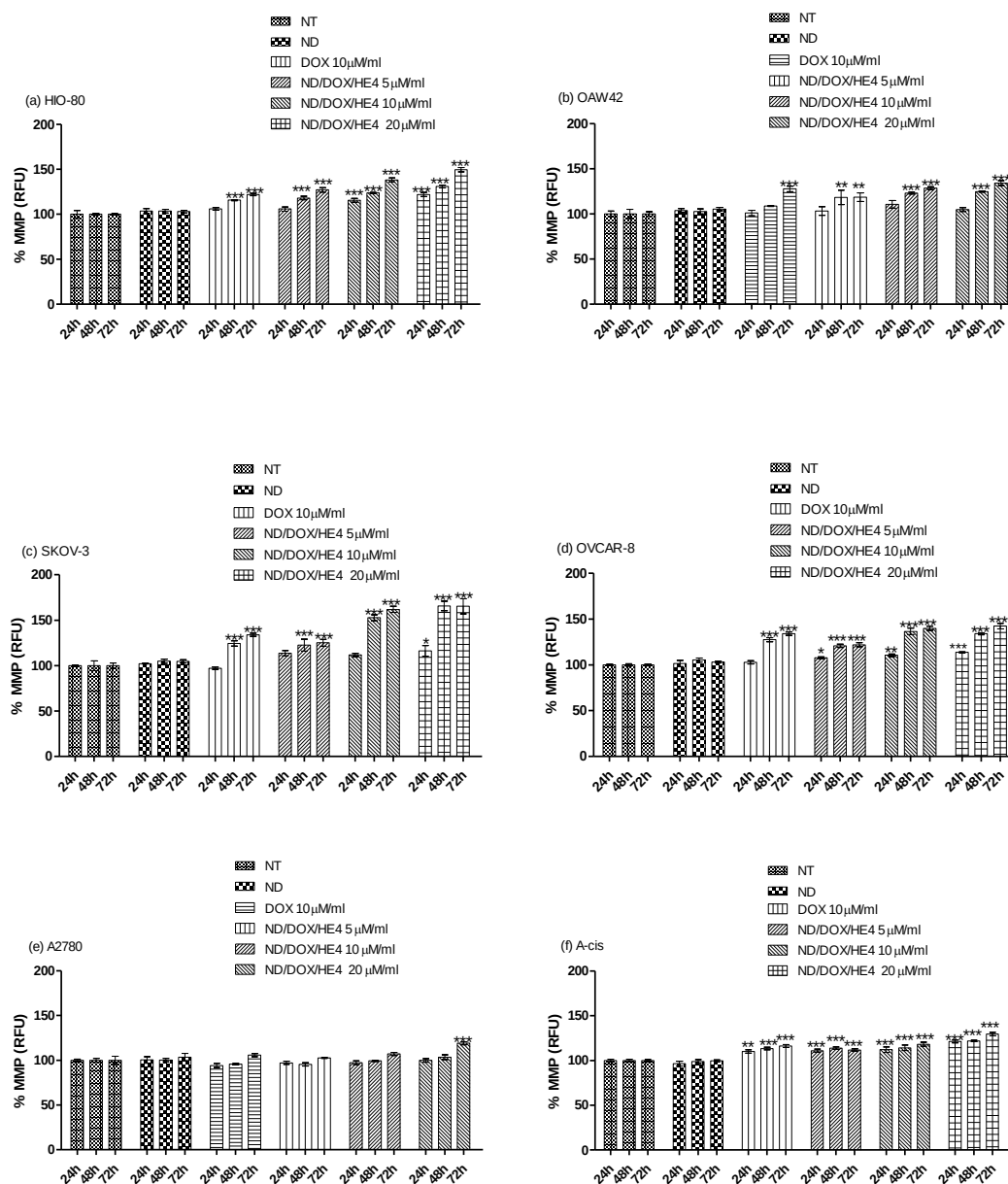


Figure 3.19: Mitochondrial membrane changes after ND/DOX/HE4 treatment

Cells were treated by ND/DOX/HE4 for three different time incubations and Cytell™ and IN cell 1000 software were used to major the mitochondrial changes (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) A2780 cells and (e) A2780 cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistically significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (DOX) doxorubicin.

Table 3.14: Percentages of MMP increases after ND/DOX/HE4 treatment.

The percentage of MMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	DOX 10µM/ml (%)	SEM	ND/DOX /HE4 5µM/ml (%)	SEM	ND/DOX /HE4 10µM/ml (%)	SEM	ND/DOX/HE4 20µM/ml (%)	SEM
HIO-80	24h	0 ± 4.2		3.6 ± 2.8		6.1 ± 1.3		5.8 ± 2.4		15.6 ± 2.2		22.1 ± 2.3	
	48h	0 ± 1.1		3.6 ± 2		15.8 ± 0.5		18.2 ± 1.8		23.9 ± 0.3		30.9 ± 1.4	
	72h	0 ± 0.9		3.1 ± 1.4		22.1 ± 1.4		27.2 ± 2.5		38.2 ± 2.2		49.7 ± 2.3	
OAW42	24h	0 ± 2.2		3.5 ± 1.5		0.9 ± 2.1		3.2 ± 3.3		10.6 ± 3		4.8 ± 1.4	
	48h	0 ± 5		2.7 ± 2.7		8.7 ± 0.5		18.3 ± 7.9		23 ± 0.9		24.6 ± 0.6	
	72h	0 ± 2.4		5.3 ± 1.9		27.7 ± 3.1		18.6 ± 4.7		28.5 ± 1.6		34.1 ± 2.7	
SKOV-3	24h	0 ± 0.7		2.4 ± 0.4		0.0 ± 1.1		13.8 ± 2.8		11.8 ± 1.6		16.2 ± 5.9	
	48h	0 ± 0.7		4.9 ± 2.2		24.4 ± 2.9		22.7 ± 6.5		52.8 ± 3.2		65.3 ± 5.1	
	72h	0 ± 2.9		4.8 ± 1.9		34 ± 1.8		25.1 ± 3.8		61.9 ± 3.4		65.4 ± 8.2	
OVCAR-8	24h	0 ± 0.6		1.7 ± 3.4		2.8 ± 1.8		7.9 ± 0.8		10.6 ± 1.5		13.9 ± 0.5	
	48h	0 ± 1.2		5.4 ± 2.2		27.6 ± 2.2		20.8 ± 1.7		36.6 ± 3.5		34.1 ± 1	
	72h	0 ± 0.8		3.1 ± 1		34.1 ± 1.7		21.9 ± 2.2		39.9 ± 2.2		42.3 ± 2.9	
A2780	24h	0 ± 1.3		0.4 ± 3.5		0.0 ± 2.2		0.0 ± 1.7		0.0 ± 2.1		0.0 ± 1.9	
	48h	0 ± 2.2		0.3 ± 1.8		0.0 ± 0.7		0.0 ± 1.8		0.0 ± 0.6		3.4 ± 2.7	
	72h	0 ± 4.3		3.6 ± 4		5.7 ± 1.7		2.5 ± 0.5		7.1 ± 1.7		19.1 ± 1.7	
A2780-cis	24h	0 ± 1.2		0 ± 2.7		10.1 ± 1.7		11.1 ± 1.6		12.1 ± 3.1		21.3 ± 1.6	
	48h	0 ± 1.4		0 ± 2		13.4 ± 1.5		13.9 ± 1.3		14.2 ± 3		21.8 ± 0.7	
	72h	0 ± 1		0 ± 0.9		16.3 ± 1.5		11.8 ± 1.2		18.2 ± 2.3		29.6 ± 1.8	

3.4.19 Induction of cell membrane permeability upon treatment with ND/DOX/HE4

A significant increase ($p < 0.001$) in CMP was seen after the cells were exposed to all nanocomplex concentrations at all time points in a cell, dose, and time dependent manner (Figure 3.20).

A2780 and OAW42 cells exhibited the highest increase in CMP after treatment with all concentrations of the nanocomplex compared to untreated cells. Increases in CMP ranging from 8.3% to 81.4% for A2780 and 8% to 64.9% for OAW42 were seen as time and concentration increased (Table 3.15). For other cell lines (A2780-cis, SKOV-3 and OVCAR-8), significant increases in CMP ($p < 0.001$) ranged between 9.6% to 36% in A2780-cis cells, 4.7% to 21% SKOV-3 cells, and 4.8% to 14.3% OVCAR-8 cells (Figure 3.20 b, c, and d). HIO-80 cells showed similar increases in CMP with 13.8% to 33.1% after treatment with the nanocomplex (Figure 3.20 a).

The mid concentration (ND/DOX/HE4 10 μ M/ml) of the nanocomplex was more effective in increasing CMP than the same dose of DOX alone (10 μ M/ml) in OAW42, SKOV-3, OVCAR-8 and HIO-80 cells with increases ranging from 4.9% to 30.2% across those cell lines compared to 2.7% to 25.6% for the DOX alone (Figure 3.20 a, b, c, and d).

Interestingly, the lowest concentration (5 μ M/ml) of the nanocomplex used in this study was more effective in increasing CMP than the clinically relevant dose of DOX (10 μ M/ml) alone in SKOV-3, OVCAR-8 and HIO-80 cell lines at all time points (Figure 3.20 a, c, and d).

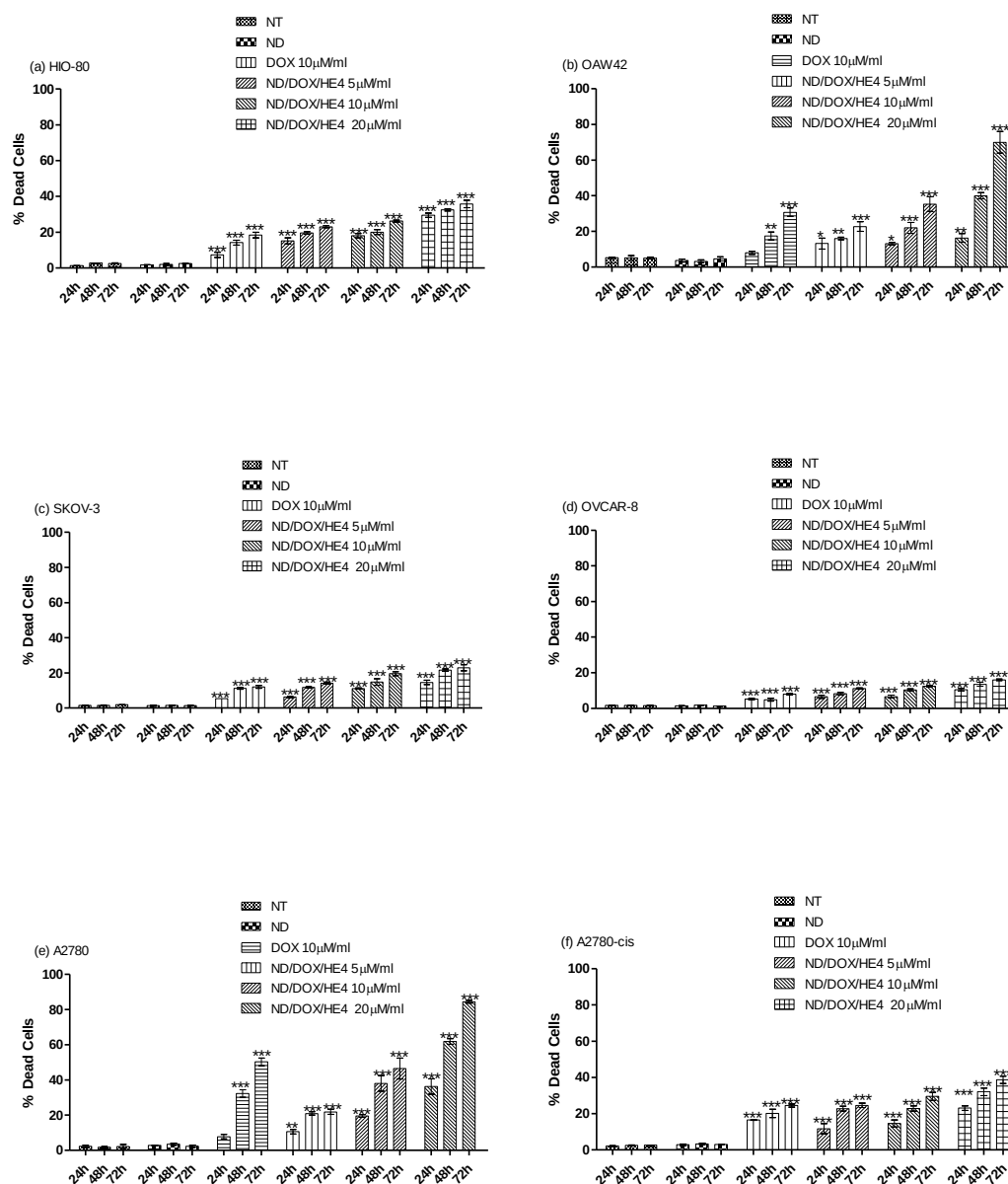


Figure 3.20: Cell membrane permeability measurement after ND/DOX/HE4 treatment

Cells were treated by NDs/DOX/HE4 and cells were incubated for 24h, 48h, and 72h and the CMP was measured by Cytell™ and INcell software, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) A2780sen cells, and (e) A2780cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (DOX) doxorubicin.

Table 3.15: Percentages of CMP increases after ND/DOX/HE4 treatment.

The percentage of CMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	DOX 10µM/ml (%)	SEM	ND/DOX/HE4 5µM/ml (%)	SEM	ND/DOX/HE4 10µM/ml (%)	SEM	ND/DOX/HE4 20µM/ml (%)	SEM
HIO-80	24h	0 ± 0.1		0.4 ± 0.1		6.0 ± 1.3		13.8 ± 1.6		16.7 ± 1.2		28.1 ± 1.1	
	48h	0 ± 0.1		0.0 ± 0.4		11.4 ± 1.3		16.8 ± 0.6		17.3 ± 1.3		29.7 ± 0.6	
	72h	0 ± 0.2		0.0 ± 0.1		15.8 ± 1.5		20.4 ± 1.6		23.6 ± 0.5		33.1 ± 2.1	
OAW42	24h	0 ± 0.3		1.5 ± 0.8		2.7 ± 0.8		8.0 ± 3.1		7.9 ± 0.6		11.0 ± 2.4	
	48h	0 ± 1.2		1.8 ± 0.7		12.3 ± 2.2		10.9 ± 0.7		16.8 ± 3		34.8 ± 1.7	
	72h	0 ± 0.5		0.4 ± 1.1		25.6 ± 2.3		17.6 ± 2.6		30.2 ± 4		64.9 ± 6.1	
SKOV-3	24h	0 ± 0.1		0.1 ± 0.2		3.9 ± 0.1		4.7 ± 0.4		9.6 ± 0.3		13.1 ± 1.2	
	48h	0 ± 0.2		0.0 ± 0.1		9.6 ± 0.5		10.2 ± 0.3		13.2 ± 1.8		20 ± 0.6	
	72h	0 ± 0.1		0.5 ± 0.1		10.1 ± 0.7		12.2 ± 0.6		17.6 ± 1.1		21 ± 1.7	
OVCAR-8	24h	0 ± 0.1		0.2 ± 0.1		3.5 ± 0.3		4.8 ± 0.8		4.9 ± 0.7		8.8 ± 0.7	
	48h	0 ± 0.2		0.1 ± 0.1		3.1 ± 0.7		6.6 ± 0.6		8.6 ± 0.6		11.8 ± 1.1	
	72h	0 ± 0.2		0.3 ± 0.1		6.4 ± 0.5		9.6 ± 0.3		10.8 ± 0.2		14.3 ± 0.4	
A2780	24h	0 ± 0.6		0.6 ± 0.2		5.5 ± 1.2		8.3 ± 1.2		17.3 ± 0.7		34 ± 4.3	
	48h	0 ± 0.1		1.4 ± 0.6		30.1 ± 2		18.8 ± 0.9		35.9 ± 4.4		59.7 ± 1.6	
	72h	0 ± 1		1.0 ± 0.6		49 ± 2.1		18.2 ± 1.4		31.9 ± 5.9		81.4 ± 0.8	
A2780-cis	24h	0 ± 0.3		0.8 ± 0.4		14.3 ± 0.2		9.6 ± 2.7		12.5 ± 1.9		21 ± 1.2	
	48h	0 ± 0.2		0.8 ± 0.4		17.6 ± 2.2		20.3 ± 1.3		20.4 ± 1.4		29.6 ± 2	
	72h	0 ± 0.1		0.4 ± 0.1		21.9 ± 0.8		22 ± 1.2		27.0 ± 2.1		36 ± 1.9	

3.4.20 Cell cycle distribution in response to ND/DOX/HE4 treatment

A significant increase ($p < 0.001$) in the number of cells in G2/M phase in OAW42, SKOV-3, OVCAR-8 and A2780 was seen cells after treatment with mid and high concentrations of the nanocomplex at all time points compared to untreated cells (Figure 3.21 c, d, and e), and the lower concentration of the nanocomplex induced a significant increase in cell number in S phase in those cells except for OAW42 which showed no significant increase in cell proportions compared to untreated cells (Figure 3.21 b). A significant increase in cell proportions in S phase were also seen in HIO-80 cells after treatment with the nanocomplex (Figure 3.21 a).

The highest percentage of cells in arrested phase was 34% and 35% in G2/M phase in OAW42 and in S phase in HIO-80 cell lines respectively. For other cells the proportions in G2/M phase were 31% in SKOV-3, 20% in OVCAR-8, 16% in A2780, and 11% in A2780-cis cell lines.

The mid concentration (ND/DOX/HE4 10 μ M/ml) of the nanocomplex was more effective in sending cells into arrest than the same dose of DOX alone (10 μ M/ml) in all cells with increases of cell proportions in arrested phase ranging from 11% to 35% across those cells compared to 5% to 30% for the DOX alone.

Cisplatin sensitive and cisplatin resistant ovarian cancer cells (A2780 and A2780-cis) exhibited similar percentages cell proportions in arrested phase after treatment with all concentrations of nanocomplex (Figure 3.21 e and f).

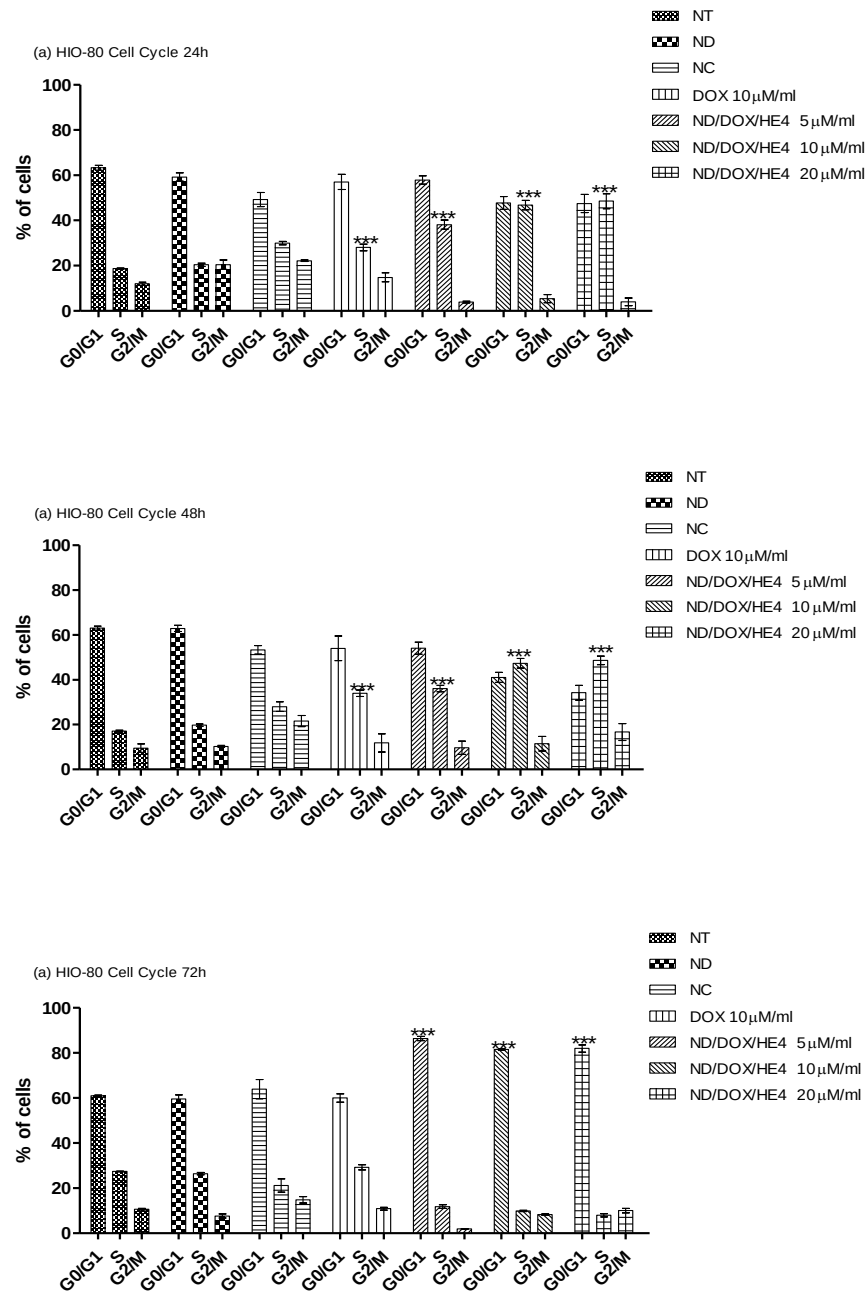


Figure 3.21 a: Cell cycle for HIO-80 cells after ND/DOX/HE4 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

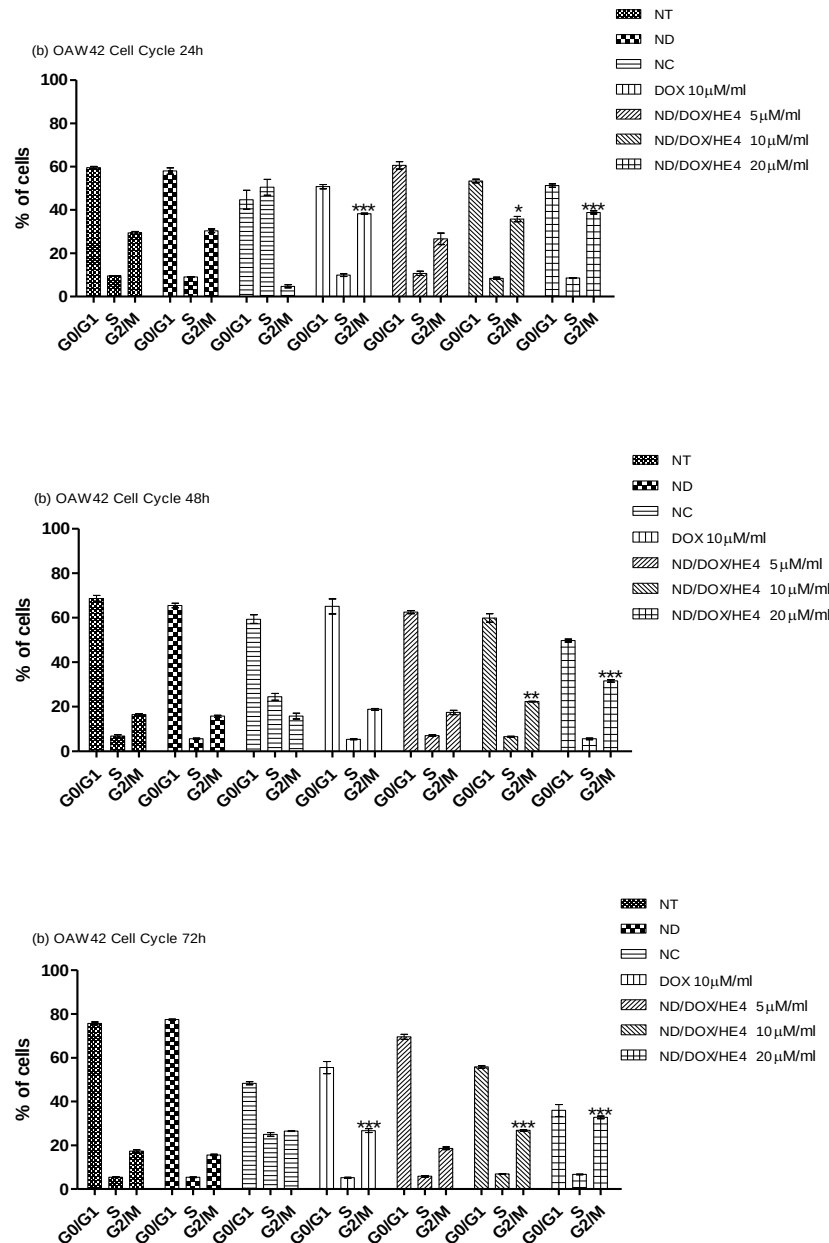


Figure 3.21 b: Cell cycle for OAW42 cells after treatment with ND/DOX/HE4

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

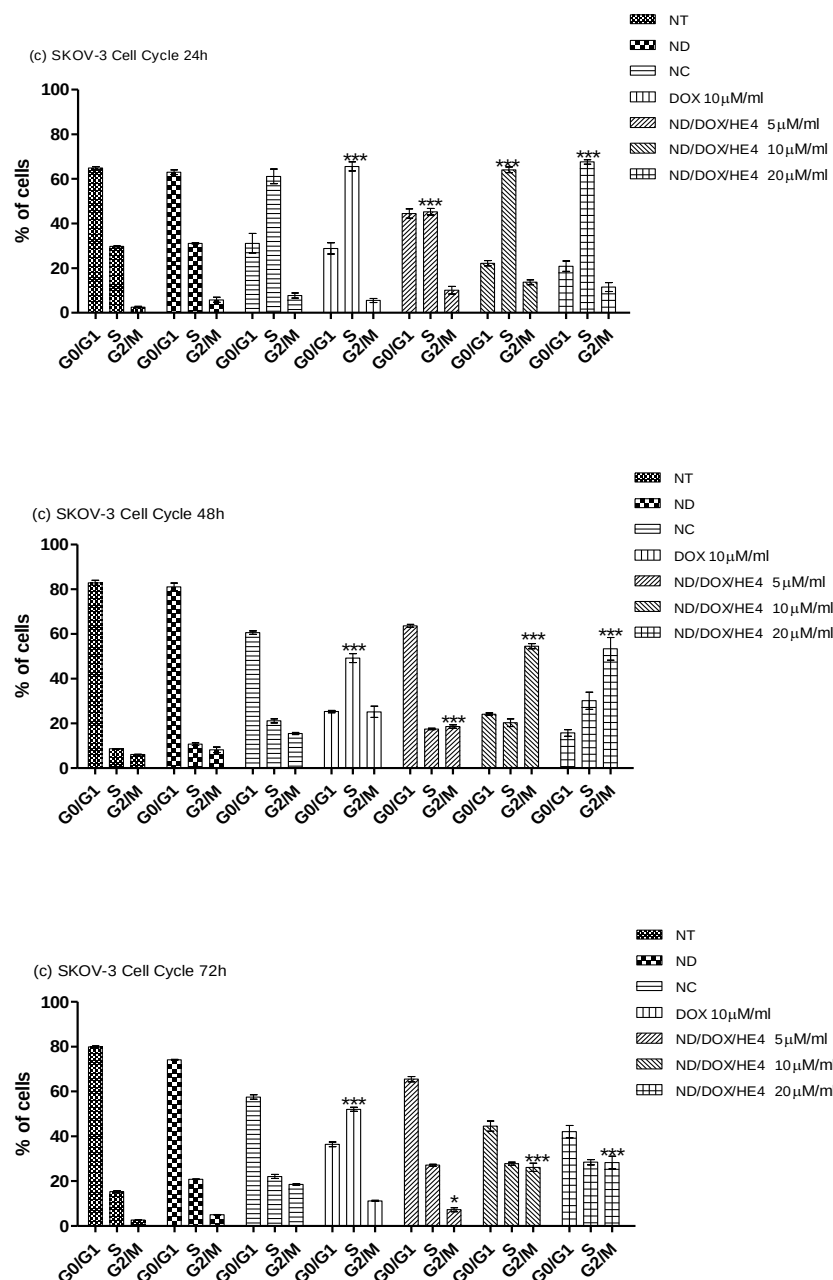


Figure 3.21 c: Cell cycle for SKOV-3 cells after treatment with ND/DOX/HE4

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

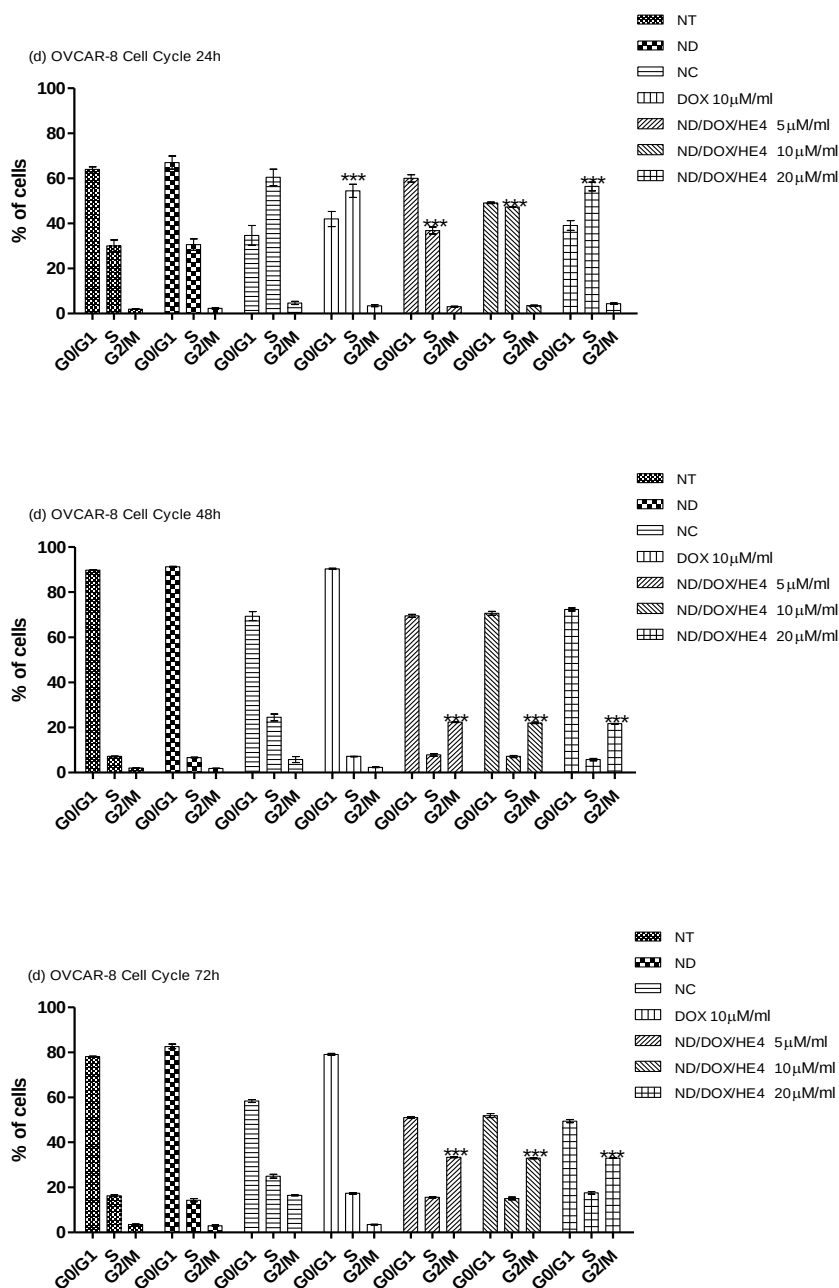


Figure 3.21 d: Cell cycle for OVCAR-8 cells after treatment with ND/DOX/HE4

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

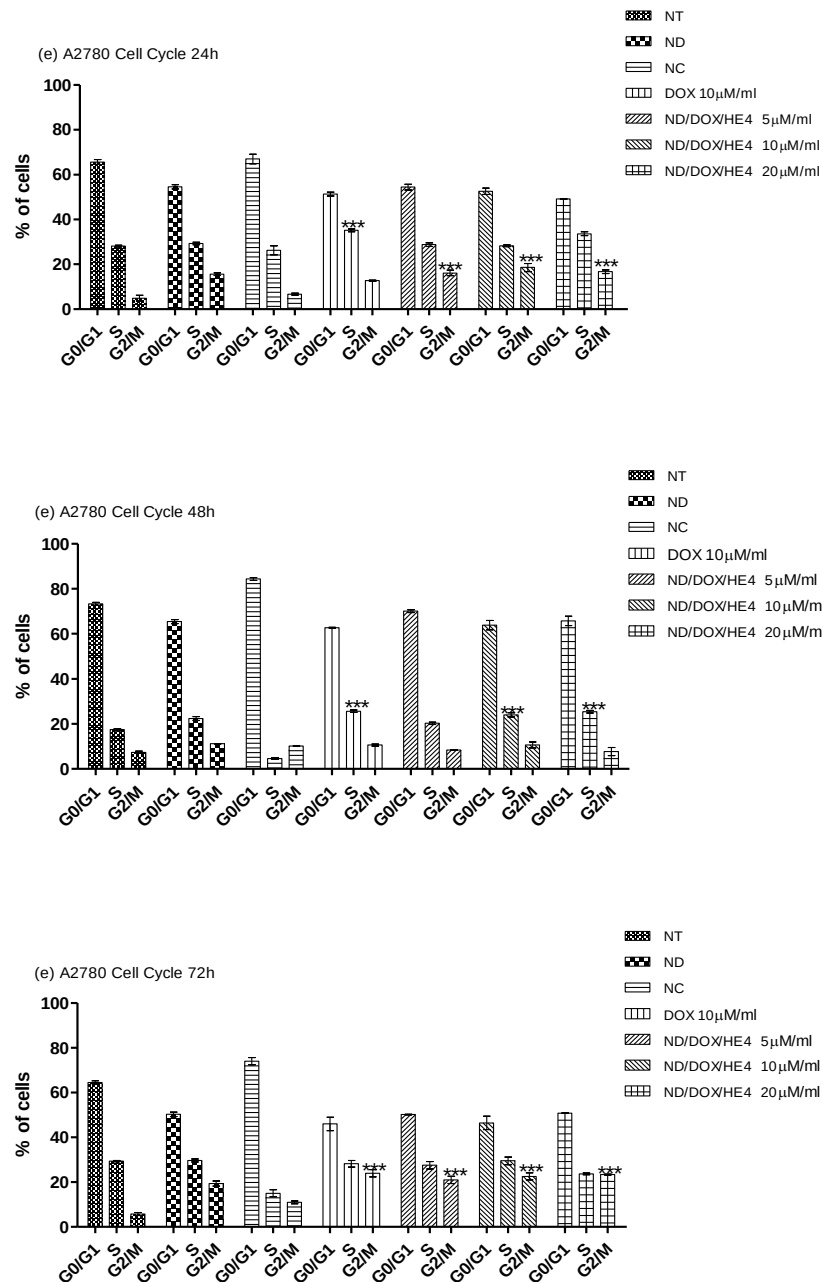


Figure 3.21 e: Cell cycle for A2780 cells after treatment with ND/DOX/HE4

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

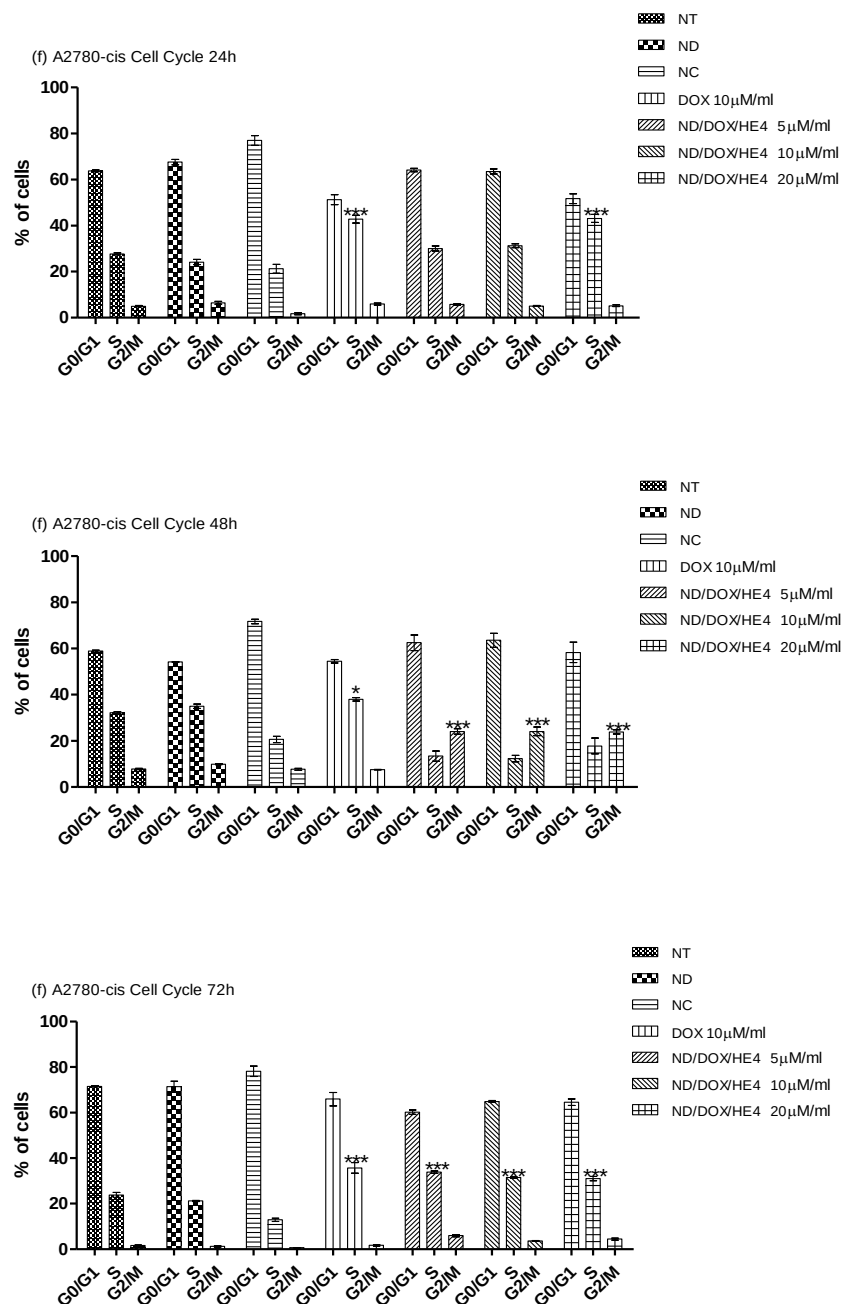


Figure 3.21 f: Cell cycle for A2780-cis cells after treatment with ND/DOX/HE4

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/HE4) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

3.4.21 Cell viability measurement in response to ND/DOX/CA125 treatment

A significant reduction ($p \leq 0.001$) in cell viability was observed after all cells were exposed to all nanocomplex concentrations and the effect was dose and time dependent compared to untreated cells (Figure 3.22).

The cisplatin resistant ovarian cancer cell line (A2780-cis) showed the highest cell viability reduction with 21.7% to 91.5% decreases over the time points compared to untreated cells (Table 3.16). The SKOV-3, OVCAR-8, and A2780 ovarian cancer cells showed similar cell viability reductions after exposure to all nanocomplex concentrations. Decreases in cell viability ranging from 28.6% to 91.1% for SKOV-3 cells, 16.1% to 87.2% for OVCAR-8 cells, and 44% to 90.2% for A2780 cells at all time intervals compared to untreated cells (Figure 3.22 c, d, and e) were seen. The OAW42 and HIO-80 cells showed significant cell viability reduction but not as high as other ovarian cancer cell lines (Figure 3.22 a and b).

The mid concentration (ND/DOX/CA125 10 μ M/ml) of the nanocomplex was more effective in reducing cell viability than the same dose of drug alone (DOX 10 μ M/ml) in all ovarian cancer cells at all time points with decreases in cell viability ranging from 27.1% to 88.5% compared to 12. %4 to 78. %5 for the drug alone (Figure 3.22 c, e, and f). Interestingly, the lowest concentration (5 μ M/ml) of the nanocomplex used in this study induced more cell viability reduction in SKOV-3, A2780, and A2780-cis cell lines than the clinically relevant dose of the drug alone at all time points (Figure 3.22 c, e, and f).

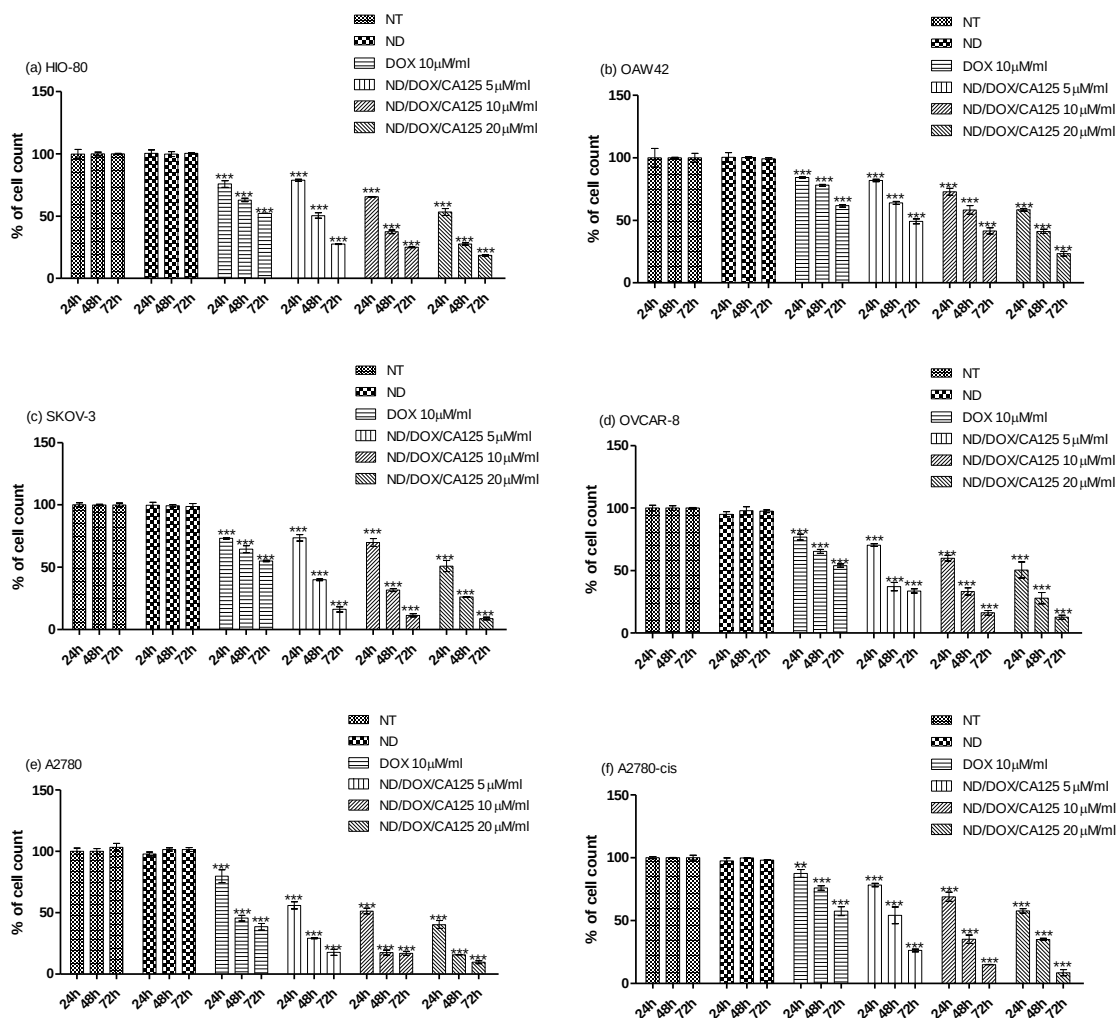


Figure 3.22: Cell viability assessment after ND/DOX/CA125 treatment

Cells were treated by ND/DOX/CA125 for three different time incubations and total cell count was measured using IN cell 1000 software, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) A2780sen cells and (e) A2780 cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistically significant between treated and untreated cells reported by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (DOX) doxorubicin.

Table 3.16: Percentages of cell viability reduction (cell kill) after ND/DOX/CA125 treatment. The percentage of cell viability reduction listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	DOX 10µM/ml (%)	SEM	ND/DOX /CA125 5µM/ml (%)	SEM	ND/DOX /CA125 10µM/ml (%)	SEM	ND/DOX/CA125 20µM/ml (%)	SEM
HIO-80	24h	0.4 ± 1		4.4 ± 0.8		17.8 ± 1.3		21.1 ± 0.7		34.3 ± 0.1		46.6 ± 2.8	
	48h	0.3 ± 0.2		1.0 ± 0.9		50.3 ± 2.4		49.5 ± 2.2		62.5 ± 1.4		72.2 ± 0.9	
	72h	0.7 ± 0.8		0.4 ± 1.4		66.4 ± 1.4		72.4 ± 0.3		75.0 ± 0.3		81.4 ± 0.6	
OAW42	24h	0.3 ± 7.4		0.2 ± 3.7		15.7 ± 0.4		11.4 ± 2.2		27.1 ± 2.7		41.5 ± 0.9	
	48h	0.2 ± 0.2		0.1 ± 0.5		21.9 ± 0.6		36.0 ± 1.1		41.6 ± 3.3		58.8 ± 1.6	
	72h	0.1 ± 3.5		0.6 ± 0.6		38.3 ± 0.9		50.8 ± 1.9		58.4 ± 2.4		76.7 ± 1.8	
SKOV-3	24h	4.7 ± 0.1		0.3 ± 0.8		23.2 ± 2.1		28.6 ± 6.3		28.4 ± 4.6		49.0 ± 4.4	
	48h	0.0 ± 1		2.9 ± 2.7		55.9 ± 3.5		60.0 ± 0.7		68.3 ± 1.1		73.8 ± 0.2	
	72h	1.6 ± 2.2		1.7 ± 0.8		78.5 ± 0.4		83.8 ± 2		88.5 ± 1.2		91.1 ± 1	
OVCAR-8	24h	0.0 ± 0.2		0.6 ± 1.9		37.9 ± 7.9		16.1 ± 6.5		36.6 ± 4.3		49.5 ± 6.4	
	48h	0.3 ± 1.3		0.1 ± 2.2		50.3 ± 5.1		62.7 ± 3.3		66.6 ± 2.8		72.0 ± 4.5	
	72h	0.0 ± 0.4		0.1 ± 1		73.5 ± 1.8		66.3 ± 1.6		83.7 ± 1.9		87.2 ± 1.6	
A2780	24h	0.9 ± 2.4		1.3 ± 1.7		39.6 ± 5.3		44.0 ± 2.9		48.6 ± 2.2		59.6 ± 3.2	
	48h	0.3 ± 3.1		0.2 ± 3.3		73.0 ± 1.3		70.7 ± 0.4		82.4 ± 1.9		84.1 ± 0.1	
	72h	0.1 ± 1.2		0.6 ± 0.5		82.3 ± 1.2		82.3 ± 2.3		83.0 ± 1.5		90.2 ± 1.2	
A2780-cis	24h	0.2 ± 0.7		2.4 ± 2.2		12.4 ± 3.1		21.7 ± 1.3		31.0 ± 3.6		42.3 ± 1.7	
	48h	0.0 ± 0.2		0.9 ± 0.1		24.0 ± 1.7		45.8 ± 6.6		64.9 ± 3.3		65.0 ± 0.8	
	72h	0.0 ± 1.9		1.8 ± 0.3		42.6 ± 3.5		73.7 ± 1		85.0 ± 0.1		91.5 ± 2.3	

3.4.22 MMP changes in response to ND/DOX/CA125 treatments

A significant increase ($p \leq 0.001$) in MMP was seen in all cells when treated with the higher concentration of the nanocomplex (20 $\mu\text{M}/\text{ml}$) at 48h and 72h compared to untreated cells (Figure 3.23). OAW42 and A2780-cis cell lines exhibited the highest MMP changes ranging between 2.8% to 126.4% for OAW42 and 5.3% to 110.7% for A2780-cis (Table 3.17).

All other ovarian cancer cells and HIO-80 cell line showed variable MMP increases ranging between 5.3% to 92.1% after all concentrations of ND/DOX/CA125 at 48h and 72h compared to untreated cells (Figure 3.23 a, c, d and e).

The cisplatin resistant to ovarian cancer cell line (A2780-cis) exhibited higher MMP increases than the cisplatin sensitive ovarian cancer cell line (A2780) after all nanocomplex treatments (Figure 3.23 e and f). The mid concentration (10 $\mu\text{M}/\text{ml}$) induced higher MMP changes in OVCAR-8, A2780 and A2780-cis than the clinically relevant dose of DOX alone (Figure 3.23 d, e and f).

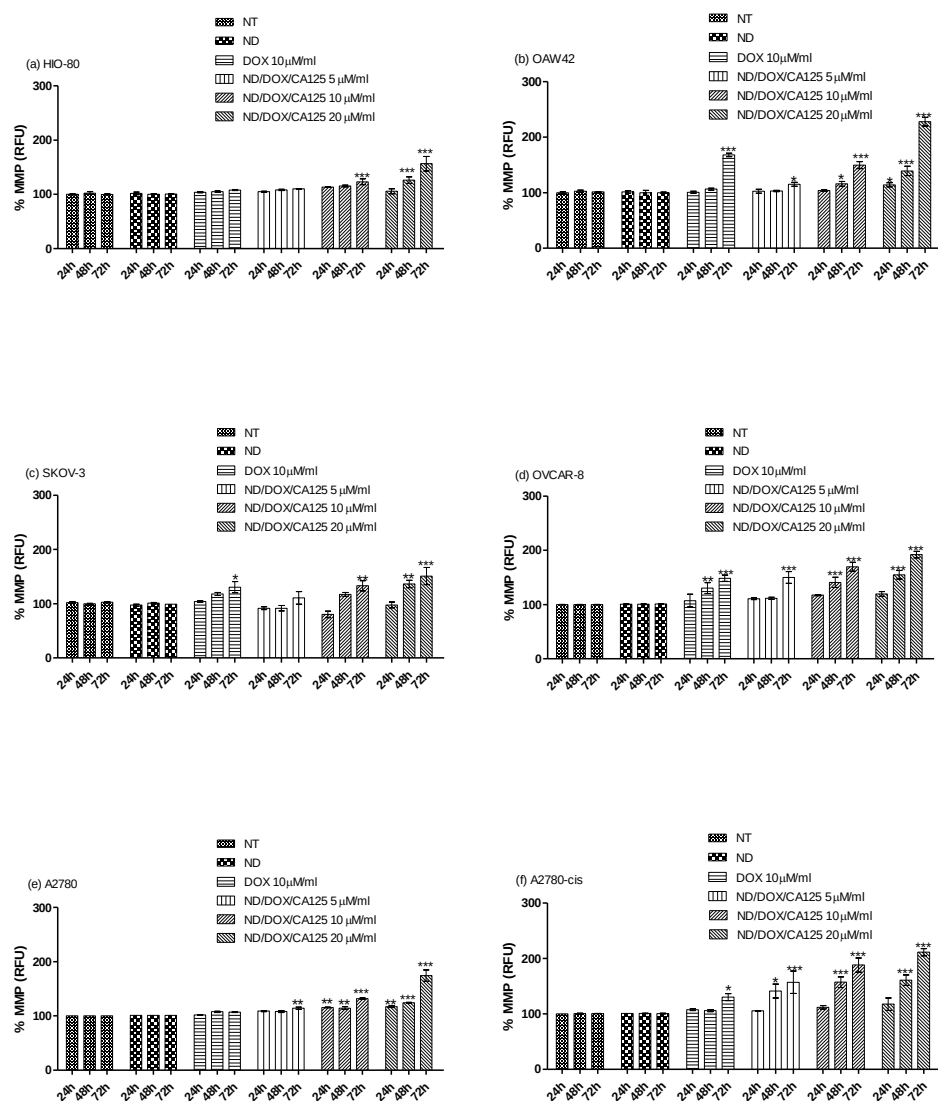


Figure 3.23: Mitochondrial membrane changes after ND/DOX/CA125 treatment

Cells were treated by ND/DOX/CA125 for three different time incubations and Cytell™ and IN cell 1000 software were used to major the mitochondrial changes (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) A2780 cells and (e) A2780 cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistically significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (DOX) doxorubicin.

Table 3.17: Percentages of MMP increases after ND/DOX/CA125 treatment.

The percentage of MMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	DOX 10 μ M/ml (%)	SEM	ND/DOX /CA125 5 μ M/ml (%)	SEM	ND/DOX /CA125 10 μ M/ml (%)	SEM	ND/DOX/CA125 20 μ M/ml (%)	SEM
HIO-80	24h	0.0 $\pm$ 0.8		1.6 $\pm$ 2.3		3.7 $\pm$ 0.9		5.0 $\pm$ 0.5		13.3 $\pm$ 0.3		5.6 $\pm$ 4.7	
	48h	0.1 $\pm$ 2.3		0.1 $\pm$ 0.3		2.6 $\pm$ 1.1		5.7 $\pm$ 1.2		12.8 $\pm$ 1.7		23.8 $\pm$ 5.9	
	72h	0.0 $\pm$ 0.9		0.6 $\pm$ 0.9		8.0 $\pm$ 0.8		10.3 $\pm$ 0.4		23.3 $\pm$ 5.4		56.7 $\pm$ 13.4	
OAW42	24h	0.0 $\pm$ 1.5		1.5 $\pm$ 2.2		0.9 $\pm$ 1.6		2.8 $\pm$ 3.3		3.7 $\pm$ 1.4		14.0 $\pm$ 3.7	
	48h	0.0 $\pm$ 2		0.0 $\pm$ 4.2		3.8 $\pm$ 1.8		0.3 $\pm$ 0.9		13.4 $\pm$ 4.1		36.5 $\pm$ 8.4	
	72h	0.0 $\pm$ 0.6		0.0 $\pm$ 1.2		109.9 $\pm$ 2.1		13.8 $\pm$ 3.5		48.3 $\pm$ 6.2		126.4 $\pm$ 7.8	
SKOV-3	24h	0.0 $\pm$ 1.6		0.0 $\pm$ 1.7		2.1 $\pm$ 1.8		0.0 $\pm$ 2.7		0.0 $\pm$ 6.2		0.0 $\pm$ 5.4	
	48h	0.0 $\pm$ 0.8		1.0 $\pm$ 1.3		18.1 $\pm$ 2.6		0.0 $\pm$ 4.9		17.4 $\pm$ 3.7		36.5 $\pm$ 6.9	
	72h	0.0 $\pm$ 1		0.0 $\pm$ 0.1		27.7 $\pm$ 10.2		7.7 $\pm$ 11.5		23.6 $\pm$ 12.7		48.1 $\pm$ 15.4	
OVCAR-8	24h	0.0 $\pm$ 0.3		0.7 $\pm$ 0.5		7.2 $\pm$ 11.7		10.8 $\pm$ 1.8		17.5 $\pm$ 1		19.7 $\pm$ 4.3	
	48h	0.1 $\pm$ 0.6		0.8 $\pm$ 0.6		30.5 $\pm$ 9.9		4.9 $\pm$ 5.4		41 $\pm$ 9.3		55.1 $\pm$ 7.6	
	72h	0.0 $\pm$ 0.4		1.0 $\pm$ 0.4		48.5 $\pm$ 5.8		49.9 $\pm$ 11		69.7 $\pm$ 8.1		92.1 $\pm$ 5.7	
A2780	24h	0.0 $\pm$ 0.1		0.7 $\pm$ 0.3		1.7 $\pm$ 0.1		8.7 $\pm$ 1.1		15.5 $\pm$ 0.8		17.1 $\pm$ 1.5	
	48h	0.1 $\pm$ 0.1		0.8 $\pm$ 0.1		7.5 $\pm$ 1		8.1 $\pm$ 1.8		14.3 $\pm$ 2.4		24.3 $\pm$ 0.7	
	72h	0.0 $\pm$ 0.2		0.7 $\pm$ 0.5		7 $\pm$ 0.5		14.2 $\pm$ 1.8		31.8 $\pm$ 1.2		74.3 $\pm$ 10.4	
A2780-cis	24h	0.0 $\pm$ 0.1		0.7 $\pm$ 0.1		7.5 $\pm$ 1.4		5.3 $\pm$ 0.4		11.7 $\pm$ 3		17.6 $\pm$ 11.3	
	48h	0.0 $\pm$ 0.6		0.6 $\pm$ 0.4		5.4 $\pm$ 1.9		40.8 $\pm$ 12.6		57 $\pm$ 9.6		60.5 $\pm$ 9.5	
	72h	0.0 $\pm$ 0.3		0.0 $\pm$ 0.8		29.6 $\pm$ 6.2		56.5 $\pm$ 20.1		87.7 $\pm$ 12.7		110.7 $\pm$ 6.4	

3.4.23 Cell membrane permeability assessment in response to ND/DOX/CA125 treatment

Significant changes in CMP ($p < 0.001$) were observed after the cells were exposed to all nanocomplex concentrations at all time points and this was time, dose and cell dependent (Figure 3.24).

The highest increase in CMP was seen in OAW42 and A2780 cells after treatment with all concentrations of the nanocomplex compared to untreated cells. Increases in CMP ranging from 2.2% to 74.4% for OAW42 and 12.8% to 70.1% for A2780 were seen as time and concentration increased (Table 3.18). For other cell lines (SKOV-3, OVCAR-8 and A2780-cis), significant increases in CMP ($p < 0.001$) ranging between 13.8% to 49.1% in SKOV-3 cells, 14% to 39.8% in OVCAR-8 cells, and 22.2% to 53.6% in A2780-cis cells (Figure 3.24 c, d and f) were seen. Similar increases in CMP were seen in HIO-80 cells after treatment with the nanocomplex with 8.7% to 41.5% (Figure 3.24 a).

The mid concentration (ND/DOX/CA125 10 $\mu\text{M}/\text{ml}$) of the nanocomplex was more effective in increasing CMP than the same dose of DOX alone (10 $\mu\text{M}/\text{ml}$) in all cells with increases in CMP ranging from 6.7% to 65.2% across those cell lines compared to 0.9% to 47.2% for DOX alone (Table 3.18). Interestingly, the lowest concentration (5 $\mu\text{M}/\text{ml}$) of the nanocomplex used in this study was more effective in increasing CMP changes than the clinically relevant dose of DOX (10 $\mu\text{M}/\text{ml}$) alone in SKOV-3, OVCAR-8, A2780, A2780-cis and HIO-80 cell lines at all time points (Figure 3.24 a, c, d, e and f).

A paired cisplatin sensitive and resistant ovarian cancer cell line (A2780 and A2780-cis) exhibited similar CMP changes after exposure to all concentrations of the nanocomplex at three time points (Figure 3.24 e and f).

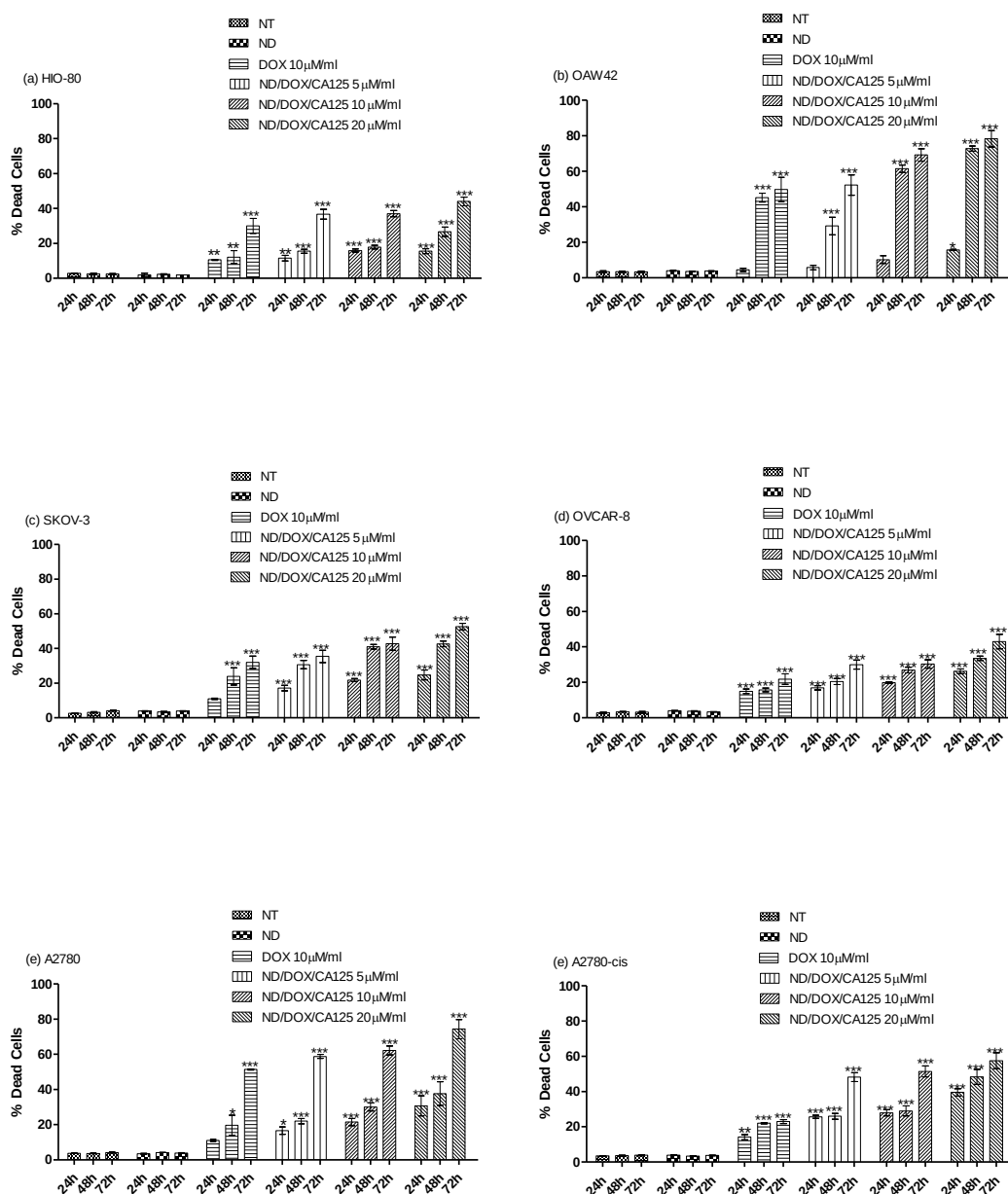


Figure 3.24: Cell membrane permeability measurement after ND/DOX/CA125 treatment

Cells were treated by ND/DOX/CA125 and cells were incubated for 24h, 48h, and 72h and the CMP was measured by Cytell™ and INcell software, (a) HIO-80 cells, (b) OAW42 cells, (c) SKOV-3 cells, (d) A2780sen cells, and (e) A2780cis cells. Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (DOX) doxorubicin.

Table 3.18: Percentages of CMP increases after ND/DOX/CA125 treatment.

The percentage of CMP increases listed are in comparison to untreated controls (NT) and the error bar represents the SEM. Values represent three independent experiments performed in triplicate.

Cells	Time	NT (%)	SEM	ND (%)	SEM	DOX 10 μ M/ml (%)	SEM	ND/DOX /CA125 5 μ M/ml (%)	SEM	ND/DOX /CA125 10 μ M/ml (%)	SEM	ND/DOX/CA125 20 μ M/ml (%)	SEM
HIO-80	24h	0 $\pm$ 0.1		0.8 $\pm$ 0.9		7.7 $\pm$ 0.2		8.7 $\pm$ 1.6		13.1 $\pm$ 0.8		12.7 $\pm$ 1.4	
	48h	0 $\pm$ 0.2		0.2 $\pm$ 0.3		9.6 $\pm$ 3.6		13 $\pm$ 1.1		15.3 $\pm$ 1		24.1 $\pm$ 2.7	
	72h	0 $\pm$ 0.2		0.5 $\pm$ 0.1		27.5 $\pm$ 4.2		34.2 $\pm$ 2.8		34.6 $\pm$ 1.7		41.5 $\pm$ 2.4	
OAW42	24h	0 $\pm$ 0.5		0.4 $\pm$ 0.2		0.9 $\pm$ 0.8		2.2 $\pm$ 1.2		6.7 $\pm$ 2.1		12.2 $\pm$ 0.3	
	48h	0 $\pm$ 0.2		0.8 $\pm$ 0.2		42.4 $\pm$ 2.4		26.4 $\pm$ 4.9		58.6 $\pm$ 2		70 $\pm$ 1.4	
	72h	0 $\pm$ 0.2		0.1 $\pm$ 0.1		45.8 $\pm$ 6.8		48.3 $\pm$ 5.6		65.2 $\pm$ 3.5		74.4 $\pm$ 4.5	
SKOV-3	24h	0 $\pm$ 0.5		0.6 $\pm$ 0.2		7.7 $\pm$ 0.3		13.8 $\pm$ 1.6		18.5 $\pm$ 0.8		21.4 $\pm$ 2.7	
	48h	0 $\pm$ 0.4		0.1 $\pm$ 0.3		20.6 $\pm$ 5		27.5 $\pm$ 2.3		37.8 $\pm$ 1.3		39.4 $\pm$ 1.5	
	72h	0 $\pm$ 0.5		0.4 $\pm$ 0.1		28.5 $\pm$ 3.6		31.9 $\pm$ 3.5		39.2 $\pm$ 3.7		49.1 $\pm$ 2	
OVCAR-8	24h	0 $\pm$ 0.4		1.1 $\pm$ 0.2		12.1 $\pm$ 1.3		14 $\pm$ 1.2		16.9 $\pm$ 0.3		23.4 $\pm$ 1.3	
	48h	0 $\pm$ 0.2		0.3 $\pm$ 0.3		12.2 $\pm$ 1		17 $\pm$ 1.7		23.5 $\pm$ 1.5		30.1 $\pm$ 1.2	
	72h	0 $\pm$ 0.3		0.0 $\pm$ 0.1		18.5 $\pm$ 2.9		26.7 $\pm$ 2.5		27 $\pm$ 2.3		39.8 $\pm$ 4	
A2780	24h	0 $\pm$ 0.2		0.3 $\pm$ 0.3		7.2 $\pm$ 0.5		12.8 $\pm$ 2.1		17.7 $\pm$ 2		27 $\pm$ 5.7	
	48h	0 $\pm$ 0.1		0.5 $\pm$ 0.1		16 $\pm$ 5.7		18.3 $\pm$ 1.5		26.4 $\pm$ 2.3		34 $\pm$ 6.7	
	72h	0 $\pm$ 0.2		0.3 $\pm$ 0.2		47.2 $\pm$ 0.3		54.6 $\pm$ 1.1		58.1 $\pm$ 2.5		70.1 $\pm$ 5.4	
A2780-cis	24h	0 $\pm$ 0.1		0.4 $\pm$ 0.2		10.6 $\pm$ 1.4		22.2 $\pm$ 0.8		24.4 $\pm$ 1.7		36 $\pm$ 2	
	48h	0 $\pm$ 0.3		0.2 $\pm$ 0.2		18.4 $\pm$ 0.3		22.4 $\pm$ 1.6		25.3 $\pm$ 2.8		44.6 $\pm$ 4.1	
	72h	0 $\pm$ 0.3		0.1 $\pm$ 0.1		19 $\pm$ 1		44.3 $\pm$ 2.6		47.5 $\pm$ 3.1		53.6 $\pm$ 4.4	

3.4.24 Cell cycle distribution in response to ND/DOX/CA125 treatment

A significant increase ($p < 0.001$) in the number of cells in S phase was observed in all cell lines after treatment with all concentrations of the nanocomplex at all time points compared to untreated cells (Figure 3.25 b, c, d, e and f). A significant increase in the cell proportion in G0/G1 phase was seen in HIO-80 cells after treatment with the nanocomplex (Figure 3.25 a).

The highest percentage of cells in arrested (S) phase was 45 % in SKOV-3. For other cell lines the cell proportions in S phase were 33% in OAW42, 26% in OVCAR-8, 29% in A2780, 36% in A2780-cis and 79% in G0/G1 in HIO-80 cell lines.

The mid concentration (ND/DOX/CA125 10 $\mu\text{M/ml}$) of the nanocomplex was more effective in increasing the cell percentage in arrested phase than the same dose of DOX alone (10 $\mu\text{M/ml}$) in OAW42, OVCAR-8, A2780 and HIO-80 cell lines with increases ranging from 28% to 77% across those cells compared to 16% to 30% for the DOX alone. Interestingly, the lowest concentration (ND/DOX/CA125 5 $\mu\text{M/ml}$) of the nanocomplex used in this study gave more efficient cell cycle arrest than the clinically relevant dose of DOX (10 $\mu\text{M/ml}$) alone in OAW42 and A2780 cell lines at all time points (Figure 3.25 b, and e).

Cisplatin resistant ovarian cancer cell (A2780-cis) exhibited higher cell cycle arrest in S phase (39%) compared to its cisplatin sensitive counterpart (29%) (Figure 3.25 e and f).

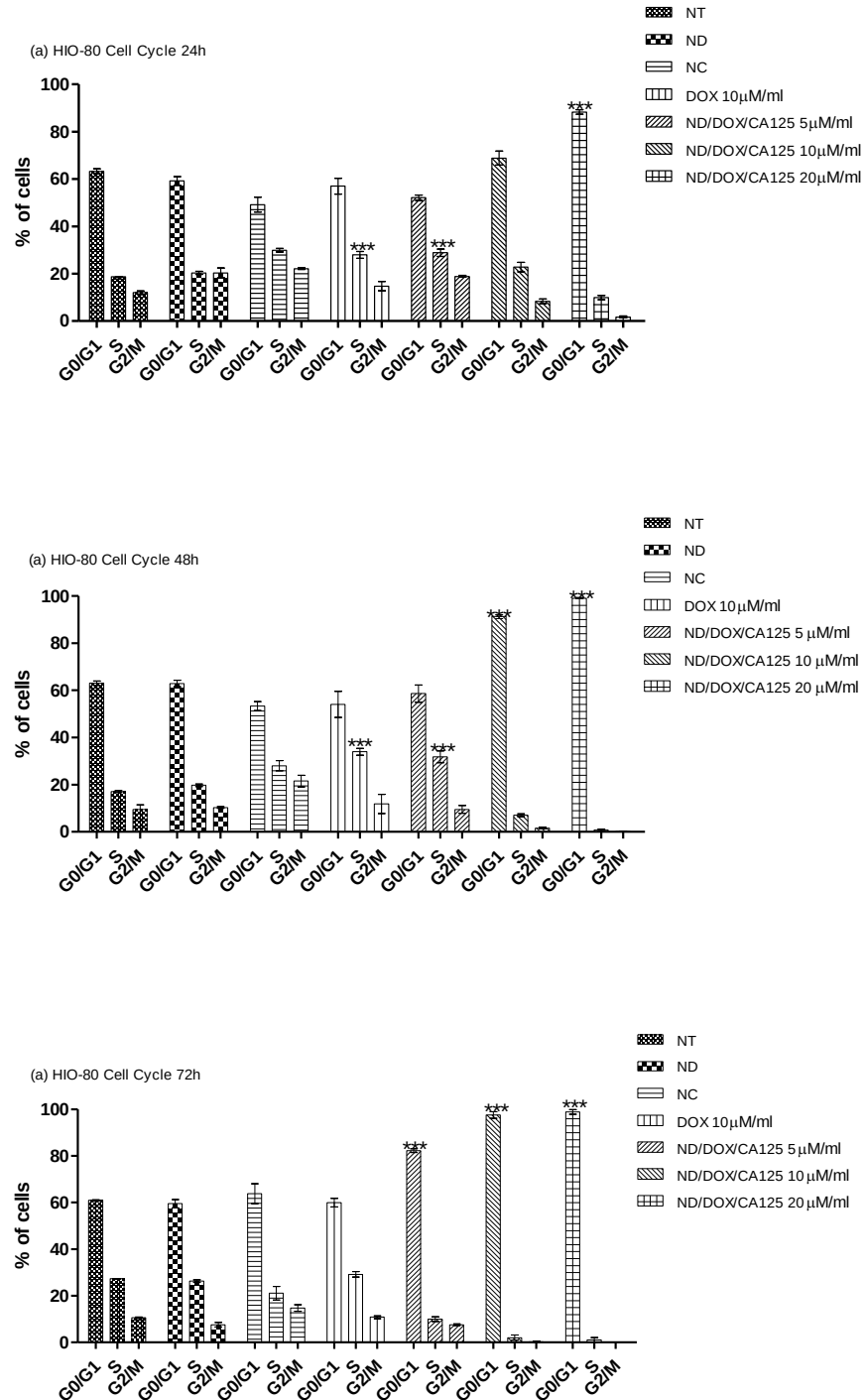


Figure 3.25 a: Cell cycle for HIO-80 cells after ND/DOX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

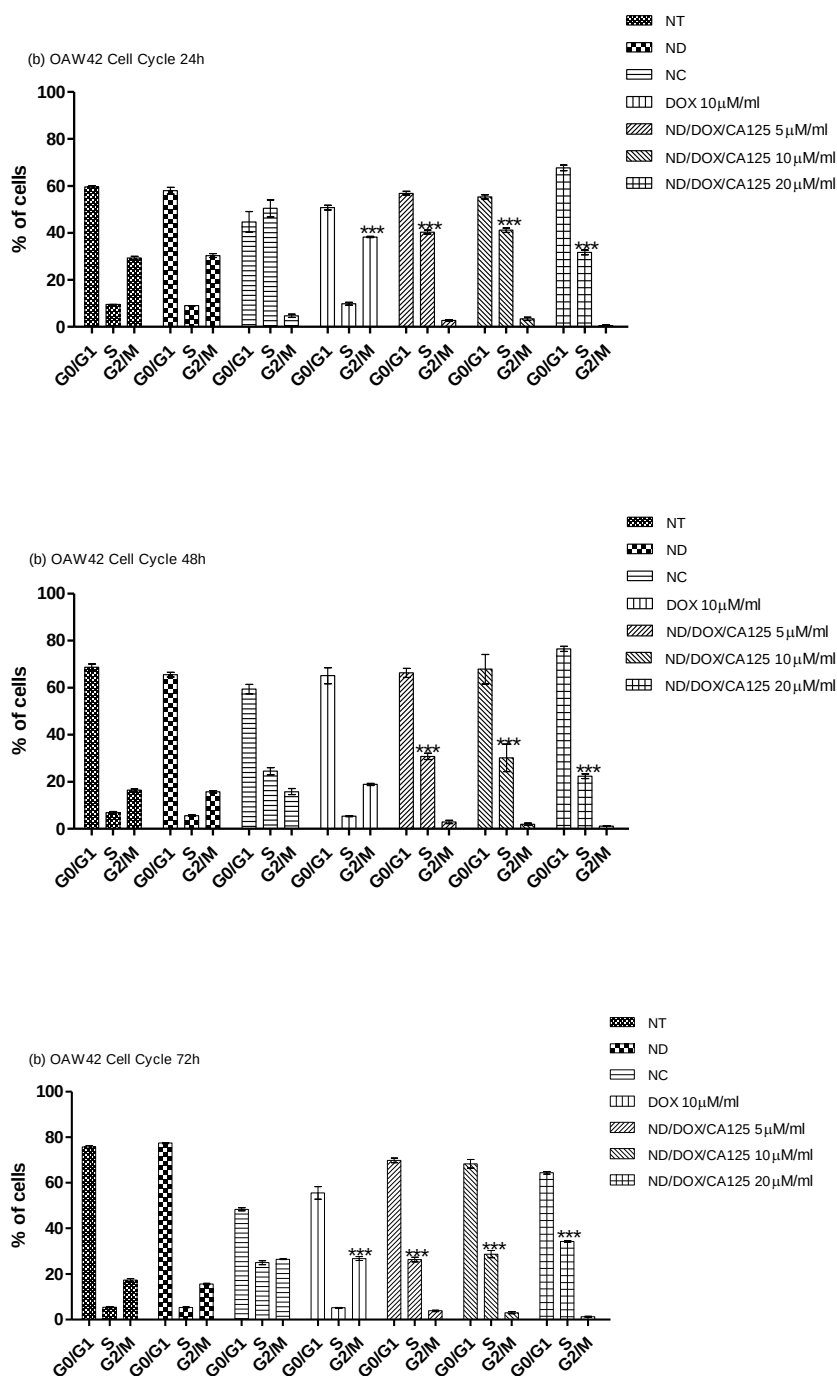


Figure 3.25 b: Cell cycle for OAW42 cells after ND/DOX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

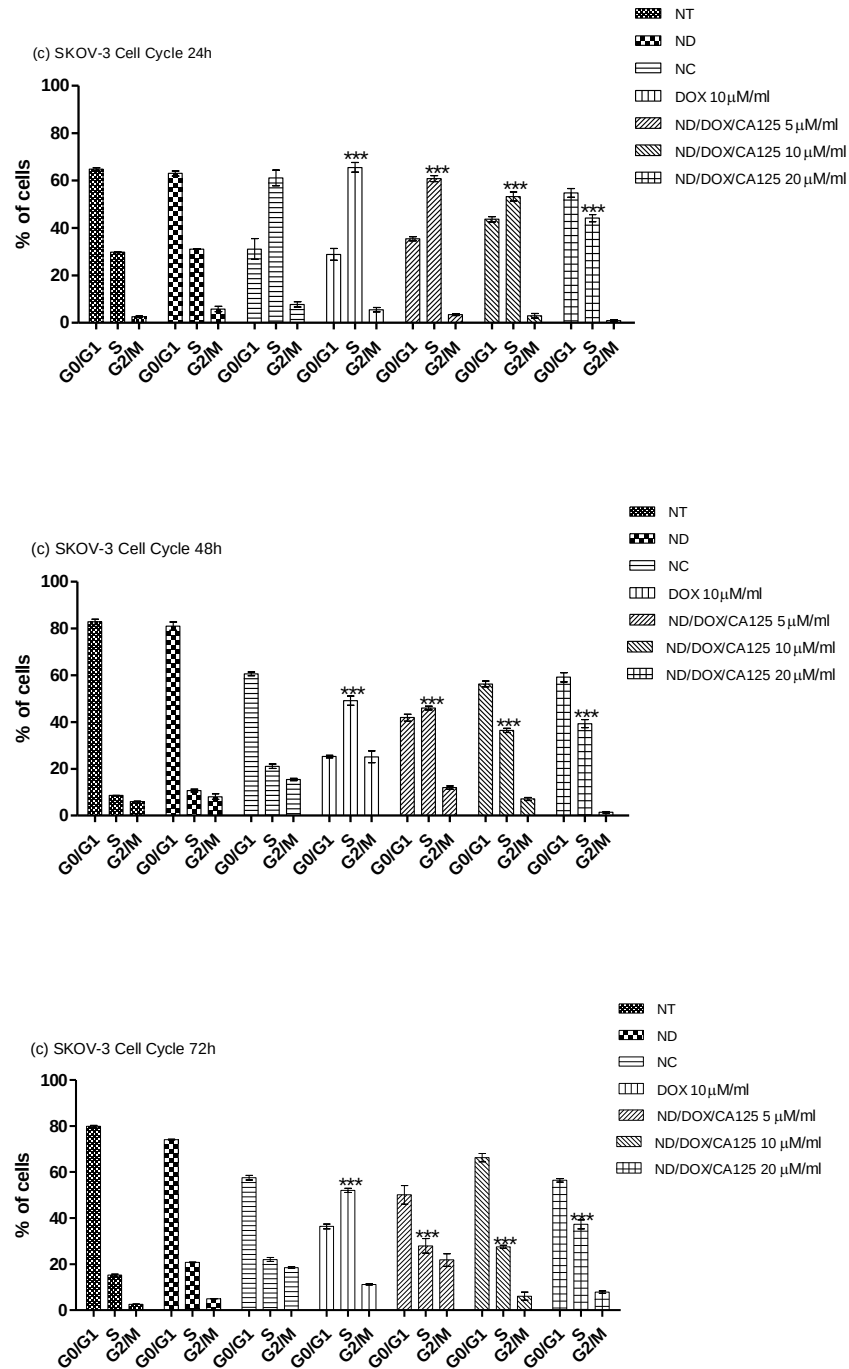


Figure 3.25 c: Cell cycle for SKOV-3 cells after ND/DOX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

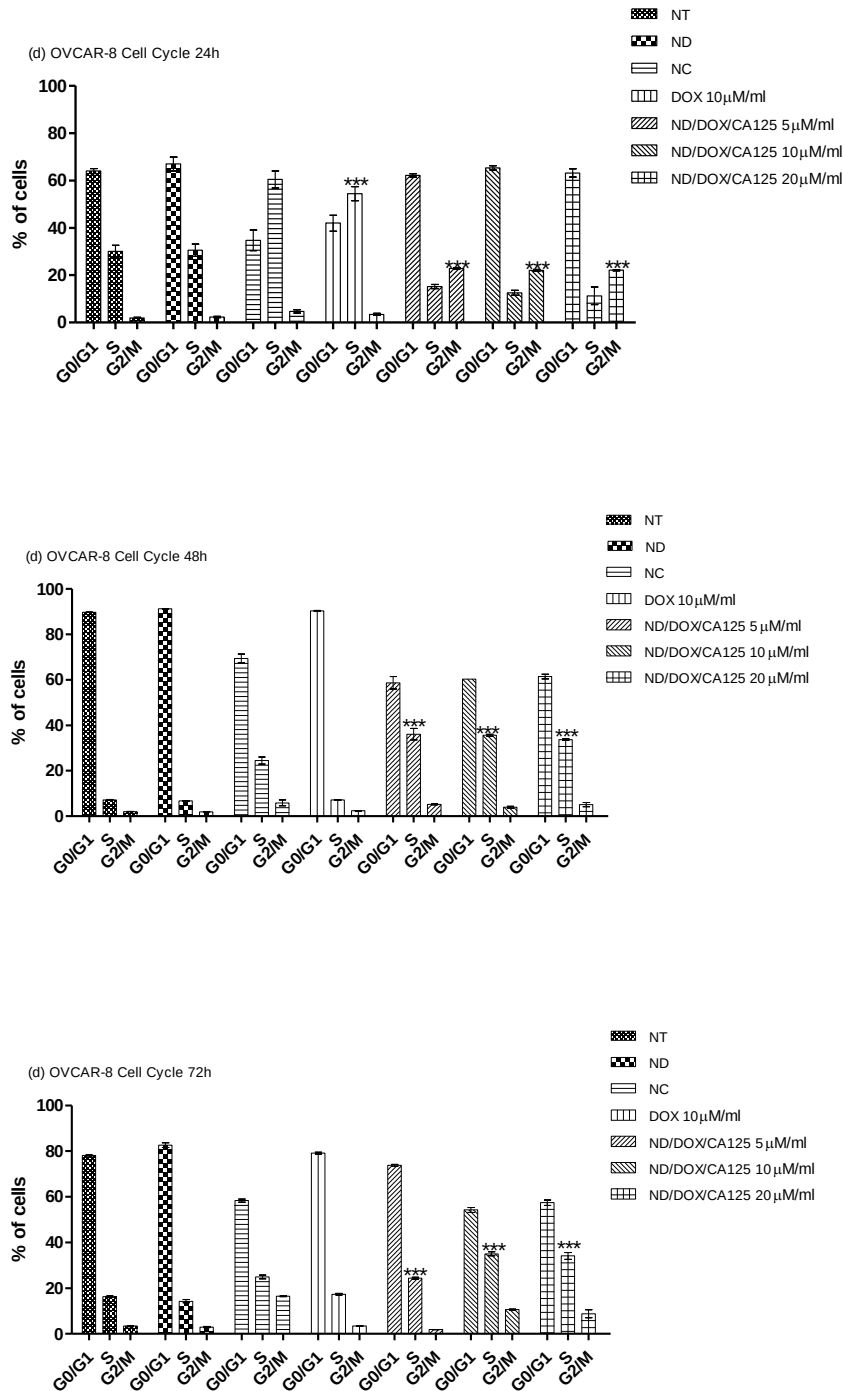


Figure 3.25: Cell cycle for OVCAR-8 cells after ND/DOX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

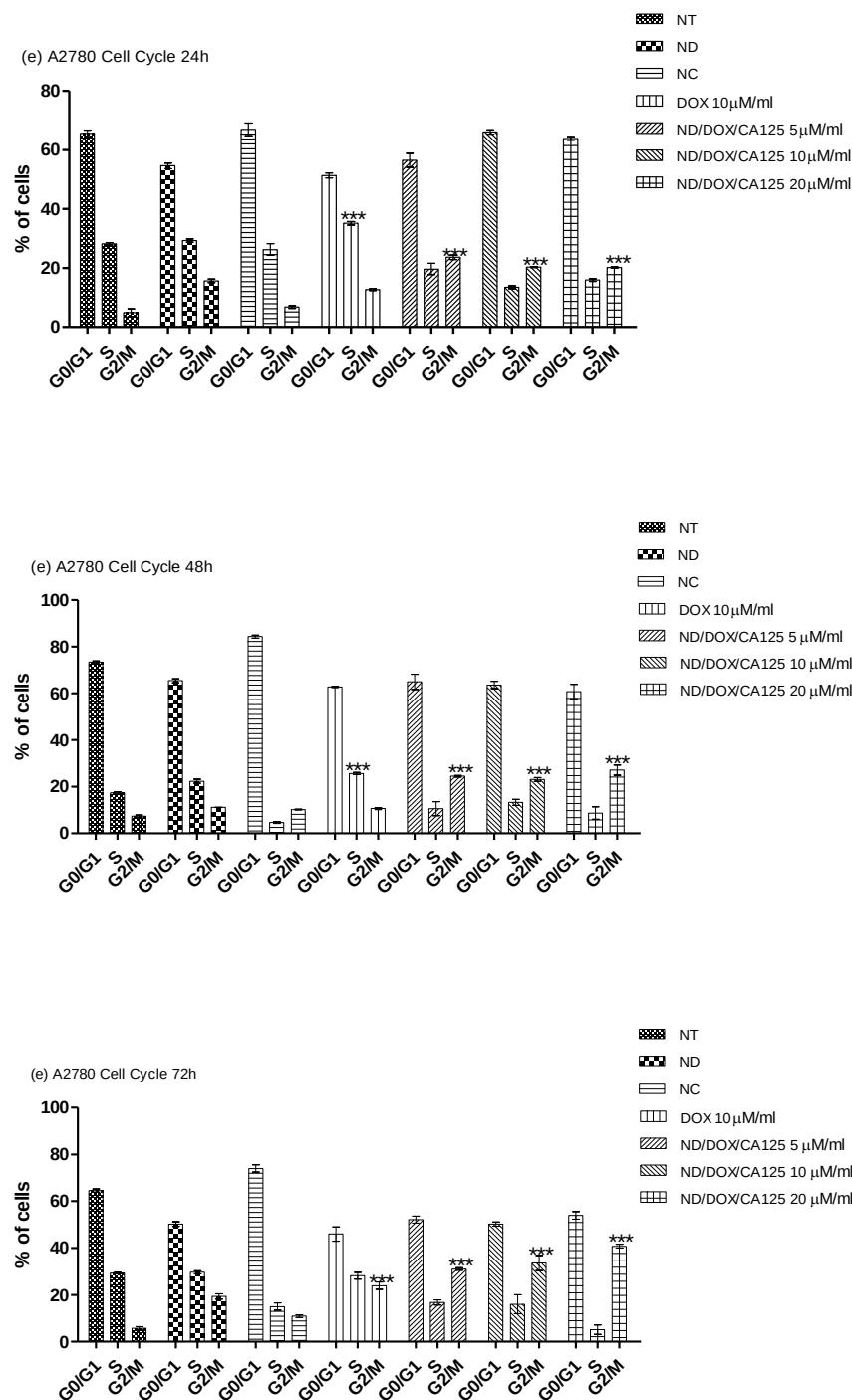


Figure 3.25 e: Cell cycle for A2780 cells after ND/DOX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

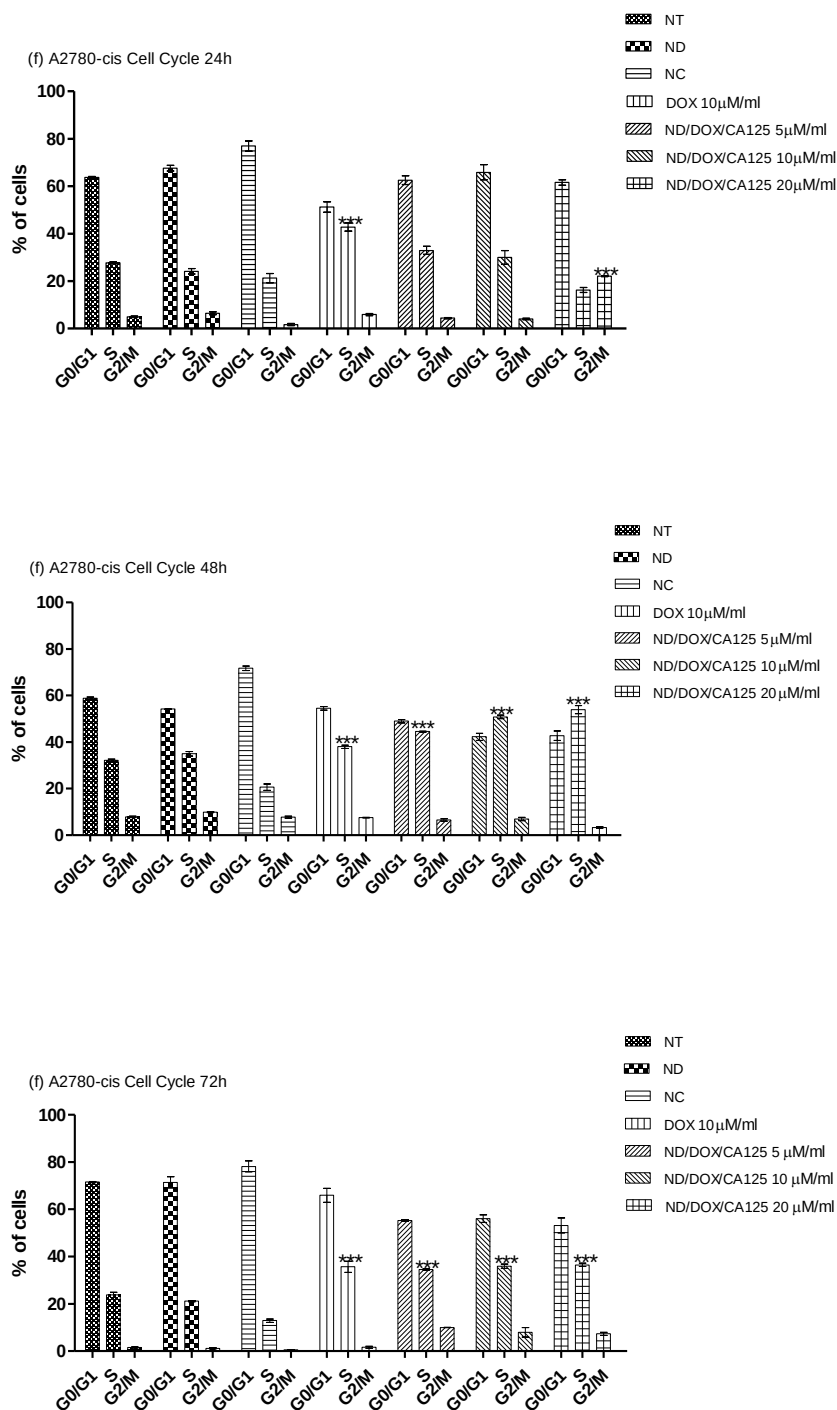


Figure 3.25 f: Cell cycle for A2780-cis cells after ND/DOX/CA125 treatment

Cell cycle analysis was performed using the Cytell™ imaging system after the cells were treated with three different concentrations of the nanocomplex (ND/DOX/CA125) and incubated for three different intervals (24h, 48h, and 72h). Data shown is normalised to an untreated control and presented as mean values of three independent experiments performed in triplicate. 2-way ANOVA with Bonferroni post-test analysis was carried out on the experimental data. Statistical significance between treated and untreated cells represented by “*” symbol, for $p < 0.05$; “**” $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) bare nanodiamonds, (NC) Nocodazole, (DOX) doxorubicin.

3.5 Summary of cell viability reduction, MMP changes and CMP changes comparing the nanocomplexes

To determine the most effective nanocomplex across the cell lines, a comparison of cell viability reduction, MMP changes and CMP changes was performed at the all-time points and with three different concentrations of the nanocomplexes and drug alone as shown in the colorimetric heat maps (figure 3.26, 3.27, and 3.28).

Ovarian cancer cells that expressed higher levels of CA125 responded better to treatment with nanocomplexes conjugated with CA125 rather than HE4 as can be seen in Figure 3.26, where OAW42, OVCAR-8, A2780, A2780-cis and HIO-80 cell lines responded better.

The nanocomplexes conjugated with HE4 induced a higher cytotoxic effect in cells that expressed higher levels of HE4 as can be seen for the SKOV-3 cells when treated with the nanocomplexes conjugated with HE4 (figure 3.26 and 3.27).

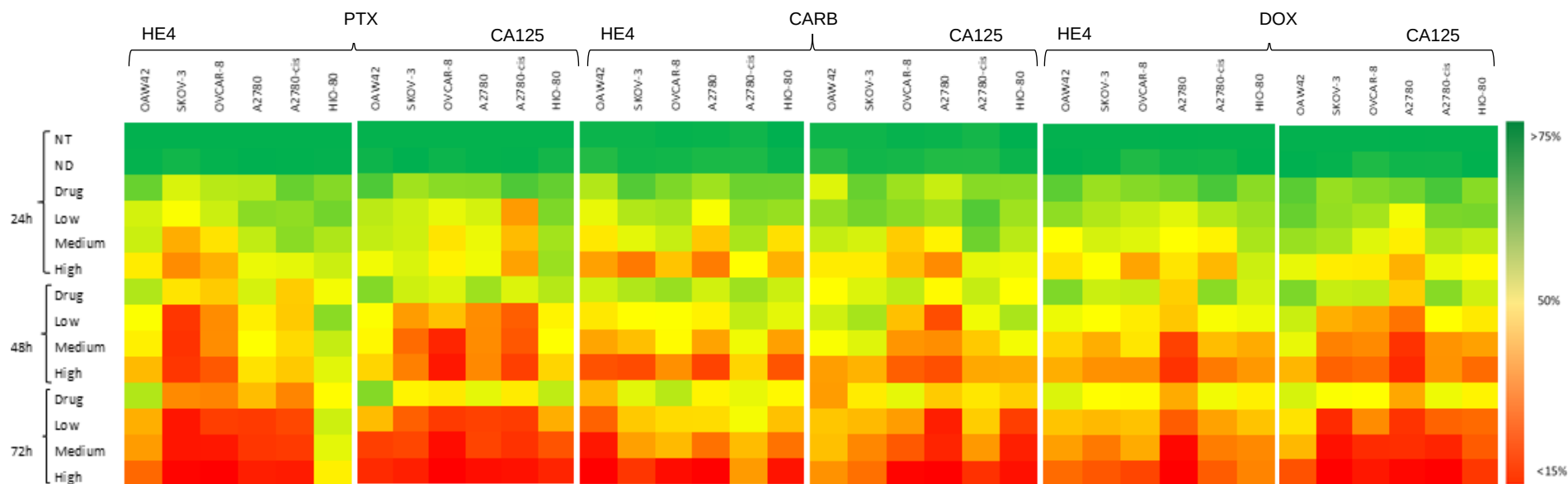


Figure 3.26: A comparison between the cellular response after treatment with drugs (PTX, CARBO, and DOX) and three concentrations of nanocomplexes (ND/PTX/HE4) and (ND/PTX/CA125), (ND/CARBO/HE4), (ND/CARBO/CA125), (ND/DOX/HE4) and (ND/DOX/CA125).

Heatmap of the high-content imaging-based results illustrating cell viability reduction in human ovarian cancer cell lines (OAW42, SKOV-3, OVCAR-8, A2780, A2780-cis, and the HIO-80 which resembles normal human surface epithelial cells). Cells were exposed to ND (10 μ g/ml), PTX (2.5 nM/ml), CARBO (14 μ M/ml), DOX (10 μ M/ml), and three concentrations of each nanocomplex (ND/PTX/HE4) and (ND/PTX/CA125) Low (1.25 nM/ml), Medium (2.5nM/ml) and High (5 nM/ml), (ND/CARBO/HE4), (ND/CARBO/CA125) Low (7 μ M/ml), Medium (14 μ M/ml) and High (28 μ M/ml), and (ND/DOX/HE4 and ND/DOX/CA125) Low (5 μ M/ml), Medium (10 μ M/ml) and High (20 μ M/ml) for 24h, 48h and 72h. Heatmaps were generated from the analysis of n = 3 experiments. Colorimetric gradient ranges are provided, the green is the lowest cell viability reduction and the red is the highest cell viability reduction.

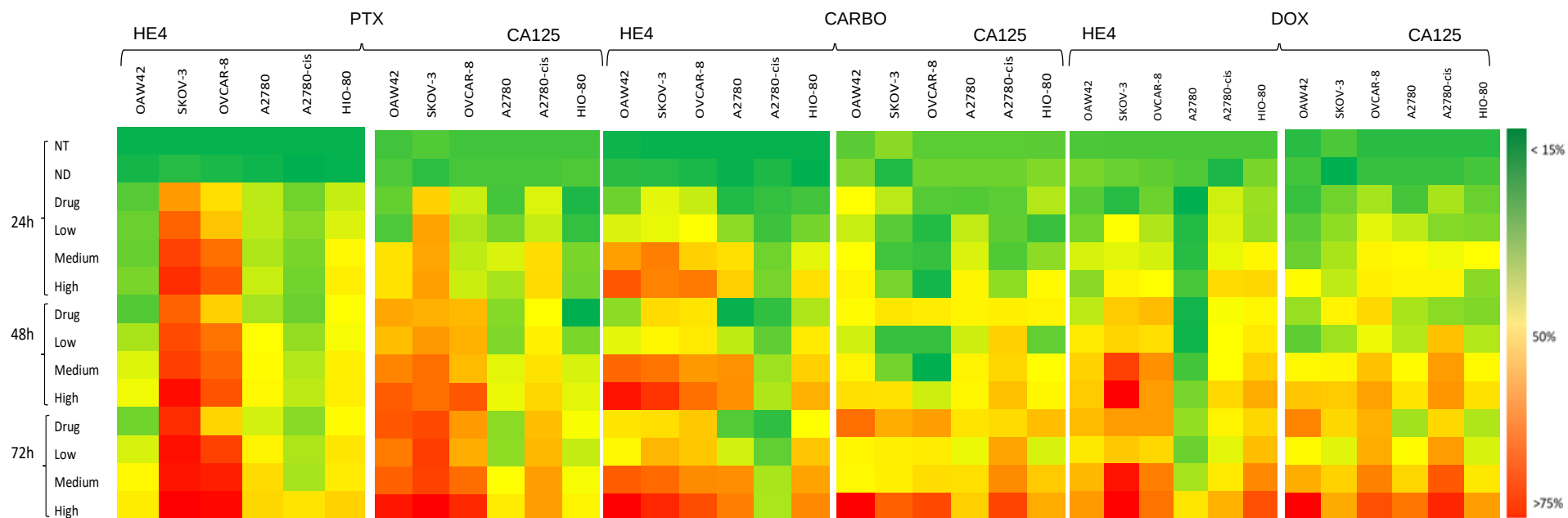


Figure 3.27:A comparison between Mitochondrial Membrane Potential (MMP) changes after treatment with drugs (PTX, CARBO, and DOX) and three concentrations of nanocomplexes(ND/PTX/HE4) and (ND/PTX/CA125), (ND/CARBO/HE4), (ND/CARBO/CA125), (ND/DOX/HE4) and (ND/DOX/CA125).

Heatmap of the high-content imaging-based results illustrating MMP changes in human ovarian cancer cell lines (OAW42, SKOV-3, OVCAR-8, A2780, A2780-cis, and the HIO-80 which resembles normal human surface epithelial cells). Cells were exposed to ND (10 μ g/ml), PTX (2.5 nM/ml), CARBO (14 μ M/ml), DOX (10 μ M/ml), and three concentrations of each nanocomplex (ND/PTX/HE4) and (ND/PTX/CA125) Low (1.25 nM/ml), Medium (2.5nM/ml) and High (5 nM/ml), (ND/CARBO/HE4), (ND/CARBO/CA125) Low (7 μ M/ml), Medium (14 μ M/ml) and High (28 μ M/ml), and (ND/DOX/HE4 and ND/DOX/CA125) Low (5 μ M/ml), Medium (10 μ M/ml) and High (20 μ M/ml) for 24h, 48h and 72h. Heatmaps were generated from the analysis of n = 3 experiments. Colorimetric gradient ranges are provided, the green is the lowest MMP changes and the red is the highest MMP changes.

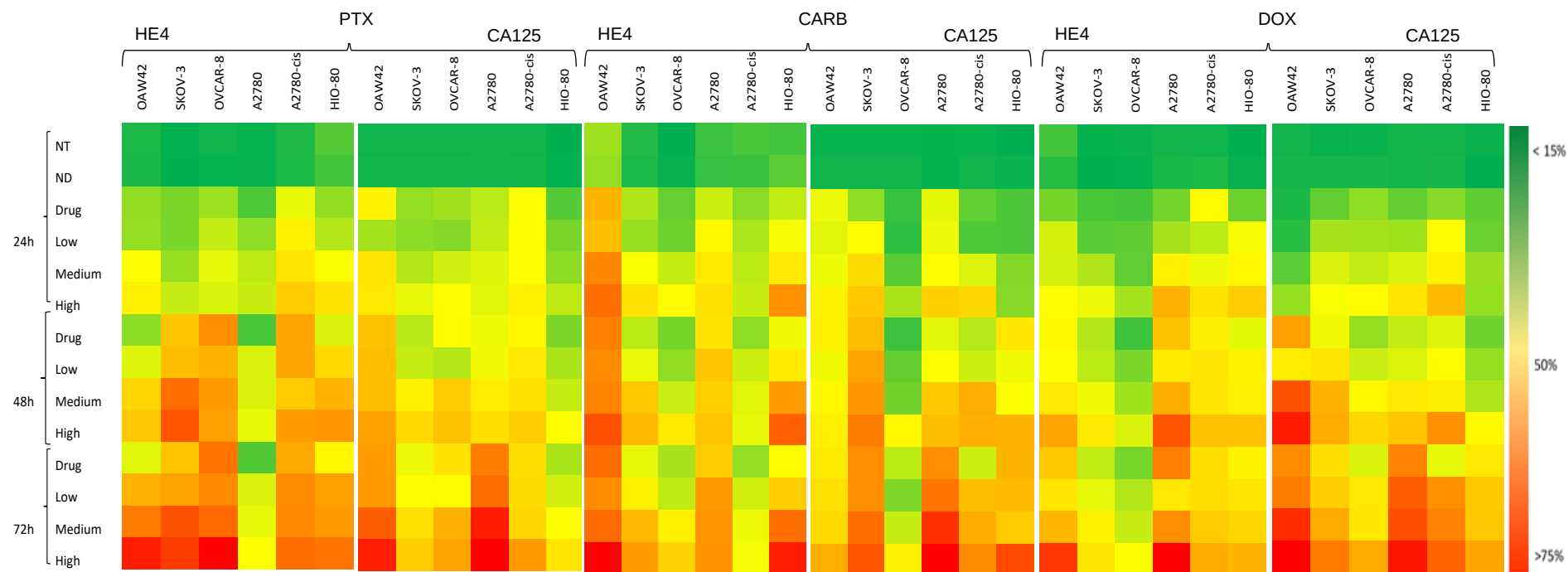


Figure 3.28: A comparison between Cell Membrane Permeability (CMP) changes after treatment with drugs (PTX, CARBO, and DOX) and three concentrations of nanocomplexes (ND/PTX/HE4) and (ND/PTX/CA125), (ND/CARBO/HE4), (ND/CARBO/CA125), (ND/DOX/HE4) and (ND/DOX/CA125).

Heatmap of the high-content imaging-based results illustrating CMP changes in human ovarian cancer cell lines (OAW42, SKOV-3, OVCAR-8, A2780, A2780-cis, and the HIO-80 which resembles normal human surface epithelial cells). Cells were exposed to ND (10 μ g/ml), PTX (2.5 nM/ml), CARBO (14 μ M/ml), DOX (10 μ M/ml), and three concentrations of each nanocomplex (ND/PTX/HE4) and (ND/PTX/CA125) Low (1.25 nM/ml), Medium (2.5nM/ml) and High (5 nM/ml), (ND/CARBO/HE4), (ND/CARBO/CA125) Low (7 μ M/ml), Medium (14 μ M/ml) and High (28 μ M/ml), and (ND/DOX/HE4 and ND/DOX/CA125) Low (5 μ M/ml), Medium (10 μ M/ml) and High (20 μ M/ml) for 24h, 48h and 72h. Heatmaps were generated from the analysis of n = 3 experiments. Colorimetric gradient ranges are provided, the green is the lowest CMP changes and the red is the highest CMP changes.

3.6 Discussion

Our results show that the chemoresistant and chemosensitive ovarian cancer cells (OAW42, SKOV-3, OVCAR-8, A2780, and A2780-cis) and the benign ovarian cells (HIO-80) displayed a good response to the nanocomplex treatments as illustrated by measuring cell viability, mitochondrial membrane changes, cell membrane permeability and cell cycle profiling. The cell cycle assessment was measured as a further biological marker to confirm the cytotoxicity effect seen by measuring CV, MMP and CMP changes after treatment with the nanocomplexes.

All nanocomplexes induced higher cytotoxic effects than the drugs alone. These findings are promising and suggest the future potential of nanotechnology-based treatment of ovarian cancer.

Up to 75% of patients with ovarian cancer have a good response rate to first line chemotherapy treatment (Lengyel 2010, Foley and Del Carmen 2013, Brasseur, Gévry et al. 2017). However, most patients relapse and develop MDR during the course of their treatment. MDR is a major clinical obstacle that critically limits the success rate of chemotherapy in ovarian cancer treatment and is the main cause of treatment failure (Penson, Oliva et al. 2004, Foley and Del Carmen 2013, Wang, Li et al. 2015, Ren, Shen et al. 2016). In recent years research has focussed on less cytotoxic drugs as alternative therapies to enhance the treatment of chemoresistant ovarian cancer (Zhou, Shen et al. 2007, Narvekar, Xue et al. 2014). In this regard, nanotechnology has been recognized as a fundamental tool in cancer research, and the potential of nanocarriers to increase drug efficacy is well described (Ferrari 2005, Heath and Davis 2008, Vergaro, Scarlino et al. 2011).

We hypothesised that targeting ovarian cancer cells with NDs complexed with 1st and 2nd line ovarian cancer drugs (PTX, CARBO and DOX) and coupled with HE4 and CA125 antibodies as docking sites (ND/PTX/HE4, ND/PTX/CA125, ND/CARBO/HE4, ND/CARBO/CA125, ND/DOX/HE4 and ND/DOX/CA125) might improve the treatment strategy of ovarian cancers, overcoming MDR development and reducing side effects.

Our findings indicated that the NDs used in this study were non-toxic, with no effect seen on ovarian cancer cells treated with different doses of bare NDs over several time

incubations. This is in agreement with previous studies by a number of groups who have shown that among all tested nanocarbon materials studied, the uptake of NDs is nontoxic to different cell lines, and shows the highest biocompatibility in ovarian cancer, lung cancer and Hela cells (Vaijayanthimala, Tzeng et al. 2009, Liu, Zheng et al. 2010, Vaijayanthimala, Cheng et al. 2012, Zhang, Hu et al. 2012, Vaijayanthimala, Lee et al. 2015, Eldawud, Reitzig et al. 2016).

Our findings confirmed that HE4 and CA125 are expressed in all studied cells and the expression level of CA125 was higher than HE4 in all cells except the SKOV-3 cells which showed higher HE4 than CA125 expression (section 2.9). The nanocomplexes coupled with a CA125 antibody (ND/Drugs/CA125) induced higher cytotoxic effects in cells with higher CA125 expression than the ones coupled with HE4. In contrast the nanocomplexes coupled with a HE4 antibody (ND/Drugs/HE4) induced higher cytotoxic effects in the SKOV-3 cells which expressed higher HE4 levels than CA125. In general, the complexes coupled with CA125 performed better which may reflect the clinical scenario as CA125 has a higher sensitivity than HE4 but HE4 has higher specificity.

We conjugated HE4 and CA125 antibodies for specific targeting, which may have given our nanocomplex an advantage over other nanocarriers as we observed more cytotoxic effects even at lower concentrations of drugs. This can be clearly seen in the cytotoxicity result chapter (chapter 3) where cells expressing higher HE4 displayed the highest cytotoxicity after treatment with the nanocomplex conjugated with HE4 compared to that seen when CA125 was used as the target and vice versa. The differences between those two nanocomplexes are presented in heat maps (figures 3.26, 3.27 and 3.28).

Using the CA125 and HE4 antibodies to guide the nanocomplexes to target ovarian cancer cells offers a great advantage for treating ovarian cancer, this is the first study reporting the use of HE4 and CA125 as docking sites to target and deliver drugs into ovarian cancer cell lines. This finding is in agreement with previous studies carried out on human ovarian carcinoma cell lines treated with different nanoparticles coupled with different drugs and conjugated with different targets such as antibodies, siRNA, folic acid and peptides (Hun, Zhang et al. 2008, Dong, Mattingly et al. 2009, Cirstoiu-Hapca, Buchegger et al. 2010, Krieger, Eckstein et al. 2010, Delie, Ribaux et al. 2012, Li, Yawata et al. 2014, Salaam, Hwang et al. 2014, Zhang, Guo et al. 2015, Son, Hong et al. 2016, Baghbani and

Moztarzadeh 2017, Lv, Zhuang et al. 2017, Ryu, Baek et al. 2017), and have shown the drugs complexed to the nanocarriers induced higher cytotoxic effects in sensitive and resistant cancer cells.

Previous studies demonstrated nanoscale drug-delivery systems with specific ligands or antibodies were developed to enable NPs to bind specific receptor. These became internalized by endocytosis, enhancing the intracellular accumulation of drugs and induced a significant increase in cytotoxicity (Vergara, Bellomo et al. 2012, Huang, Yi et al. 2014). This is in agreement with our findings which show that all nanocomplexes exhibited more potent anti-proliferative activity against both chemoresistant and chemosensitive ovarian cancer cells. Thus, it seems administration of these lower concentrations of chemotherapeutic agents using biocompatible nanoparticle-based delivery systems with antibodies (HE4 and CA125) for specific targeting could offer the clinical possibility of reducing systemic side effects, overcoming the issue of dose limitation, and reducing the chances of development of the MDR tumour phenotype.

In agreement with our findings where our developed nanocomplexes induced higher cytotoxic effects in A2780 and A2780-cis cells, Huynh *et al.*, 2013, developed three ND based complexes coupled with cisplatin for A2780 and A2780-cis cells treatment. They demonstrated that all three ND-complexes increased the cell toxicity and displayed lower IC50 compared to free cisplatin (Huynh, Pearson et al. 2013).

In other cancer systems, NDs have shown great efficacy when complexed with various targets such as PTX, DOX, and amonafide. They effectively improved drug delivery, facilitated the treatment of chemoresistant cancers, significantly inhibited the cancer cell viability and induced higher cytotoxic effects than drugs alone (Chow, Zhang et al. 2011, Zhang, Lam et al. 2011, Salaam, Hwang et al. 2014, Vijayanthimala, Lee et al. 2015, Zhao, Lai et al. 2016, Zhao, Lu et al. 2018).

The nanocomplexes developed in our laboratory induced cell cycle arrest in S phase and/or G2/M phase in all studied ovarian cancer cell lines. Similarly, a study by Zhang et al., 2015, showed an increase in cell proportion in G2/M phase after OVCAR-5 cells were

treated with mesoporous silica nanoparticles coupled with bevacizumab and conjugated with tumour endothelial marker 1 (BMSN/Ab), which was found to increase the cellular uptake and intracellular drug distribution in OVCAR-5 cancer cells (Zhang, Guo et al. 2015). Another study by Liu et al., 2010 who developed a ND/PTX complex and treated lung cancer (A549) cell lines found that ND/PTX induced a significant cell viability reduction and both mitotic arrest and apoptosis induction. Furthermore, the ND/PTX significantly inhibited the tumour growth of lung cancer cells in xenograft SCID mice (Liu, Zheng et al. 2010).

Kobayashi *et al.*, 2013, showed DOX/dextran-lipid NPs induced 5 - to 10 fold higher antiproliferative activity at the IC₅₀ than free DOX in ovarian tumour cells. DOX/NP also showed two-fold higher activity than poly (ethylene glycol)-modified liposomal DOX in ovarian MDR tumour cells (Kobayashi, Iyer et al. 2013). This is in agreement with our findings where all nanocomplexes induced a higher cytotoxic effect than drugs alone when different biological parameters were investigated.

In addition, as most ovarian cancers will eventually become multidrug resistant, we have shown that targeted delivery of our developed nanocomplexes is capable of overcoming drug resistance by using a lower dose of drug than the clinically relevant dose, which has great potential in the treatment of ovarian cancer. Others have found the use of mesoporous silica nanoparticles complexed with DOX and Bcl-2 siRNA had enhanced efficacy in multidrug resistant A2780/AD human ovarian cancer cells. It could effectively silence the Bcl-2 mRNA and overcome the resistance phenomenon. DOX was internalized to the nuclear region and enhanced the cytotoxic effect (Chen, Zhang et al. 2009).

Others have used different nanomaterials complexed with DOX and found similar cytotoxic effects in breast cancer cells (Peng, Su et al. 2014). They investigated a class of high-performance silicon nanowire (SiNWs) based drug nanocarriers for MDR applications, which was highly efficacious for reversing drug resistance. In particular, drug-resistant cells could be largely inhibited by the nanocarriers and exhibited a huge reduction in cell viability following 72h incubation with the SiNWs-DOX, which was in

sharp contrast to the DOX-treated cells. We have seen similar improved cytotoxicity with our nanocomplex was coupled to lower doses of DOX and HE4/CA125.

Liposomal doxorubicin is already available in the clinic for recurrent ovarian cancer in the form of Doxil® and it would be interesting to compare this to our findings. Doxil® has been shown to decrease some of the toxicity associated with doxorubicin. However, liposomes are not without their disadvantages such as low encapsulation efficiency, poor storage stability, easy oxidation of liposomal phospholipids and short release time (Jurj, Braicu et al. 2017). We do, however, plan to compare Doxil® to our other nanocomplexes in future studies.

In contrast, others have demonstrated that NDs complexed with DOX were less toxic than DOX alone in 2D breast cancer cells models (Zhao, Lai et al. 2016). However, our developed nanocomplexes showed higher cytotoxic effect even at lower concentration in 2D ovarian cancer models.

To date, NDs are one of the most inert materials for drug delivery as other NPs have shown disappointing results. For instance, several publications have reported that silica NPs are suitable for drug delivery. However, a recent publication reports that silica NPs trigger tumour markers and induce tumorigenesis (Xie, Zhou et al. 2019). In addition, many of the above studies used folate/folic acid for targeting but clinical trials have now demonstrated no benefit in using drugs complexed with the folate receptor over conventional drugs. The forward 1 trial in ovarian cancer did not meet its primary endpoint of progression free survival. <http://investor.immunogen.com/node/18696/pdf>. These studies highlight the importance of our study and the use of nanodiamonds as the drug delivery vehicle and also the novel use of HE4 and CA125 as targets

In conclusion, the results of our study showed the benefit of using nanotechnology for ovarian cancer treatment rather than using drugs alone. Cytotoxic effects were evaluated using different biological parameters such as cell viability, mitochondrial membrane changes, cell membrane permeability and cell cycle profiling. The changes in the biological parameters indicated the advantages of functionalised NDs for drug delivery. Moreover,

the nanocomplexes induced a greater effect even at lower drug concentrations. Further work is needed to understand the mechanisms involved in the nanocomplex induced cytotoxicity.

The nanocomplex developed in our laboratory might enhance the uptake and increase the transport of anticancer drugs in ovarian cancer cells and this needs to be further investigated. It is also enhancing the cytotoxicity against both chemosensitive and chemoresistant ovarian cancer cells. To the best of our knowledge this is the first time that NDs have been loaded with first and second line ovarian cancer drugs (PTX, CARBO and DOX) and coupled with HE4 and CA125 antibodies.

Chapter 4

Investigation of the Mechanisms of Action of Nanocomplexes in Ovarian Cancer Cell Lines

4 Investigation of the Mechanisms of Action of Nanocomplexes in Ovarian Cancer Cell Lines

4.1 Introduction

As described in chapter 3, all nanocomplexes induced higher cytotoxic effects in all studied ovarian cancer cell lines than the drugs alone as measured by four biological markers (cell viability, MMP, CMP and cell cycle). Ovarian cancer cell lines used in this work exhibited cell viability reduction and MMP changes after treatment with the nanocomplexes. The MMP changes observed in chapter 3 are an indicator of apoptosis and caspase-3 was selected because it is involved in both apoptotic pathways (intrinsic and extrinsic).

The synchronization between the cell viability reduction and the MMP changes was obvious, suggesting the involvement of the mitochondria in the ovarian cancer cell lines apoptotic responses to the nanocomplexes. It has been widely accepted that the mitochondria play an important role in the process of the cellular apoptotic pathways (intrinsic and extrinsic pathways) (Kerr, Wyllie et al. 1972, Fladmark, Brustugun et al. 1999, Elmore 2007, Tsujimoto and Shimizu 2007). Both apoptotic pathways involve the activation of a series of cysteine proteases collectively known as caspases (Nicholson and Thornberry 1997). There are at least 14 caspases identified in mammalian cells, which are synthesized as inactive precursor molecules (procaspases) and are converted by proteolytic cleavage to active caspases (Thornberry and Lazebnik 1998). The caspase family are generally divided into two groups: the initiators, which are involved in upstream regulatory events (i.e. caspase 2, 8, 9, 10) (Jänicke, Ng et al. 1998, Sandri, El Meslemani et al. 2001), and the effectors, which are directly responsible for proteolytic cleavage that lead to cell disassembly (caspase 3, 6, and 7) (Porter and Jänicke 1999).

Caspases form a proteolytic network within the cell whereby upstream initiator caspases are activated early in the apoptotic process (caspase-8 and caspase-9) and then activate other downstream caspases such as (caspase-3 and caspase-7). The downstream caspases

are largely responsible for cleavage of many other cellular proteins, leading to the morphological demonstration of apoptosis (Henkels and Turchi 1999, Blanc, Deveraux et al. 2000). Caspase-3 is a member of the caspases family found in the cytoplasm, and probably the best understood of the mammalian caspases in terms of its specificity and role in apoptosis (Porter and Jänicke 1999). It is also widely accepted that caspase-3 activity is controlled by upstream regulatory caspases, such as caspase-8 or caspase-9, which modulate the extrinsic or intrinsic pathways of apoptosis, respectively (Nakagami, Morishita et al. 2001, Movsesyan, Yakovlev et al. 2002, Sarisozen, Dhokai et al. 2016). Caspase-3 was selected for evaluation in this chapter following the evidence from chapter 3. The downstream caspases are largely responsible for cleavage of many other cellular proteins, leading to the morphological demonstration of apoptosis (Henkels and Turchi 1999, Blanc, Deveraux et al. 2000). Therefore, we decided to investigate the downstream activity by examining caspase 3 expression.

Our other findings presented in chapter 3 indicated successful cellular responses (in both chemosensitive and chemoresistant ovarian cancer cell lines) to our developed nanocomplexes. These findings supported our hypotheses that using drug-functionalised nanomaterials might lead to overcoming the drug resistance phenomena. Thus, we aimed to examine the effect of our developed nanocomplexes on one of the molecules known to be communally involved in the development of drug resistance phenomena; P-glycoprotein (P-gp) (Di Pietro, Dayan et al. 1999, Thomas and Coley 2003).

Resistance to a wide spectrum of chemotherapeutic drugs in ovarian cancer is known “multidrug resistance” (MDR) which is a major challenge in chemotherapy treatments (Auner, Sehouli et al. 2010). MDR occurs during or after ovarian cancer treatment and it is caused by several mechanisms including an increase in drug efflux and the inhibition of the apoptosis signalling pathway by the induction of antiapoptotic molecules (Katayama, Noguchi et al. 2014).

The ATP-binding cassette (ABC) transporter proteins are involved in MDR, and they are the key molecules involved in drug uptake and efflux. The human ABC transporters consist of 48 or 49 family members, among these is P-glycoprotein (ABCB1) (P-gp) (Di Pietro,

Dayan et al. 1999, Thomas and Coley 2003). P-gp facilitates the efflux of various anticancer drugs, and its expression is significantly elevated in drug-resistant tumours resulting in the pumping out of various anticancer drugs (Lehne 2000, Katayama, Noguchi et al. 2014). Previous studies revealed that the most important mechanism in MDR cells was their active discharge of drug due to overexpression of P-gp which is the first human ABC transporter that was identified (Johnatty, Beesley et al. 2013, Gou, Liu et al. 2015, Fletcher, Williams et al. 2016).

Other studies that have interrogated the mechanism of cytotoxicity with nanocomplexes have largely reported apoptotic mechanisms and a role for P-gp. (Table 1.3 in the introduction chapter) summarises different nanocarriers being investigated as anti-cancer treatments and the mechanisms by which they induce their effect.

To assess the functional mechanisms involved in the observed increased cytotoxicity outlined in chapter 3, this chapter focussed on assessing the expression of caspase-3 and P-gp to confirm if the observed changes induced by the nanocomplexes were evident at the protein level.

4.2 Methods

Briefly, the cells were exposed to ND and treated with (2.5nM/ml of PTX, 14 μ M/ml of CARBO, and 10 μ M/ml of DOX) and the medium concentration of all nanocomplexes (which appears to induce the desired effect in all cells compared to the cells treated with drugs alone), (2.5nM/ml of ND/PTX/HE4, 2.5nM/ml of ND/PTX/CA125, 14 μ M/ml ND/CARBO/HE4, 14 μ M/ml of ND/CARBO/CA125, 10 μ M/ml ND/DOX/HE4 and 10 μ M /ml ND/DOX/CA125), then incubated for 48h at 37°C and 5% CO₂. Proteins were extracted from all ovarian cancer cell lines as previously described (Chapter-2, section 2.5).

Equal amounts of extracted protein from each treatment were loaded (30 μ g) and separated by 12% SDS-PAGE and transferred onto a PVDF membrane. After blocking with 5% non-fat dry milk, membranes were probed with specific antibodies caspase-3 (1: 1000) or p-gp (1:1000) overnight at 4°C, followed by incubation with the relevant secondary antibody. Antibody binding was detected with the luminol detection reagent as described in section 2.5.7. Western blots were quantified using Image J software after normalizing to corresponding loading controls (β -Actin).

4.3 Statistical Analysis

Western blot data was normalised to β -actin abundance. Total protein was normalised to control and expressed as fold changes. Data are expressed as the mean $\pm$ standard error of the mean (S.E.M.) and were analysed using analysis of variance (ANOVA) with *post hoc* analysis (Bonferroni or Dunnett; GraphPad Prism version 5). A value of $P < 0.05$ was considered statistically significant.

4.4 Results

4.4.1 Evaluation of caspase-3 activation up on ND/PTX/HE4 and ND/PTX/CA125 treatment exposure

ND/PTX/CA125 induced significant increase in caspase-3 expression in all cell lines used in this work compared with PTX alone (Figure 4.1), while ND/PTX/HE4 induced significant increase in caspase-3 expression in all cell lines except A2780 and A2780-cis cell lines (Figure 4.1 a, b, c, and d).

A significant increase in caspase-3 activation was observed in most but not all of the chemosensitive and chemoresistant cell lines upon exposure to the nanocomplexes (ND/PTX/HE4 and ND/PTX/CA125) in comparison to the unexposed (NT) cells (Figure 4.1). This expression was also statistically significant in comparison to the PTX-treated cells. The HIO-80 (control cells) and the A2780 which is a chemosensitive cell line showed the highest increase of caspase-3 expression compared to the other cell lines (Figure 4.1). A2780 cells are the only cells that responded to PTX alone (Figure 4.1 e). As expected, bare ND did not induce activation of caspase-3 in all examined cells.

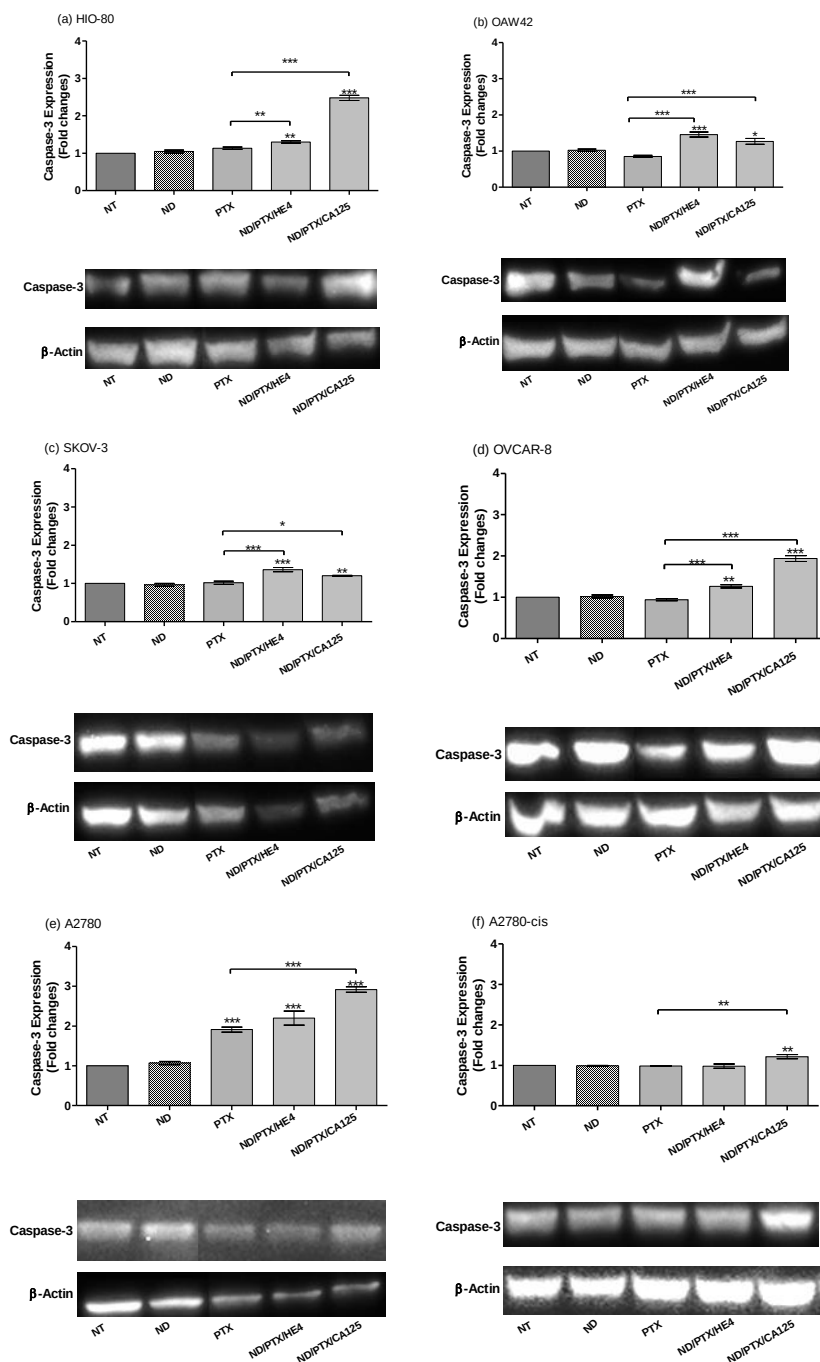


Figure 4.1: The effect of ND/PTX conjugated with HE4 and CA125 on the expression of caspase-3 in all ovarian cancer cell lines.

Caspase-3 was quantified after normalization to β -actin and indicated as fold changes of caspase-3 expression, (a) HIO-80, (b) OAW42, (c) SKOV-3, (d) OVCAR-8, (e) A2780 and (f) A2780-cis. Data shown is normalised to untreated control and presented as mean values of three independent experiments performed in triplicate. Representative data and blots from one of the three separate experiments is shown. 2-way ANOVA with Bonferroni post-test analysis was carried out, normalised to controls, and statistical significance is documented by asterisks "*" symbol, for $p < 0.05$; "***" for $p < 0.01$; "****" for $p < 0.001$. (NT) untreated controls, (ND) nanodiamond, (PTX) paclitaxel.

4.4.2 Investigation of caspase-3 activation in response to ND/CARBO/HE4 and ND/CARBO/CA125 nanocomplexes

The results showed that bare ND did not induce caspase-3 expression in all examined ovarian cancer cells. Significant caspase-3 expression was observed after OAW42 and SKOV-3 cell lines were treated with both ND/CARBO/HE4 and ND/CARBO/CA125. The ND/CARBO/HE4 induced higher caspase-3 expression in SKOV-3 cells than the ND/CARBO/CA125, while OAW42 showed higher caspase-3 expression after treatment with ND/CARBO/CA125 than ND/CARBO/HE4 (Figure 4.2 b and c).

No significant caspase-3 expression was seen in OVCAR-8, A2780 and A2780-cis cell lines (Figure 4.2 d, e and f). The HIO-80 and SKOV-3 cell lines exhibited a significant increase in caspase-3 expression after CARBO alone treatment (Figure 4.2 a and c). OAW42 and SKOV-3 cell lines showed a significant increase in caspase-3 expression after ND/CARBO/HE4 treatment compared to CARBO alone (Figure 4.2 b and c), and ND/CARBO/CA125 induced a significant increase in caspase-3 expression in OAW42 cells compared to CARBO alone (Figure 4.2 b).

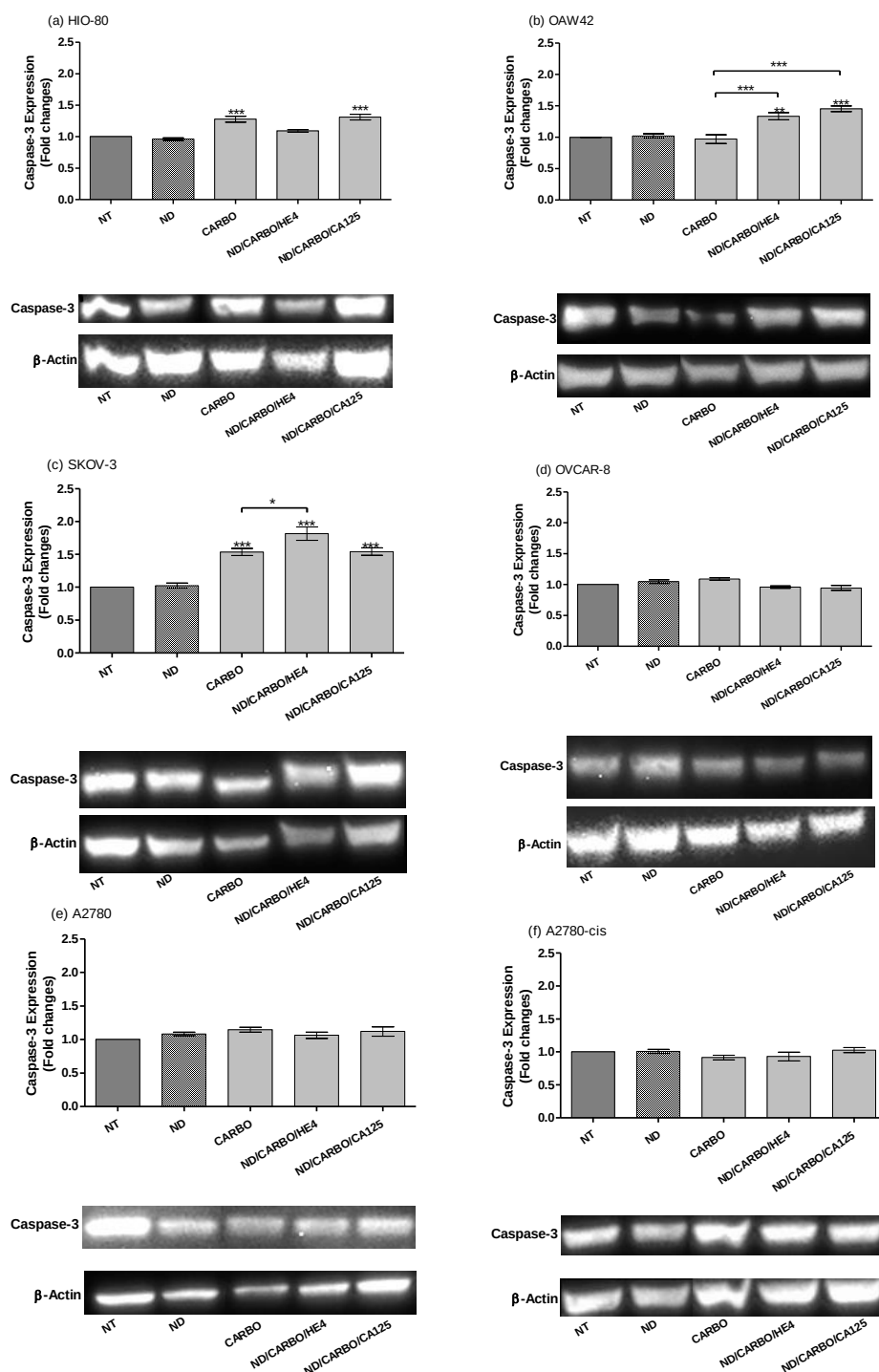


Figure 4.2: The effect of ND/CARBO conjugated with HE4 and CA125 on the expression of caspase-3 in all ovarian cancer cell lines.

Caspase-3 was quantified after normalization to β -actin and indicated as fold changes of caspase-3 expression, (a) HIO-80, (b) OAW42, (c) SKOV-3, (d) OVCAR-8, (e) A2780 and (f) A2780-cis. Data shown is normalised to untreated control and presented as mean values of three independent experiments performed in triplicate. Representative data and blots from one of the three separate experiments is shown. 2-way ANOVA with Bonferroni post-test analysis was carried out, normalised to controls, and statistical significance is documented by asterisks “*” symbol, for $p < 0.05$; “***” for $p < 0.001$. (NT) untreated controls, (ND) nanodiamond, (CARBO) carboplatin.

4.4.3 The effect of ND/DOX/HE4 and ND/DOX/CA125 on caspase-3 activation

Exposure to ND/DOX/CA125 and ND/DOX/HE4 induced a significant caspase 3 expression in SKOV-3, OVCAR-8 and A2780 cell lines in comparison to the unexposed/or untreated cells (Figure 4.3 c, d and e). A higher expression of caspase 3 induced by ND/DOX/HE4 in SKOV-3 and A2780 cell lines compared to ND/DOX/CA125 (Figure 4.3 c and e). In contrast, HIO-80 responded to ND/DOX/CA125 but not to the ND/DOX/HE4 (Figure 4.3 a).

Neither ND/DOX/HE4 nor ND/DOX/CA125 induce caspase 3 expression in OAW42 and A2780cis cells (Figure 4.3 b and f). Interestingly, treatment of these ovarian cancer cells lines with DOX showed no changes in the caspase 3 expression, whereas HIO-80 cells were significantly responded to it (Figure 4.3 a).

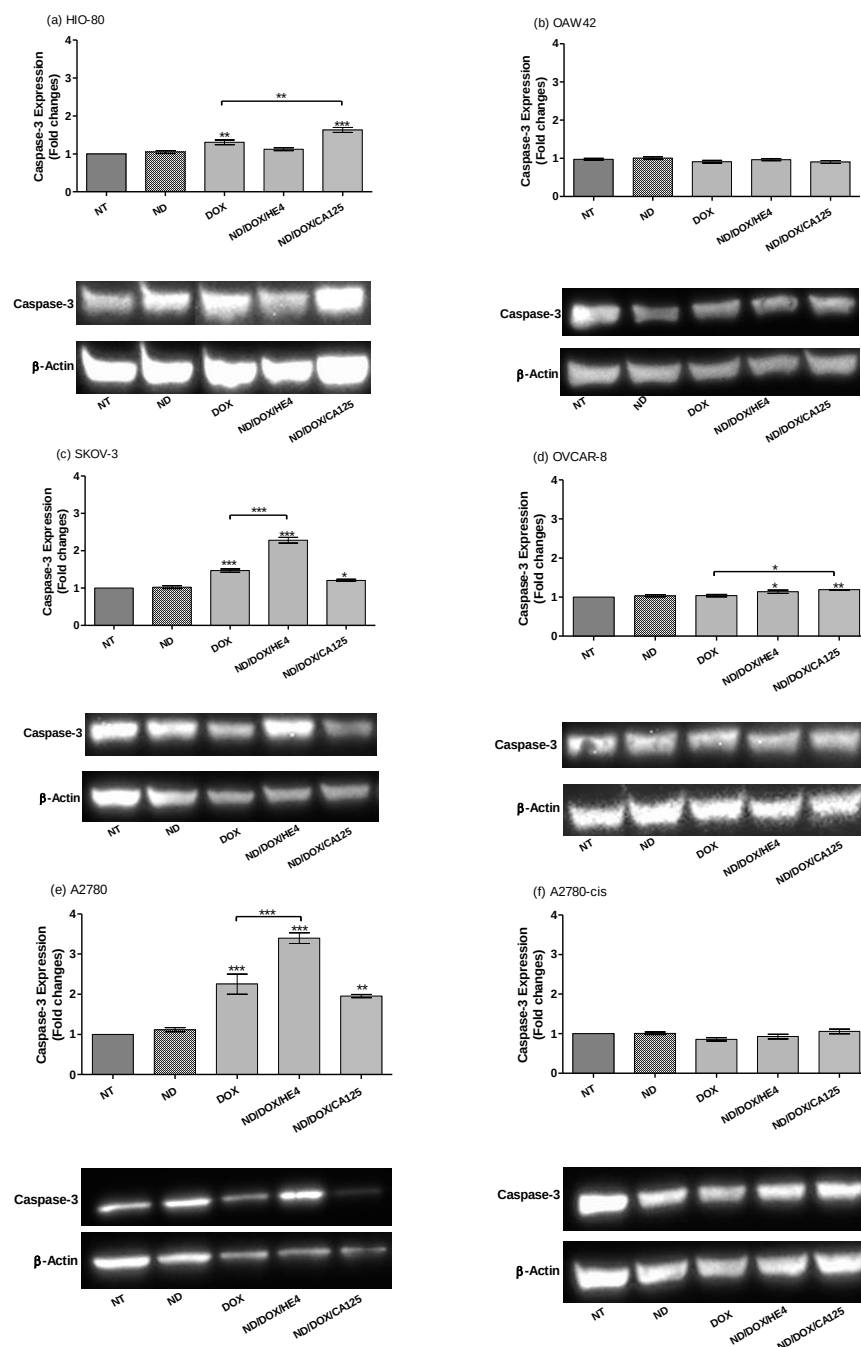


Figure 4.3: The effect of ND/DOX conjugated with HE4 and CA125 on the expression of caspase-3 in all ovarian cancer cell lines.

Caspase-3 was quantified after normalization to β -actin and indicated as fold changes of caspase-3 expression, (a) HIO-80, (b) OAW42, (c) SKOV-3, (d) OVCAR-8, (e) A2780 and (f) A2780-cis. Data shown is normalised to untreated control and presented as mean values of three independent experiments performed in triplicate. Representative data and blots from one of the three separate experiments is shown. 2-way ANOVA with Bonferroni post-test analysis was carried out, normalised to controls, and statistical significance is documented by asterisks “*” symbol, for $p < 0.05$; “**” for $p < 0.01$; “***” for $p < 0.001$. (NT) untreated controls, (ND) nanodiamond, (DOX) doxorubicin.

4.4.4 The nanocomplexes mediated MDR reversal at the P-gp expression level

We further investigated P-gp enhancement in MDR cells upon treatment with our lab developed nanocomplexes. In order to do that, we first examined the P-gp expression in chemoresistant and chemosensitive ovarian cell lines, P-gp was detected only in OAW42 and SKOV-3 cell lines (Figure 4.4).

Upon treatment with chemotherapeutic agents such as PTX, CARBO, and DOX, we observed that P-gp expression was stimulated in OAW42 and SKOV-3 cells and showed 2, 1.2 and 1.2 fold increases in OAW42 cells, and 2.4, 1.1, and 2.5 fold increases in SKOV-3 respectively (Table 4.2).

Interestingly, nanocomplexes including ND/PTX/HE4, ND/PTX/CA125 ND/DOX/HE4 and ND/DOX/CA125 did not stimulate P-gp expression in OAW42 cell lines (Figure 4.5 a, Figure 4.7 a), whereas these cells responded to the ND/CARBO/HE4 nanocomplex and showed a significantly higher level of P-gp expression compared to untreated cells (Figure 4.6 a).

P-gp expression was enhanced and showed statistically significant increases in SKOV-3 cell lines following exposure to ND/PTX/HE4, and ND/CARBO/HE4, ND/CARBO/CA125 and ND/DOX/HE4 and ND/DOX/CA125 nanocomplexes (Figure 4.5 b, Figure 4.6 b and Figure 4.7 b), while no response was observed when these cells were exposed to the ND/PTX/CA125 nanocomplex in comparison to the untreated controls (Figure 4.5).

Interestingly, P-gp was not expressed in OVCAR-8, A2780, and A2780-cis cell lines (Figure 4.4) even upon treatment with the chemotherapeutic drugs (PTX, CARBO and DOX).

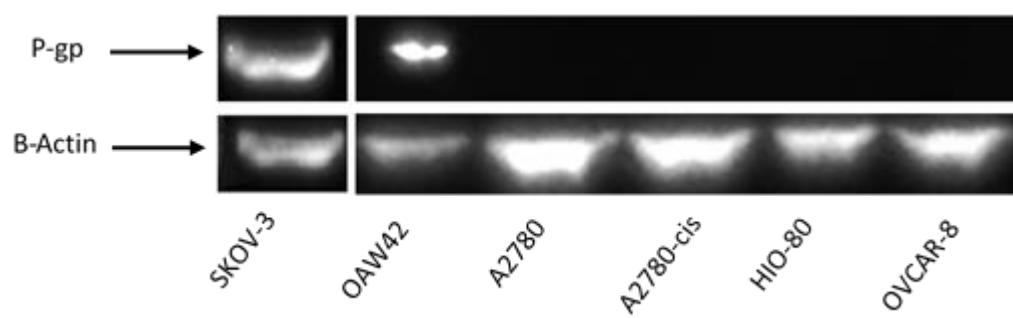


Figure 4.4: P-gp expression in ovarian cancer cell lines.

OAW42 cell line was used as positive control, the other cells were not expressed P-gp.

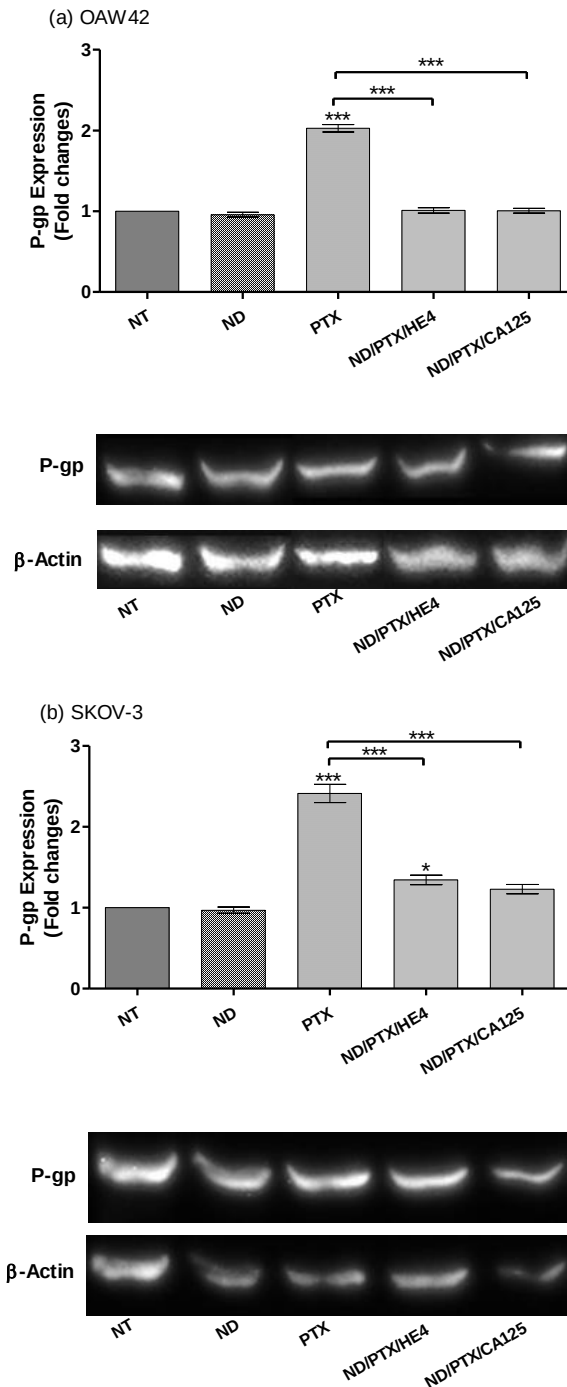


Figure 4.5: The effect of ND/PTX conjugated with HE4 and CA125 on P-gp expression in OAW42 and SKOV-3 cell lines.

P-gp was quantified after normalization to β -actin and indicated as fold changes of p-gp expression, (a) OAW42 and (b) SKOV-3. Data shown is normalised to untreated control and presented as mean values of three independent experiments performed in triplicate. Representative data and blots from one of the three separate experiments is shown. 2-way ANOVA with Bonferroni post-test analysis was carried out, normalised to controls, and statistical significance is documented by asterisks “*” symbol, for $p < 0.05$; and “***” for $p < 0.001$.

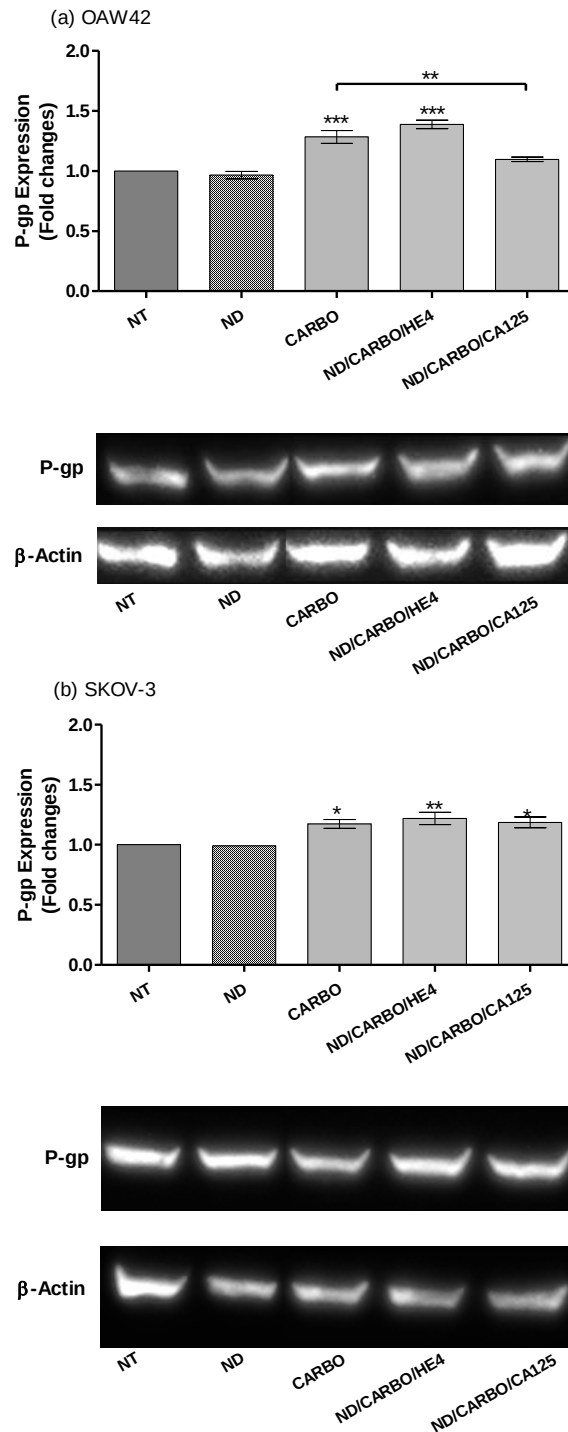


Figure 4.6: The effect of ND/CARBO conjugated with HE4 and CA125 on P-gp expression in OAW42 and SKOV-3 cell lines.

P-gp was quantified after normalization to β -actin and indicated as fold changes of p-gp expression, (a) OAW42 and (b) SKOV-3. Data shown is normalised to untreated control and presented as mean values of three independent experiments performed in triplicate. Representative data and blots from one of the three separate experiments is shown. 2-way ANOVA with Bonferroni post-test analysis was carried out, normalised to controls, and statistical significance is documented by asterisks “*” symbol, for $p < 0.05$; “**” for $p < 0.01$; “***” for $p < 0.001$.

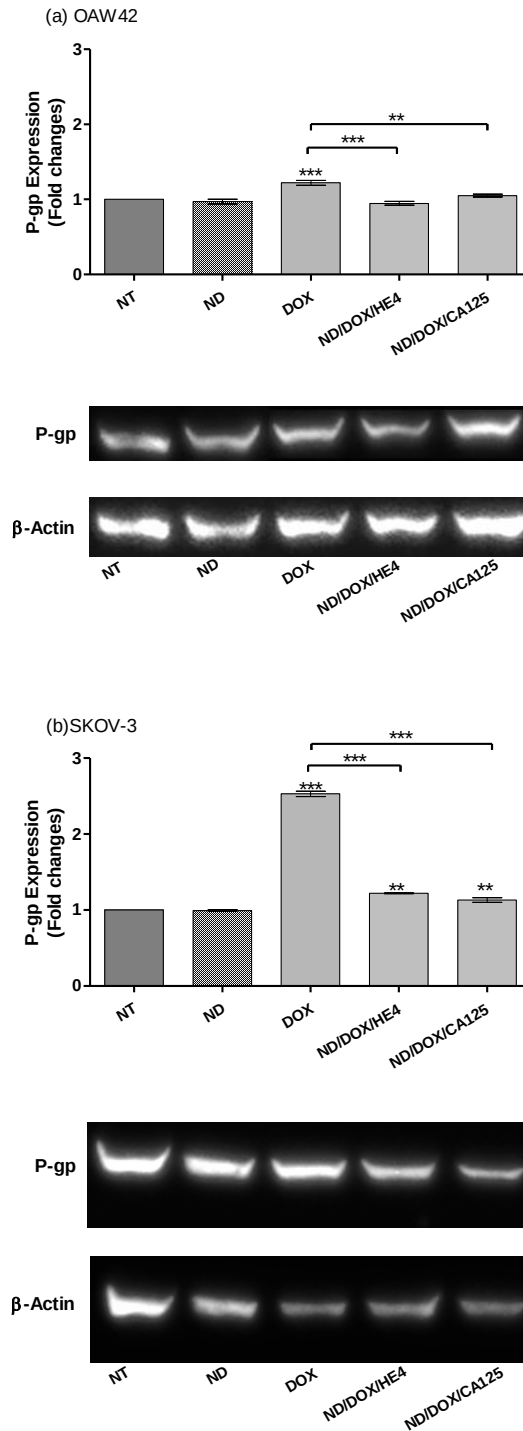


Figure 4.7: The effect of ND/DOX conjugated with HE4 and CA125 on P-gp expression in OAW42 and SKOV-3 cell lines.

P-gp was quantified after normalization to β -actin and indicated as fold changes of p-gp expression, (a) OAW42 and (b) SKOV-3. Data shown is normalised to untreated control and presented as mean values of three independent experiments performed in triplicate. Representative data and blots from one of the three separate experiments is shown. 2-way ANOVA with Bonferroni post-test analysis was carried out, normalised to controls, and statistical is documented by asterisks, “***” for $p < 0.01$; “****” for $p < 0.001$.

Table 4.1: Fold changes of P-gp expression in OAW42 and SKOV-3 cell lines after treatment with drugs and nanocomplexes compared with untreated cells.

Statistical significance between cells treated with drugs alone (PTX, CARBO, and DOX) and cells treated with the nanocomplexes are documented by asterisks, “***” for $p < 0.01$; “****” for $p < 0.001$.

Cells	NT	ND	PTX	ND/PTX/HE4	ND/PTX/CA125
OAW42	1	0.9	2.0	1.0 ***	1.0 ***
SKOV-3	1	0.9	2.4	1.3 ***	1.2 ***
	NT	ND	CARBO	ND/CARBO/HE4	ND/CARBO/CA125
OAW42	1	0.9	1.2	1.3	1.0 **
SKOV-3	1	0.9	1.1	1.2	1.1
	NT	ND	DOX	ND/DOX/HE4	ND/DOX/CA125
OAW42	1	0.9	1.2	0.9 ***	1.0 **
SKOV-3	1	0.9	2.5	1.2 ***	1.1 ***

4.5 Discussion

We demonstrated that the nanocomplexes developed in our lab effectively enhance sensitivity in the chemoresistant ovarian cancer cells via induction of apoptosis and overcoming chemoresistance. The mechanism of apoptosis appears to be mediated by caspase-3 activation.

In this study, we demonstrated that caspase-3 was significantly upregulated after the chemoresistant and chemosensitive ovarian cancer cells were treated with the nanocomplex compared to the drug alone. Similar to our findings, where we saw an increase in caspase-3 when SKOV-3 cells were treated with the nanocomplex, Narvekar *et al.*, 2014, found the use of an All-trans-retinoic-acid - polymer-oil nanostructured carrier induced extensive apoptotic cell death as measured via caspase-3 in an ovarian cancer cell line SKOV-3PR which can withstand high concentrations up to 300 nM of paclitaxel compared to PTX alone treatment (Narvekar, Xue et al. 2014).

The novel use of a specific docking site such as HE4 and CA125 in this study increased the cytotoxicity of the nanocomplex and induced higher caspase-3 expression than the drug alone. Others have used the folate receptor as a docking site to target ovarian cancer cells and found that the use of folate-conjugated capsaicin-loaded lipid nanoparticles induced significant apoptosis via caspase-3 compared to that of the nontargeted nanoparticle system in the SKOV-3 cell line (Lv, Zhuang et al. 2017).

Similarly, other researchers using different nanocomplexes as drug carriers to treat ovarian cancer reported that functionalized nanoparticles induced cellular apoptosis in the OVCAR-5 cell line and ovarian cancer stem cells by increasing the cellular drug uptake and intracellular distribution (Zhang, Guo et al. 2015, Abou-ElNaga, Mutawa et al. 2017). One study used mesoporous silica nanoparticles conjugated with (Ab-/scFv) and coupled with bevacizumab (Zhang, Guo et al. 2015), and the second study demonstrated the use of PTX-loaded folic acid/poly lactic-co-glycolic acid nanoparticles (Abou-ElNaga, Mutawa et al. 2017).

Increases in apoptosis via caspase-3 have also been reported in the literature in breast, lung and glioma cancer cell systems when treated with Oleanolic acid-PLGA, ND-paclitaxel and micelle nanocarriers respectively (Liu, Zheng et al. 2010, Sarisozen, Dhokai et al. 2016, Sharma, Sharma et al. 2017). These findings indicated that the higher cytotoxic effect induced after the ovarian cancer cell lines were treated with different nanocomplexes could be due to the increased cellular uptake, intracellular drug distribution and slow drug release from the nanocomplexes.

Others have found different mechanisms to be involved. In particular, a study on the use of NDs coupled with a high concentration of PTX induced necrosis in cervical and lung cancer cells (Lim, Jung et al. 2016), whereas the NDs coupled with PTX developed in our lab induced apoptosis and did not stimulate P-gp in ovarian cancer treatment. However, our cytotoxicity results indicated that necrosis may also be taking place, but this would need to be confirmed in further studies.

Our results demonstrated that P-gp was stimulated after treatment of ovarian cancer cells with PTX, CARBO, and DOX alone in the chemoresistant OAW42 and SKOV-3 cells only. However, when treated with the nanocomplexes developed in our lab, we did not observe the stimulation of P-gp. This is in agreement with a study carried out by Abou-ElNaga, *et al.*, 2017, using poly (lactic-co-glycolic acid) nanoparticles incorporated with the same anti-cancer drug, PTX, which demonstrated an upregulation in P-gp when ovarian cancer stem cells were treated with free PTX while the level of P-gp was downregulated after these cells were treated with PTX incorporated with folic acid - poly (lactic-co-glycolic acid) nanoparticles (Abou-ElNaga, Mutawa et al. 2017). Our findings are also in agreement with a previous study which demonstrated that doxorubicin and paclitaxel-loaded lipid-based nanoparticles containing the polyoxymethylene 20-stearyl ether overcame P-gp-mediated drug resistance in the P-gp-overexpressing human ovarian carcinoma (NCI/ADR-RES) cell line (Dong, Mattingly et al. 2009).

In addition, we demonstrated that DOX alone stimulates P-gp in chemoresistant ovarian cancer cell lines (OAW42 and SKOV-3), while the nanocomplex incorporated with DOX did not stimulate P-gp. Similarly, previous studies using doxorubicin loaded nanomaterials, doxorubicin loaded lipid-functionalized dextran-based biocompatible nanoparticles and

poly (ethylene glycol) (PEG)-modified liposomal DOX (PLD) in ovarian cancer cells and their doxorubicin-resistant P-gp-overexpressing analogue cell lines, observed that the nanocarriers not only effectively modulate the downregulation of P-gp in MDR cells by providing an alternative route of delivery of doxorubicin to P-gp-overexpressing cancer cells but also may improve the uptake of the nanocomplexes by endocytosis activation (Arora, Jensen et al. 2012, Kobayashi, Iyer et al. 2013).

While many studies have utilised different nanocomplexes, there are very few studies describing the use of nanodiamonds for drug delivery and the mechanisms involved. As outlined in (Table 1.3 in the introduction chapter), there were several studies which evaluated different nanoparticles as drugs carriers for cancer treatment and found similar mechanisms to ours involved in cytotoxicity induction.

In agreement with our findings, a previous study investigated the use of NDs coupled with DOX to treat murine liver tumour and mammary carcinoma models, and demonstrated that they significantly overcame drug efflux and increased apoptosis compared to DOX alone (Chow, Zhang et al. 2011).

In addition, a similar study demonstrated the use of NDs coupled with a different anticancer drug (mitoxantrone) and the result showed enhancement in the sensitivity of drug-resistant breast cancer cells by inhibition of the drug transporter protein (P-gp) after ND-MTX treatment compared to the use of mitoxantrone alone, providing strong evidence for the use of the ND–MTX complex as an effective drug delivery platform (Toh, Lee et al. 2014).

Previous studies have reported that the cellular uptake of drug loaded NPs is an ATP-mediated action. Therefore, NPs are thought to act as a competitive inhibitor to P-gp's action, which would be responsible for the drug uptake increase and MDR reversal in multi-drug resistant cells (Zhou, Shen et al. 2007, Singh, Tammam et al. 2017).

The stimulation of P-gp has also been seen in other cancer systems where, breast cancer and human melanoma cell lines showed higher P-gp expression after chemotherapy

treatment (Kang, Seok et al. 2016, Nemcova-Furstova, Kopperova et al. 2016), which is in agreement with our findings where the drug alone stimulates P-gp expression.

A previous study used gold nanoparticles complexed with DOX for treatment of human hepatoma cell lines and showed efficient release of the anticancer drug, enhancing the cytotoxicity against MDR cancer cells and highlighting the potential of using gold nanoparticles for overcoming MDR in cancer chemotherapy (Gu, Cheng et al. 2012). Our results are in agreement with these findings suggesting that the nanocomplexes facilitated the intracellular accumulation of conjugated drugs (PTX, CARBO, and DOX) in MDR ovarian cancer cell lines without stimulation of P-gp.

Others have demonstrated the importance of P-gp in improving chemosensitivity using nanomaterials complexed with siRNA. One study demonstrated the use of self-assembled nanoscale coordination polymers linked with cisplatin coated with a cationic lipid layer followed by siRNAs adsorption for targeting three MDR genes (survivin, Bcl-2, and P-gp). The result showed down-regulating the MDR genes led to an enhancement of chemotherapeutic efficacy in ovarian cancer cell lines (He, Liu et al. 2015).

In addition, the use of mesoporous silica nanoparticles functionalized with DOX and P-gp siRNA for cervical adenocarcinoma cell lines has been reported previously. The results demonstrated that knock down of P-gp gene expression improved the sensitivity to chemotherapeutic drug (Meng, Liong et al. 2010). These findings are in parallel with ours, as the nanocomplex coupled with DOX enhanced the sensitivity in chemoresistant ovarian cancer cells via non-stimulation of P-gp. These studies further strengthen the central role of P-gp in overcoming chemoresistance.

Although the advantages of using nanocarriers complexed with different drugs to reverse MDR by non-stimulation of P-gp, a number of P-gp inhibitors have been tested in clinical trials. Due to their toxicity, drug metabolism and interaction with other transporters, none of them have been clinically approved yet (Palmeira, Sousa et al. 2012, Syed, Arya et al. 2017, Nanayakkara, Follit et al. 2018).

In conclusion, our developed nanocomplexes improved cytotoxicity in chemosensitive and chemoresistant ovarian cancer cell lines by activation of the apoptosis pathway through caspase-3 upregulation and non-stimulation of the drug transporter protein P-gp. Therefore, the nanocomplexes developed in our lab are a promising platform to effectively deliver anti-cancer chemotherapeutics to chemoresistant cancer cells that over express drug transporter proteins (P-gp). Further work on ovarian tumour explants (*Ex vivo*) is warranted to find if these nanocomplexes are able to deliver drugs to the ovarian tumour explants and induce similar cytotoxic effects to that induced in 2D models.

Chapter 5

***Ex vivo* case study of the nanocomplexes efficacy**

5 *Ex vivo* case study of the nanocomplexes efficacy

5.1 Introduction

Ovarian cancer cell lines within three-dimensional (3D) structures behave more closely to their *in vivo* environment than ovarian cancer cells which have been selected to survive on two-dimensional (2D) tissue culture surfaces (Roskelley and Bissell 1995, Prestwich 2007, Legendre, Froment et al. 2010, Loessner, Stok et al. 2010, Dusinska, Runden-Pran et al. 2012, Cheung and Ewald 2014, Kenny, Lal-Nag et al. 2015). All 2D cultures are associated with specific limitations, such as lack of the tumour microenvironment which has a significant impact on tumour biology and therapeutic response, and the use of established cancer cell lines in drug development studies for many passages, where they may no longer represent the cells in their originating tumour (Cree, Glaysher et al. 2010, Dean, McClendon et al. 2012, Sarafray-Yazdi, Gorelick et al. 2015). These problems highlight the importance of the use of primary cells obtained from tumour tissue at surgery and cultured *ex vivo* in order to bring the interpretation of results from an *in vitro* model of a disease closer to those expected in the *in vivo* condition.

To account for the effect of the microenvironment, 3D *ex vivo* ovarian cancer models are frequently being used as alternative. *Ex vivo* models closely resemble tumour phenotype and heterogeneity and represent the model between *in vitro* and *in vivo*. The main function of 3D culture is to maintain the structural and physiologic balance within the surrounding environment (Centenera, Gillis et al. 2012, Dusinska, Runden-Pran et al. 2012, Centenera, Raj et al. 2013, Werooha, Becker et al. 2014, Liu, Palakurthi et al. 2017, Ricciardelli, Lokman et al. 2018). The *ex vivo* explant assay for predicting response to existing or novel therapeutics has great potential for personalizing cancer treatment and improving ovarian cancer patient survival (O'toole, Sheppard et al. 2001, Dean, McClendon et al. 2012, Centenera, Raj et al. 2013, Saadeh, Norris et al. 2013, Yao, Li et al. 2018).

Traditional chemotherapy has many disadvantages such as nonspecific tumour targeting, high toxicity, and poor therapeutic efficiency (Dean, McClendon et al. 2012, Yao, Li et al. 2018). In order to overcome such limitations, we developed a novel nano-carrier drug

delivery system (nanocomplex). Successful targeting and drug delivery using nanocarriers into tumour explant (*ex vivo*) has been reported previously, where fructose coated NDs complexed with anti-cancer drugs such as amonafide or DOX were successfully delivered into 3D breast cancer spheroid models and induced significant cytotoxic effects (Zhao, Lai et al. 2016, Zhao, Lu et al. 2018).

Here we describe the establishment and utility of an *ex vivo* explant assay to assess drug response using fresh ovarian cancer tissue. As a proof of concept, we assessed response to the standard 1st and 2nd line chemotherapy drugs, paclitaxel, carboplatin, doxorubicin and the novel nanocomplexes developed in our laboratory.

Our results so far in this thesis have indicated that the nanocomplexes induced cytotoxic effects in chemosensitive and chemoresistant ovarian cancer cell lines and the effect appeared to be mediated through activation of the apoptosis pathway and overcoming chemoresistance via non-stimulation of P-gp. To confirm our results described in chapter 3 and chapter 4, we further investigated the effect of our developed nanocomplexes on fresh ovarian tumour tissue obtained from a patient attending the Gynaecological Oncology Service and scheduled for surgery at St James's Hospital.

The aim of this case study was to evaluate if:

1. The nanocomplexes were taken up by the ovarian tumour explants.
2. The nanocomplexes induced a cytotoxic effect in the ovarian tumour explants and to assess the mechanism of cytotoxicity.
3. The nanocomplexes induced the same cytotoxicity effect as the drugs alone.

5.2 Method

5.2.1 Ethical Approval

Tissue samples were obtained from patients recruited through the DISCOVERY bioresource. Having given full and informed consent, patients attending the Gynaecological Oncology Service and scheduled for surgery at St James's Hospital (by Dr Feras Abu Saadeh and his group) were invited to donate tissue samples. Fresh tumour biopsies were obtained following pathology examination. The study was approved by the St. James's Hospital Ethics committee.

5.2.2 Visualisation of the uptake of the nanocomplexes into the ovarian tumour explants

Several ovarian tumour explants from one patient were cultured following surgical resection as described in chapter 2 section 2.6. Briefly, the biopsies were cut into 1 – 2 mm³, and cultured in 96 well plates using supplementary RPMI media for 7 days. To confirm the nanocomplex is taken up by the cells and as we already had a labelled HE4 antibody, we chose this for this aspect of the study. The ovarian tumour explants were treated with the nanocomplexes conjugated with FITC labelled HE4 to determine uptake of our developed nanocomplexes. Cells were fixed with 3% PFA, stained with DNA binding dye (Hoechst) and MMP tracker and imaged using an inverted microscope.

5.2.3 Measurement of Lactate Dehydrogenase (LDH) release in ovarian tumour explants

The cytotoxic effect of the drugs (PTX, CARBO and DOX) and nanocomplexes on ovarian tumour explants was evaluated by measuring LDH release into the supernatant after 48h of treatment using the LDH Cytotoxicity Detection Kit (Cat. # MK401, Takara Bio, USA) and 2% Triton® X-100 was used as positive control. The lactate dehydrogenase (LDH) assay measures cellular enzyme activity as damaged cells release LDH into the supernatant (Šimaga, Osmak et al. 2005, Lee, Lilly et al. 2009, Dusinska, Runden-Pran et al. 2012, Miao, Sheng et al. 2013).

5.2.4 Evaluation of Caspase-3 expression in ovarian tumour explants

Protein was extracted after the ovarian tumour explants were treated with the nanocomplexes and drugs, then caspase-3 expression was measured by western blot as described in chapter 2 section 2.5.

5.3 Result

5.3.1 Uptake of the nanocomplexes in ovarian tumour explants

We demonstrated that nanocomplexes conjugated with FITC labelled HE4 antibody were successfully taken up by ovarian tumour explants as displayed in Figure 5.1 where cancer cells display the localisation of FITC labelled HE4 antibody conjugated to the nanocomplexes. The explant was made up of cancer cells, fibroblast cells and immune cells. No uptake was observed in the fibroblast cells as can be seen by the yellow arrow in Figure 5.1.

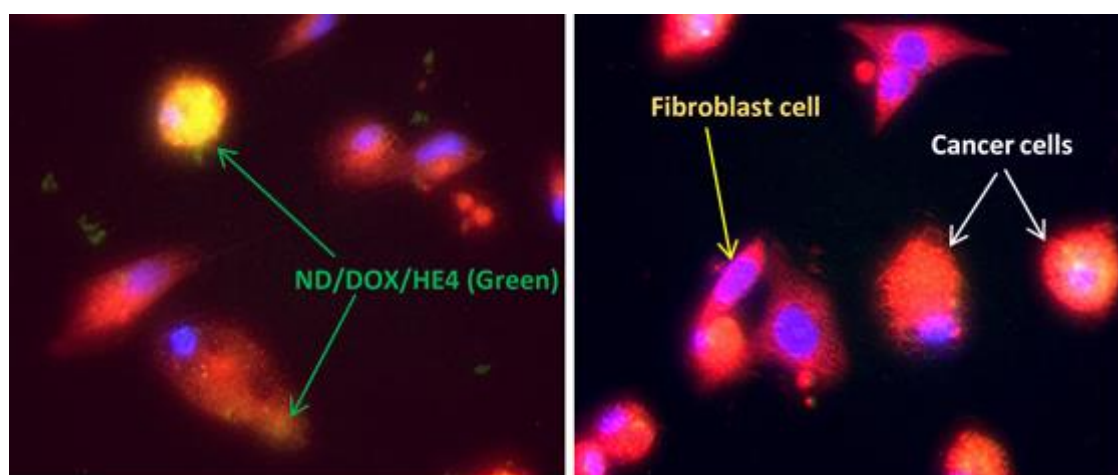


Figure 5.1: Representative images obtained using Inverted microscope.

The nanocomplexes were taken up by ovarian tumour explants marked with green arrows. Ovarian tumour explant cells are marked with white arrows, fibroblast cells are marked with a yellow arrow, nucleus is labelled with Hoechst dye (blue colour) and mitochondria is labelled with mitochondrial tracker dye (red colour).

5.3.2 Evaluation of the cytotoxic effect of nanocomplexes on ovarian tumour explants

The LDH assay was used to assess the ability of the nanocomplexes to induce cytotoxicity in the *ex vivo* ovarian tumour explants. This assay measures release of LDH into the culture medium. The result showed a significant increase of LDH release from ovarian tumour explants after treatment with all nanocomplexes and drugs (PTX, CARBO, and DOX) and the effect was concentration dependent compared with untreated control (Figure 5.2 a, b and c).

Interestingly, the lower concentration of all nanocomplexes induced significant increase of LDH release in comparison to untreated control, and no LDH leakage was observed after the ovarian tumour explants were exposed to bare ND compared to untreated control.

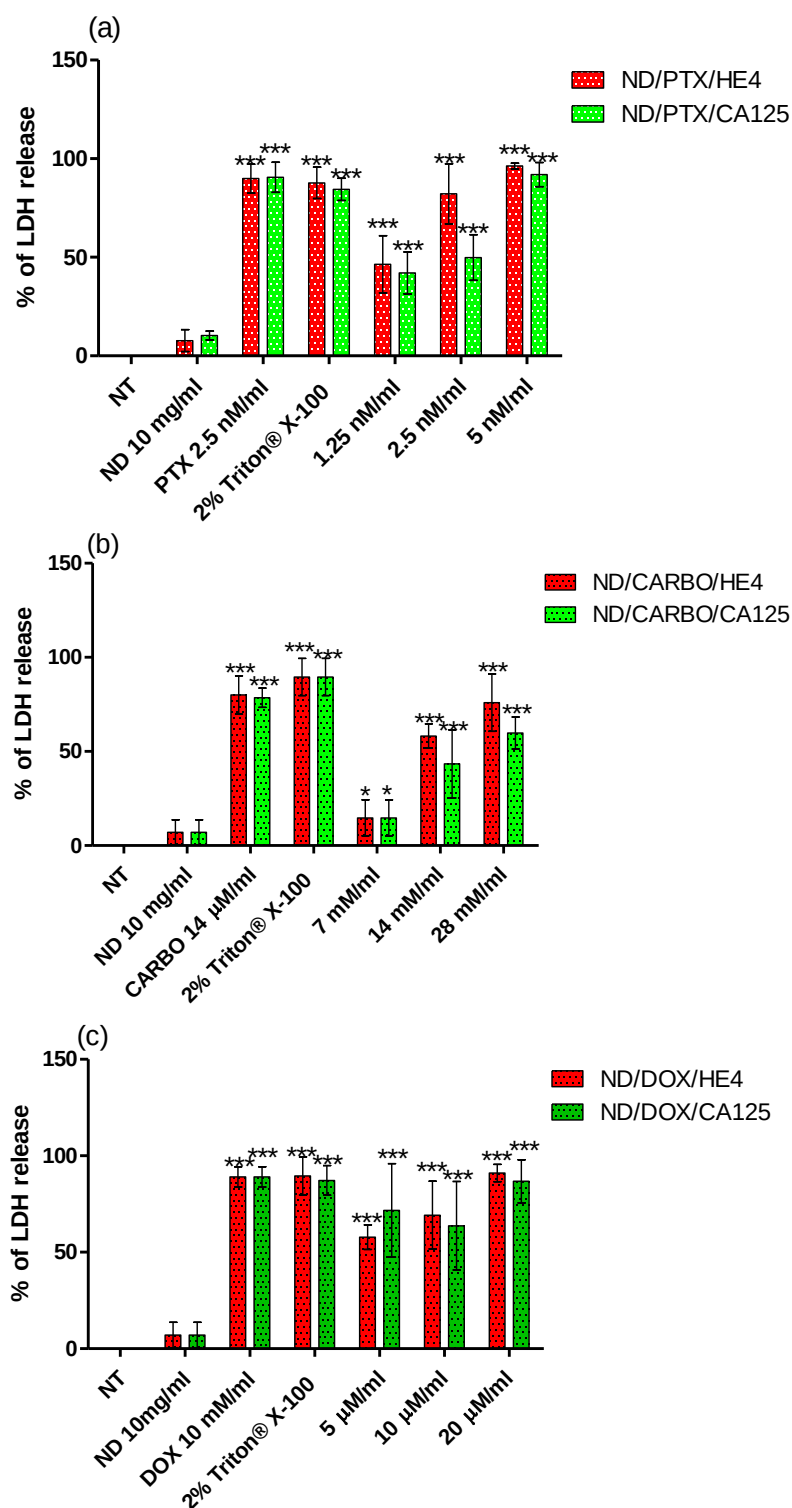


Figure 5.2: LDH release in ovarian tumour explant following nanocomplexes treatments.

(a) percentage of LDH released after the ovarian tumour explants were treated with ND/PTX/HE4 and ND/PTX/CA125. Data shown is normalised to untreated control and presented as mean values of three independent experiments performed in triplicate, (b) percentage of LDH released after the ovarian tumour explants were treated with ND/CARBO/HE4 and ND/CARBO/CA125 and (c) percentage of LDH released after the ovarian tumour explants were treated with ND/DOX/HE4 and ND/DOX/CA125. Red curve represents ND/drug/HE4 and blue curve represents ND/drug/CA125. NT (untreated control) was normalised to (0) and the treatments were normalised to untreated control. The positive control used in this study is (Triton® X-100). Statistical significance is documented by asterisks as “*” for $p < 0.05$ and “***” for $p < 0.001$, the asterisks indicated for the significant of ND/drugs/HE4 and the p-values indicated the significant of ND/drug/CA125.

5.3.3 Caspase-3 activity after ovarian tumour explant treated with drugs and nanocomplexes

Significant caspase-3 activation was seen after the ovarian tumour explants were treated with ND/DOX/HE4, ND/DOX/CA125, ND/CARBO/HE4 and ND/CARBO/CA125 (Figure 5.3). Conversely, no significant caspase-3 expression was seen after treatment with all drugs alone (PTX, CARBO and DOX), ND/PTX/HE4 and ND/PTX/CA125 compared to untreated controls. Interestingly, no increase in caspase-3 expression was seen after ovarian tumour explants were exposed to bare ND (Figure 5.3).

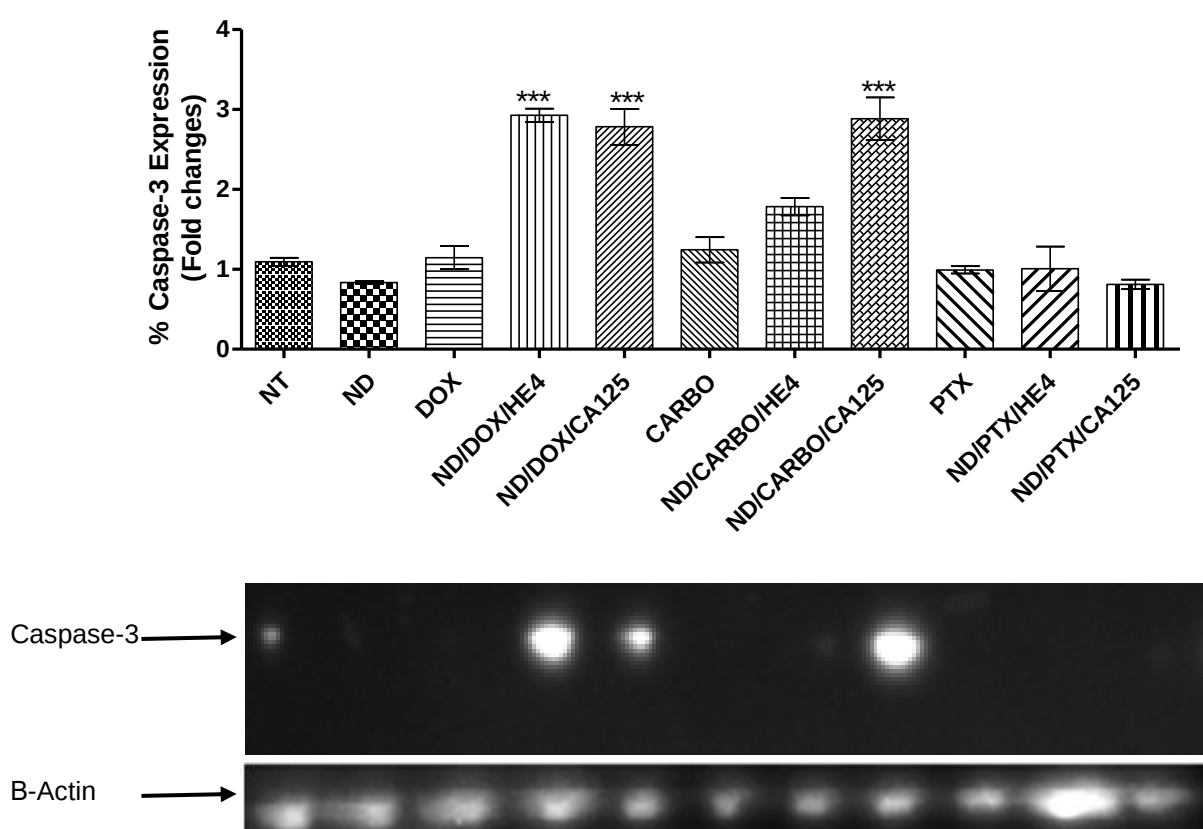


Figure 5.3: Caspase-3 expression in ovarian tumour explant following nanocomplexes treatments.

Caspase-3 was quantified after normalization to β -actin and indicated as fold change of caspase-3 expression. Data shown is normalised to untreated control and presented as mean values of three independent experiments performed in triplicate. Representative data and blots from one of the three separate experiments is shown. 2-way ANOVA with Bonferroni post-test analysis was carried out, normalised to controls, and statistical significance is reported as "***" $p < 0.01$; "****" for $p < 0.001$.

5.4 Discussion

We have reported that LDH activity was elevated in ovarian tumour explants following exposure to drugs (PTX, CARBO and DOX) and the nanocomplex treatments but not in untreated control ovarian tumour explants. We have also found that the nanocomplexes coupled with CARBO and DOX induced caspase-3 upregulation in the ovarian explants compared with the drugs alone.

We demonstrated that the nanocomplexes were successfully taken up by the ovarian tumour explant cells. Others have examined uptake of nanodiamonds complexed with DOX in 2D and 3D breast cancer models and overexpression of GLUT-5 receptors on the surface could play a central role for nanocomplex uptake (Zhao, Lai et al. 2016, Zhao, Lu et al. 2018). Some studies have shown that nanodiamonds are taken up by cells via endocytosis (Vaijayanthimala, Tzeng et al. 2009, Perevedentseva, Hong et al. 2013, Eldawud, Reitzig et al. 2016, Son, Hong et al. 2016). While we demonstrated successful uptake of the complexes into the cancer cells, we did not interrogate the mechanism of uptake and this will be examined by our group in future studies.

Our developed nanocomplexes induced significant cytotoxic effects in ovarian tumour explants which more closely resembles the *in vivo* environment. Others have shown similar findings using a different nanocomplex in spheroid cultures from an ovarian cancer cell line (Perche, Patel et al. 2012). They used doxorubicin-loaded PEG–PE micelles linked to an antinucleosomal antibody (2C5-MDOX) and DOX alone on 3D ovarian cancer cell spheroids. They observed an increase in drug distribution, cell death induction and specific tumour targeting. The cytotoxic effect induced after 2C5-MDOX treatment was measured in a similar way to our study by assessing the LDH released into culture media. Since spheroids like clusters have been detected in peritoneal cavities of ovarian cancer patients and were proposed as metastasis intermediates, intraperitoneal administration of nanocomplexes represent a promising treatment for ovarian cancer.

In addition, previous studies have reported that LDH was significantly upregulated after ovarian tumour explants were treated with chemotherapy (Šimaga, Osmak et al. 2005, Koukourakis, Kontomanolis et al. 2009). This is in agreement with our finding, indicating

that all the chemotherapy drugs used in our study induced significant LDH activity. Similar to ovarian tumour explants, LDH overexpression has also been seen in other cancer systems (breast, colorectal, uterus, urinary bladder, large intestine, endometrial and lung carcinomas) following chemotherapy treatment (Koukourakis, Giatromanolaki et al. 2003, Giatromanolaki, Sivridis et al. 2006, Koukourakis, Giatromanolaki et al. 2006, Koukourakis, Kontomanolis et al. 2009).

We reported the ability of the nanocomplexes to induce cytotoxicity in an *ex vivo* setting by measuring LDH activity which is a marker of cell membrane integrity that is released from damaged cells into the culture media. While no significant differences were seen between drugs and nanocomplexes, we demonstrated that the complex is taken up in the explant system. We also recognise the limitation of this study in terms of numbers and efforts are ongoing to increase the number of explants treated with the nanocomplex.

Our findings also indicated that the nanocomplexes induced significant caspase-3 upregulation in the ovarian tumour explants after the treatment with ND/DOX/HE4, ND/DOX/CA125, while the drugs alone did not. A similar mechanism was observed in a study by Seokyung *et al.*, 2015, using pH-sensitive gold nanoparticles for targeting and treating mesenchymal tumour stem cells. They showed that pH-sensitive gold nanoparticles induced a higher tumour targeting efficacy, increased cytotoxicity via caspase-3 activation and decreased the proliferation rate of the targeted tumour cell lines (Kang, Bhang et al. 2015).

In a different cancer system, Ristorcelli *et al.*, 2008, found the use of nanoparticles derived from SOJ-6 pancreatic cells (Exosomes) for pancreatic tumour cell treatment, reduced cell proliferation of SOJ-6 pancreatic cells and other pancreatic tumour cells (Ristorcelli, Beraud et al. 2008). This correlated with the induction of apoptosis similar to the mechanism observed in our study.

In addition, others used *ex vivo* ovarian tissue explants and treated them with the combination of chemotherapy drugs such as 4-methylubelliferone and carboplatin and showed a significant induction of apoptosis via caspase-3 (Ricciardelli, Lokman et al.

2018). Flanagan *et al.* 2017, also found the use of 5-Fluorouracil based chemotherapy for colorectal tissue explants induced an increase in apoptosis via upregulation of caspase-3 expression (Flanagan, Meyer *et al.* 2017).

Surprisingly, our nanocomplexes coupled with PTX and conjugated with HE4 and CA125 did not induce caspase-3 activation in ovarian tumour explants. This is in contrast to Lilian *et al.*, 2010, who found the use of ceramide nanoparticles in combination with PTX for treatment of ovarian and breast tumour cell lines induced increases in apoptosis through caspase-3 activation (van Vlerken, Duan *et al.* 2010). This could be due to the dose of PTX (1 μ M) coupled with ceramide nanoparticles which is higher than the dose of PTX (2.5nM) coupled with the ND used in our study.

Other studies have reported different mechanisms of cell death such as necrosis. Ormosil nanoparticles loaded with Near Infrared dye and coupled with DOX were used to treat ovarian cancer spheroids and induced necrosis after incubation with functionalised ormosil nanoparticles and exposure to laser, no cytotoxic effect was seen after laser exposure (Nagesetti, Srinivasan *et al.* 2017). Necrosis was not examined as a mechanism of cell death in our model, but it is possible that necrosis is also taking place in the explants and this will be evaluated in future studies.

A study by Zhao *et al* 2016, demonstrated that NDs complexed with DOX were less toxic than DOX alone in 2D breast cancer cells models, but showed higher cytotoxic effect than DOX alone in 3D models (Zhao, Lai *et al.* 2016). However, our developed nanocomplexes showed higher cytotoxic effect even at lower concentration in ovarian cancer cell lines and ovarian tumour explant.

In conclusion, the novel nanocomplexes developed in our laboratory were successfully taken up by ovarian tumour explant cells and specifically delivered the desired drugs into targeted ovarian tumour explants which more closely resemble the *in vivo* situation. This is the first time that NDs complexed with ovarian cancer drugs and coupled with HE4 and CA125 antibodies have shown efficacy in an explant system. The mechanism of apoptosis observed seems to be via the caspase-3 pathway, but further evaluation is needed in a larger cohort of explant samples.

Chapter 6

General Discussion /Conclusion and Future Work

6 General Discussion /Conclusion and Future Work

6.1 General discussion

NDs complexed with ovarian cancer chemotherapy drugs (PTX, CARBO and DOX) and conjugated with HE4 and CA125 antibodies increased cytotoxicity in chemosensitive and chemoresistant ovarian cancer cells. In addition, the nanocomplexes increased apoptosis via upregulation of caspase-3 expression and overcame chemoresistance by non-stimulation of P-gp. Interestingly, nanocomplexes coupled with lower concentrations of chemotherapy drugs induced a higher cytotoxic effect than the clinically relevant dose of the drug alone. In an *ex vivo* model, the nanocomplexes were specifically delivered into ovarian cancer explants and only ND/DOX/HE4, ND/DOX/CA125 and ND/CARBO/CA125 significantly activated caspase-3 and induced apoptosis, while all drugs used in this study did not induce caspase-3 activation in an *ex vivo* model.

Generally, patients with ovarian cancer respond well to first line chemotherapy. However, most patients relapse and develop chemoresistance during the course of their treatment. Chemoresistance is a major clinical obstacle that critically limits the success rate of chemotherapy in ovarian cancer treatment, and it is the main cause of treatment failure (Lengyel 2010, Foley and Del Carmen 2013, Ren, Shen et al. 2016, Brasseur, Gévry et al. 2017). Research has focussed on overcoming this chemoresistance and looking at alternative therapies to enhance the treatment of chemoresistant ovarian cancer (Zhou, Shen et al. 2007, Narvekar, Xue et al. 2014). In this regard, nanotechnology has been recognized as a fundamental tool in cancer research, and the potential of nanocarriers to increase drug efficacy is well described (Ferrari 2005, Heath and Davis 2008, Vergaro, Scarlino et al. 2011).

The application of nanotechnology in the field of cancer has experienced exponential growth in the past few years. Nanoparticles provide opportunities for designing and tuning properties that are not possible with other types of therapeutic drugs and therefore have a bright future as a new generation of cancer therapeutics (Misra, Acharya et al. 2010, Eldawud, Reitzig et al. 2016).

Nanotechnology-based approaches highlighted an improvement in drug delivery for cancer treatment and successes have already been seen with PEGylated liposomal nanoencapsulation of doxorubicin, which was approved by the FDA for use in recurrent ovarian cancer (Gordon, Fleagle et al. 2001, Gordon, Tonda et al. 2004, Engelberth, Hempel et al. 2014). Other nano-formulations have shown promising results in preclinical *in vivo* studies such as polymeric micelles which are moving into clinical trials and highlight novel nanotherapeutic strategies (Engelberth, Hempel et al. 2014, Hare, Lammers et al. 2017).

Although the benefit has been shown of using nanoparticles in cancer treatment, some studies using different nanoparticles such as aluminium oxide, copper oxide, zinc oxide, silica, silver, titanium oxide, single-walled and multi-walled carbon nanotubes for *in vitro* and *in vivo* treatment of different cancer types, have shown that some nanoparticles can induce undesirable toxic effects (Hussain, Hess et al. 2005, Magrez, Kasas et al. 2006, Herzog, Casey et al. 2007, Chen, Yokel et al. 2008, Liu, Yin et al. 2009, Ahamed, Siddiqui et al. 2010, Osman, Baumgartner et al. 2010, Haase, Tentschert et al. 2011, Sun, Li et al. 2011, Alshatwi, Vaiyapuri Subbarayan et al. 2012, Guan, Kang et al. 2012, Sharma, Singh et al. 2012), highlighting the need for nanocarriers with less toxicity.

Nanodiamonds are an emerging class of engineered carbon-based nanomaterial known for their small primary particle size with narrow size distribution and high surface functionalization area with high adsorption capability including bio-conjugation, light weight, chemical and thermal stability, as well as biocompatibility (Schrand, Hens et al. 2009, Eldawud, Reitzig et al. 2016). Their ability to allow multi-surface functionalization by bio-conjugation with nucleic acids, proteins and therapeutic agents gives NDs an attracted interest in biomedical applications such as drug delivery platforms in cancer treatment (Schrand, Hens et al. 2009, Williams, Hees et al. 2010, Mansoorianfar, Shokrgozar et al. 2013, Perevedentseva, Hong et al. 2013, Perevedentseva, Lin et al. 2013, Toh, Lee et al. 2014, Vijayanthimala, Lee et al. 2015, Eldawud, Reitzig et al. 2016). Due to their inert profile, NDs were chosen as the drug carrier for this study.

We hypothesised that targeting ovarian cancer cells with a nanocarrier complex containing nanodiamond loaded with PTX, CARBO and DOX and coupled with HE4 and

CA125 antibodies (ND/PTX/HE4, ND/PTX/CA125, ND/CARBO/HE4, ND/CARBO/CA125, ND/DOX/HE4 and ND/DOX/CA125) might improve the treatment strategy of ovarian cancers, overcoming MDR development and reducing side effects.

It is well documented that HE4 and CA125 are overexpressed in ovarian cancer cells and they have been established as tumour markers for diagnosis and monitoring of ovarian cancer (Boivin, Lane et al. 2009, Theriault, Pinard et al. 2011, Lu, Sun et al. 2012, Zhu, Gao et al. 2013, Chudecka-Głaz, Cymbaluk-Płoska et al. 2014, Lokich, Hill et al. 2015). HE4 and CA125 were selected to be conjugated with NDs for targeting and drug delivery to ovarian cancer cell lines.

We demonstrated that nanocomplexes were successfully taken up by the ovarian cancer cells and ovarian tumour explants. Others have examined uptake of nanodiamonds complexed with drugs in cancer cells and tumour models (Vaijayanthimala, Tzeng et al. 2009, Perevedentseva, Hong et al. 2013, Eldawud, Reitzig et al. 2016, Son, Hong et al. 2016, Zhao, Lai et al. 2016, Zhao, Lu et al. 2018), and have shown that NDs were up taken through energy-dependent clathrin-mediated endocytosis pathway.

We demonstrated in our laboratory that the nanocomplexes induced greater cytotoxic effects in chemosensitive and chemoresistant ovarian cancer cell lines than ovarian cancer chemotherapy treatments alone in terms of CV reduction, MMP and CMP changes and cell cycle arrest. Several studies report the importance of nanotechnology for drug delivery and targeting in cancer therapy and have shown their ability to induce significant cytotoxicity (Chen, Zhang et al. 2009, Dong, Mattingly et al. 2009, Dietrich, Greenberg et al. 2010, Krieger, Eckstein et al. 2010, Arora, Jensen et al. 2012, Delie, Ribaux et al. 2012, Gu, Cheng et al. 2012, Vergara, Bellomo et al. 2012, Vaijayanthimala, Lee et al. 2015, Baghbani and Moztarzadeh 2017).

To assess the functional mechanisms involved in the observed increased cytotoxicity outlined in chapter 3, we further investigated the expression of caspase-3 and P-gp to confirm if the observed changes induced by the nanocomplexes were evident at the protein level. The synchronization between the cell viability reduction and the MMP changes was obvious, suggesting the involvement of the mitochondria in the ovarian cancer cell lines apoptotic responses to the nanocomplexes.

We demonstrated that the nanocomplexes developed in our laboratory effectively enhance sensitivity in the chemoresistant ovarian cancer cells via induction of apoptosis and overcoming the chemoresistance phenomenon. The mechanism of apoptosis appears to be mediated by caspase-3 activation, as it is significantly upregulated after treatment with the nanocomplexes compared to untreated controls. The overcoming of the MDR mechanism seems to be mediated by non-stimulation of P-gp expression, where the nanocomplexes did not stimulate the expression of P-gp in the treated cells compared to the drugs alone which induced significant P-gp stimulation.

Our findings are similar to other studies which have reported the use of nanocarriers to treat ovarian and other cancer cell lines and the results showed different nanocarriers induced cytotoxic effects which appeared to be mediated via apoptosis through caspase-3 activation (Henkels and Turchi 1999, Kolfsooten, Hulscher et al. 2002, Narvekar, Xue et al. 2014, Zhang, Guo et al. 2015, Sarisozen, Dhokai et al. 2016, Abou-ElNaga, Mutawa et al. 2017, Lv, Zhuang et al. 2017), and/or overcoming the MDR induced chemoresistance through P-gp downregulation (Zhou, Shen et al. 2007, Dong, Mattingly et al. 2009, Arora, Jensen et al. 2012, Gu, Cheng et al. 2012, Kobayashi, Iyer et al. 2013, Toh, Lee et al. 2014, Kang, Seok et al. 2016, Nemcova-Furstova, Kopperova et al. 2016, Abou-ElNaga, Mutawa et al. 2017, Singh, Tammam et al. 2017). Currently, no approved drugs are available for clinical use in cancer treatment to reverse MDR by inhibiting P-glycoprotein. A number of P-gp inhibitors have been tested in clinical trials, but their use has been limited for different reasons such as toxicity, interaction with other transporters and drug metabolizing enzymes, resulting in an unpredictable pharmacokinetics (Palmeira, Sousa et al. 2012, Syed, Arya et al. 2017, Nanayakkara, Follit et al. 2018). While P-gp plays an important role, further work is needed to fully understand the mechanism involved in P-gp inhibition.

While the majority of studies have shown the biocompatibility of nanodiamonds, one study by Xing *et al.*, 2011, demonstrated that NDs and oxidized NDs activated DNA repair proteins in mouse embryonic stem cells. The NDs slightly increased the expression of DNA repair proteins (p53 and MOGG) after incubation with embryonic stem cells, while the

oxidized nanodiamonds induced more DNA damage than bare NDs (Xing, Xiong et al. 2011). However, the DNA damage induced by either NDs or oxidized NDs was much less than those induced by multiwalled carbon nanotubes in mouse embryonic stem cells. The accumulation of this nanocomplex also induced apoptosis and activated the tumour suppressor protein p53 after 2 hours of exposure (Zhu, Chang et al. 2007). These results suggest that it is important to consider the genotoxicity of nanomaterials even when they show very little toxicity at the cellular level.

We further investigated the effect of the nanocomplexes on ovarian tumour explants (chapter 5), using an *ex vivo* explant system which behaves more closely to the *in vivo* environment. As a proof of concept, we assessed the response of ovarian tumour explants to the standard 1st and 2nd line chemotherapy drugs (paclitaxel, carboplatin and doxorubicin) and the nanocomplexes developed in our laboratory. Our findings indicated that the nanocomplexes and drugs induced a cytotoxic effect in the ovarian tumour explants, with LDH significantly raised in supernatant even after the treatment with nanocomplexes coupled with lower concentrations of chemotherapy drugs compared to untreated controls. These findings are similar to other studies which have reported the level of LDH overexpressed in ovarian, breast, colorectal and lung tumours after chemotherapy treatments (Koukourakis, Giatromanolaki et al. 2003, Šimaga, Osmak et al. 2005, Giatromanolaki, Sivridis et al. 2006, Koukourakis, Giatromanolaki et al. 2006, Koukourakis, Kontomanolis et al. 2009, Perche, Patel et al. 2012).

We also demonstrated that caspase-3 was upregulated after the nanocomplexes were coupled with CARBO and DOX treatments compared to the drugs alone and untreated control. This is in agreement with a study carried out by Chow *et al.*, 2011, who developed a biocompatible ND-based drug delivery agent (ND/DOX) which enhanced the chemotherapeutic efficiency through improved drug delivery and facilitated the treatment of chemoresistant cancers, such as recurrent mammary tumours and liver cancer (Chow, Zhang et al. 2011). Other studies demonstrated an induction of apoptosis in ovarian, pancreatic and colorectal tumours after chemotherapy treatments (Ristorcelli, Beraud et al. 2008, Flanagan, Meyer et al. 2017, Ricciardelli, Lokman et al. 2018).

In an *in vivo* study, mouse models were injected with NDs complexed with anthracyclines (Epirubicin). The drug was effectively delivered to the tumour cells, with limited drug found outside of the tumour and hence resulting in less side effects. The mouse models that received the nanocomplex lived longer than mouse models that only received the Epirubicin treatment (Wang, Low et al. 2014).

6.2 Conclusion

We have developed a novel nanocarrier using NDs coupled with 1st and 2nd line ovarian cancer chemotherapy (PTX, CARBO and DOX) and conjugated with ovarian cancer biomarkers (HE4 and CA125 antibodies) to deliver desired drugs to targeted cancer cells. The results of our study showed the nanocomplexes developed in our laboratory were successfully taken up by ovarian cancer cell lines and ovarian tumour explants.

Different biological parameters such as cell viability, mitochondrial membrane changes, cell membrane permeability and cell cycle profiling were used to evaluate the cytotoxic effects. The changes of the biological parameters indicated the advantages of functionalised NDs for drug delivery.

NDs complexed with ovarian cancer chemotherapy drugs (PTX, CARBO and DOX) and conjugated with HE4 and CA125 antibodies increased cytotoxicity in chemosensitive and chemoresistant ovarian cancer cells. In addition, the nanocomplexes activated the apoptosis pathway through caspase-3 upregulation and non-stimulation of the drug transporter protein P-gp. Interestingly, nanocomplexes coupled with lower concentrations of chemotherapy drugs induced a higher cytotoxic effect than the clinically relevant dose of the drug alone.

The nanocomplexes developed in our lab are a promising platform to effectively deliver anti-cancer chemotherapeutics to chemoresistant cancer cells that over express drug transporter proteins (P-gp).

We have reported that LDH activity was elevated in ovarian tumour explants following exposure to drugs (PTX, CARBO and DOX) and the nanocomplex treatments, but not in untreated control ovarian tumour explants. We have also found that the nanocomplexes

coupled with CARBO and DOX induced caspase-3 upregulation in the ovarian explants compared with the drugs alone. Interestingly, the lower concentration of all nanocomplexes induced significant increase of LDH release in comparison to untreated control, and no LDH leakage was observed after the ovarian tumour explants were exposed to bare ND compared to untreated control.

The use of NDs complexed to cytotoxic drugs and conjugated with specific targets offers promise in improving the response of ovarian cancers to treatment and requires further investigation with *in vivo* studies.

6.3 Future work

In this thesis, we demonstrated successful functionalisation of NDs with 1st and 2nd line chemotherapy treatments and coupled with HE4 and CA125 antibodies as docking sites to target and deliver this nanocomplex into cancer cells. We demonstrated that the nanocomplexes developed in our laboratory were taken up by ovarian cancer cells and induced cytotoxic effects in both chemoresistant and chemosensitive ovarian cancer cell lines.

Further work is warranted to establish the mechanism by which these nanocomplexes are taken up by the ovarian cancer cells and to establish the cellular compartment that processes the nanocomplex. If the nanocomplex is endocytosed, then it will be important to see how it is presented to the endosomal/lysosomal biogenesis process and whether this is pH dependent. Our group would like to further investigate the mechanisms involved in uptake of the nanocomplex. Mechanisms of sustainability in the cell and ultimate excretion of the nanodiamonds needs to be further investigated. Understanding these mechanisms will enable us to interrogate the observed biocompatibility of nanodiamonds and ensure they are exploited as effective nanovehicles.

We have demonstrated that the nanocomplex induces caspase-3 upregulation and does not stimulate P-gp. Further work is needed to further elucidate the pathways involved in these mechanisms. Other mechanisms of cell kill such as autophagy, anoikis and necrosis may also be playing a role here and require further investigation.

We further demonstrated that the nanocomplexes developed in our laboratory induced cytotoxic effects in an ovarian tumour explant via LDH release and apoptosis. The mechanism of apoptosis observed seems to be via the caspase-3 pathway. Further evaluation is needed in a larger cohort of primary and recurrent ovarian explant samples.

An extension of the present work to *in vivo* studies (animal models) is warranted using NDs as a drug carrier. It is proposed to use the DICER/PTEN double knock out mouse, which is a good model for HGSOC, the most common and lethal subtype of ovarian cancer. Fluorescent probes could be used for tumour targeting and early diagnosis using NDs which may also be constructed as *in vivo* biosensors by attaching photonic crystals that change colour when targeted molecules bind the sensor surface.

We would of course like to test the possibility of combination treatment with two drugs or more to be complexed to NDs and conjugated with specific antibodies, but it was beyond the scope of this thesis.

Nanotechnology has been used in the treatment of recurrent ovarian cancer, in the form of Doxil (Polyethylene glycol PEGylated liposomal doxorubicin). Further work is needed to compare our developed nanocomplexes with Doxil for MDR ovarian cancer.

Clinicians have administered standard chemotherapies directly via the IP route for decades and demonstrated a therapeutic benefit, but patients still exhibit systemic toxicity on IP administration. However, this study has shown the nanocomplexes induced higher cytotoxic effects with lower doses of chemotherapy than the drugs alone, which may improve the toxicity profile via the IP route, offering a significant advance in ovarian cancer therapeutics.

This study has the potential to transform ovarian cancer therapeutics by focussing on targeted drug delivery, bioavailability, reduced bio-dispersion and offering a significant evidence base for nanodiamond driven IP chemotherapy.

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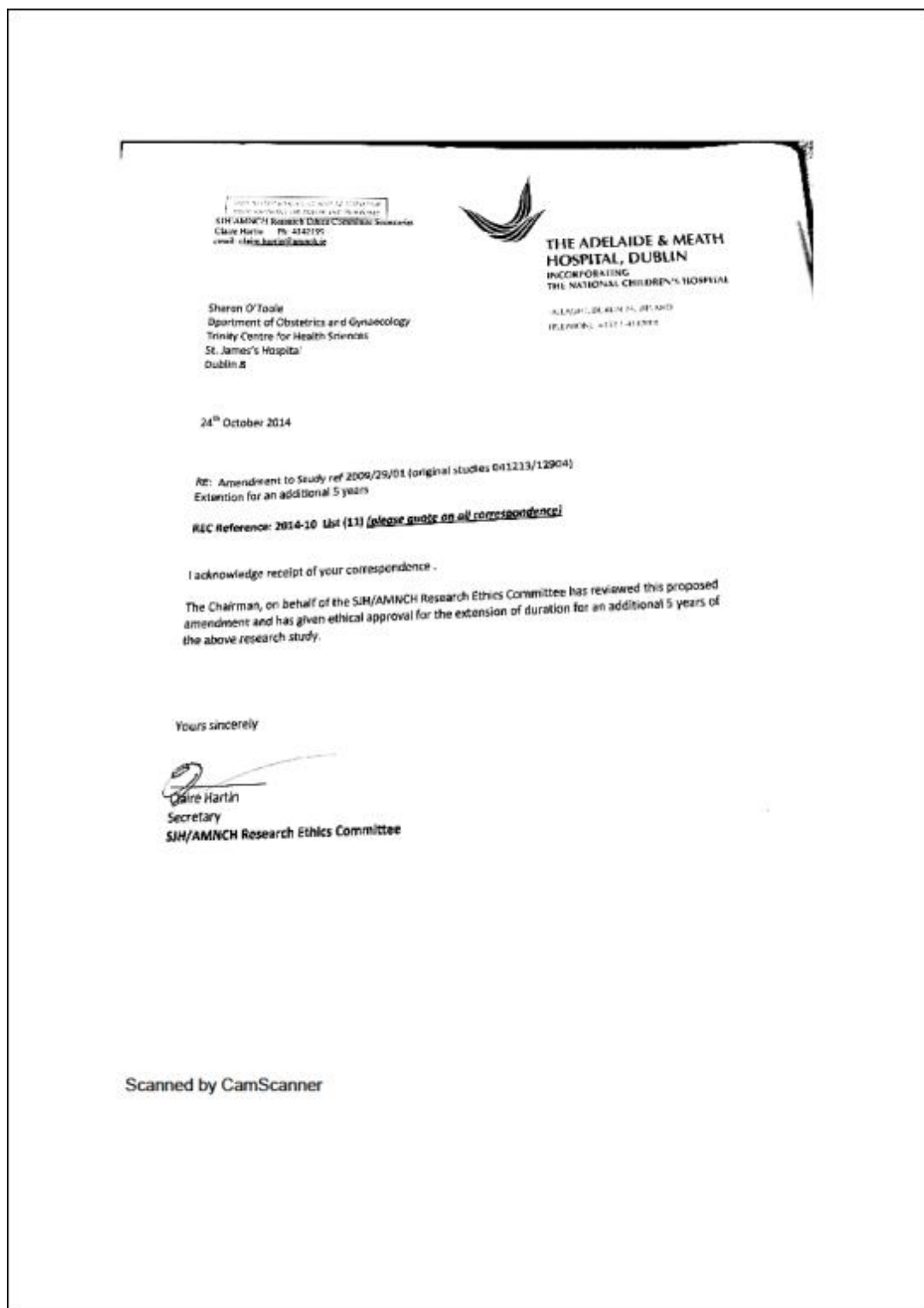
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8 Appendix

8.1 Ethics approval



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Dr. Sharon O'Toole
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Trinity Centre for Health Sciences
St. James's Hospital
James Street
Dublin 8

August 13th 2009

Re: Amendment to Study 041213/12904

Please quote this reference in any follow up to this letter: 2009/29/01

Dear Dr. O'Toole,

Thank you for your letter dated July 15th 2009 and enclosures in which you request ethical approval of an amendment to the above referenced study.

The Chairman, on behalf of the Research Ethics Committee, has given ethical approval to your request to use some of the cervical tissue for explant culture techniques in a study entitled: "Molecular Pharmacological Targeting of a Viral Transforming Protein in Cervical Serum and Tissue". Approval has also been given to extend the Bioresource to collect tissue and serum for the next 5 years and to include the collection of ascites from ovarian cancer patients.

Yours sincerely

Daniel R. Lynch,
Secretary,
SJH/AMNCH Research Ethics Committee

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Professor Brian. L. Sheppard
Department of Obstetrics & Gynaecology
Trinity Centre for Health Sciences
St. James's Hospital
James Street
Dublin 8

April 5th 2007.

Re: Amendments to Studies 06/06/01, 2006/21/06 and 04123/2904

Please quote this reference in any follow up to this letter: 2007/05/17

Dear Professor Sheppard

Thank you for your letter dated January 10th 2007 in which you requested approval for an amendment to the above referenced studies.

The Chairman, on behalf of the Ethics Committee has reviewed your request and given his ethical approval for the use of archival formalin-fixed paraffin embedded tissue that correspond to ovarian cancer biopsies collected (or to be collected) from consented patients. He has also given ethical approval for Dr. Alex Laois to use archival formalin fixed paraffin embedded tissue from primary ovarian cancers and recurrent ovarian cancers which will be used for the examination of proteins.

Yours sincerely,

Daniel R. Lynch,
Secretary,
SJH/AMNCH Research Ethics Committee.

THIS DOCUMENT MUST NOT BE USED FOR
RESCRIPTIONS OR INVOICING PURPOSES

Dan Lynch, Secretary, SJH / AMNCH Research Ethics Committee.
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Simon / Alex

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21st December 2004

**Re: To identify protein patterns and potential biomarkers in the sera of women with
ovarian cancer.**

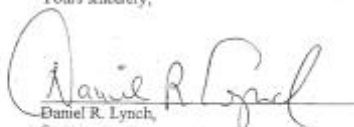
Please quote this reference in all communications regarding this study: 041213/12904

Dear Professor Sheppard,

The SJH / AMNCH Research Ethics Committee reviewed the proposal to conduct the
study under the above title at its meeting on 8th December 2004.

It was agreed to give ethical approval to this study.

Yours sincerely,


Daniel R. Lynch,
Secretary,
SJH / AMNCH Research Ethics Committee.

8.2 Materials

Table 8.1: Consumables

Product	Manufacturer
96 well plates	Sarstedt, Germany
6 well plates	Sarstedt, Germany
12 well plate	Sarstedt, Germany
48 well plate	Sarstedt, Germany
T-25 cell culture flasks, filter cap	Nunc, USA
T-75 cell culture flasks, filter cap	Nunc, USA
Micro 96 well plates	Sarstedt, Germany
BioPointe Pipettes tips	BioPointe Scientific, USA
12 well plates	Sarstedt, Germany
50 mL tubes	Sarstedt, Germany
15 mL tubes	Sarstedt, Germany
Cell scraper	Fisher Scientific, UK
Cryogenic vials	Corning, USA
Serological pipettes	Corning, USA
MicroAmp Optical 8-Cap strip/cap	Applied Biosciences, USA
Hybond-P, Hydrophobic polyvinylidene difluoride membrane (PVDF)	GE Healthcare, UK

Table 8.2: Equipment

Equipment	Manufacturer
Analytical balance	Mettler, AE240, USA
Autoclave	Dixon, UK
Automated pipettes	Medical Supply Company (MSC), IRELAND
Centrifuge	Hettich Zentrifugen, EBA 12R/mikro 22R, UK
High content screening system (HCS)	GE Healthcare, UK
Cytell [™] imaging system	GE Healthcare, UK
Digital imaging	Fusion FX imaging system, Vilber
Freezer (-80)	Thermofisher Scientific, Revco Valure Plus, USA
Gel electrophoresis system	Bio-Rad, Mini-Protean, USA
Heat block	Thermofisher Scientific, USA
Incubator (37 °C, 5% CO₂)	HERAcell 240i, USA
Inverted microscope	VWR, VistaVision [™] , USA
Laminar flow hood	Mason Technology, BioBan, USA
Microplate reader	BioTek ELX 808, USA
pH meter	Mettler-Toledo Inc., MP320, USA

