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Translational studies to support the use of pitavastatin as a cancer therapeutic

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Abstract

Ovarian cancer is the fifth leading cause of death associated with cancer. Initially, the disease responds to surgical cytoreduction, followed by chemotherapy. The primary response rate for chemotherapy is about 80%. Unfortunately, most patients relapse and tumours eventually become frontline therapy refractory. At this point, the lack of widely successful treatments contributes to a low 5-year survival. New therapeutic agents or therapy approaches are therefore required. Statins exert anticancer modality by cause cancer cell death by preventing the synthesis of geranylgeraniol an intermediate of mevalonate pathway. Moreover, we found that exogenous geranylgeraniol can suppress the pro-apoptotic activity of pitavastatin *in vitro.* Thus, suggesting that strict dietary regimen should be maintained to achieve correct pitavastatin therapeutic efficacy. Likewise, supplementation of diet with geranylgeraniol observed to suppress the pitavastatin-induced regression of ovarian cancer xenografts in mice. Several human foods have already been shown to contain geranylgeraniol. We tested 30 organic solvent foods extracts to see their ability to suppress anticancer activity of pitavastatin *in vitro*. The foods included eight oils, several types of solid foods and eleven fruits and vegetables. The IC_{50s} of growth inhibition of pitavastatin were (5.2 μM, 8.2 μM) for Ovcar-4 and Fuov1 ovarian cancer cell line respectively. Pitavastatin anticancer activity is blocked completely by adding of geranylgeraniol or solvent extracts from sunflower oil. Some food extracts from lettuce, corn oil, ground nut oil, grape seed oil black bean and oats partially blocked the effect of pitavastatin, whereas the other foods did not. This research identified several foods apparently lack geranylgeraniol and which patients could eat while enrolled in clinical trials of pitavastatin.

Among the most effective and commonly used cancer treatment agents are natural and synthetic compounds that disrupt the dynamics of microtubules. However, there is still a lack of reliable markers that disrupt the dynamics of microtubules can decide the sensitivity of cancer cells to targeting agents of microtubules and play a role in tumour cell resistance to these agents. This growing family of microtubule-associated proteins (MAP) includes products from oncogenes`

apoptosis regulators, suggesting that altering the dynamics of microtubules may be one of the critical events in tumour origin and tumour progression. The objective of this study is to integrate microtubule-targeting therapies and relevance with highlight MAP7-tubulin-pitavastatin interactions as a new avenue for isoprenoid-related activity to inhibit mevalonate production. We found highly express of MAP-7 in sensitive cells to pitavastatin (OVCAR-8 & OVSAHO) and the sensitivity is decreased after gene transfection of MAP-7 especially in OVSAHO cell line while no significant changes with KIF5-B on the sensitivity of cancer cells. Interestingly, we noticed a significant change in tubulin level after course time of treatment (24,48,72h) of ovarian cancer cell lines with single /double IC_{50} of pitavastatin. Consequently, we tried the CNP role in rescuing of mevalonate pathway which show significant change as well, based on the available evidence, we suggest that the isoprenoid (GGOH & FARNSOL) has important role in microtubules changes at its carboxyl-terminals but does not binding directly, CNP make association between isoprenoid and Cytoskeleton of cancer cell.

Repurposing statins for use in oncology is an attractive strategy, while legitimate concerns about the drug's potential for myopathy. In addition, certain pharmacological agents inhibiting the ovarian cancer cell therefore we evaluated causes of ovarian cancer cell death synergistically. To identify additional drugs that could interact synergistically with pitavastatin, it was identified ivermectin that potentiated pitavastatin activity and/or had notable activity as a single agent. In several cell growth and viability assays, this study confirmed the synergistic interaction between ivermectin and pitavastatin. These data suggest that inhibiting drug combinations can increase the pitavastatin therapeutic window and offer potential treatment for ovarian cancer.

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Abbreviation

ATM	Ataxia-telangiectasia mutated
ATR	Ataxia telangiectasia and RAD3-related
BER	Base excision repair
Cdks	Cyclin dependent kinases
CRH	Corticotrophin-releasing hormone
DRC	Dose response curve
ECACC	European collection of authenticated cell cultures
ERCC1	Excision repair cross-complementing group 1
FDPS	Farnesyl diphosphate synthase
FOH	Farnesol
FTase	Farnesyl transferase
GAPs	GTPase activating proteins
GDIs	Guanine nucleotide dissociation inhibitors
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GGOH	Geranylgeraniol
GGPPS	Geranylgeranyl pyrophosphate synthase
GGTII-β	Geranylgeranyl Transferase-II beta subunit
GGTI-β	Geranylgeranyl Transferase-I beta subunit
GR	Glucocorticoid receptor
GTP	Guanosine-5-triphosphate
HIDS	Hyperimmunoglobulinaemia D syndrome
PPC	Primary peritoneal carcinoma
GnRH	Gonadotropin-releasing hormone
FSH	Follicle-stimulating hormone
LH	luteinizing hormone
LMP	Borderline low-malignant potential
HGSC	High grade serous ovarian cancer
PPC	Primary peritoneal carcinoma
EOPPC	Serous surface papillary carcinoma, extra-ovarian and primary peritoneal carcinoma
FR	Folate receptor alpha
PFS	Progression-free survival
LGSOC	low-grade serous OC
CDK	cycline-dependent kinase
MDSCs	myeloid-derived suppressor cells
IGF	Insulin-like growth factor
FAK	Focal adhesion kinase
TNF-α	tumour necrosis factor-alpha
PCOS	Polycystic ovary syndrome
PID	pelvic inflammatory disease

IUD	Intrauterine device
HRT	Hormone replacement therapy
ET	Oestrogen therapy
BMI	Body mass index
SNPs	Single nucleotide polymorphisms
IARC	International Cancer Research Agency
GSTM 1	glutathinine S-tansfase M1
GSTT1	GSS-transferase T1
NSAIDS	non-steroidal anti-inflammatory drugs
OSE	ovarian surface epithelium
Erk	extracellular signal-regulated kinase
HNPCC	Hereditary non-polyposis colorectal cancer syndrome
MOC	mucinous *ovarian carcinoma*
PET	positron emission tomography
CT	computed tomography
CTR1	copper transporter-1
DSS	Disease-specific survival
ATCC	American Tissue Culture
RIPA	Radioimmunoprecipitation assay

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Chapter 1:

Introduction

1. Introduction

1.1. Female Reproductive System

The female reproductive system consists of organs outside and inside the body. The uterus, ovaries, and fallopian tubes made up the internal organs used in the reproductive system of women. The ovaries develop, store and release needed eggs for reproduction. An ovulated egg passes across the fallopian tube and down the uterus. It may be fertilised by sperm during this period, implant into the uterus lining and grow into a foetus. [1].

1.1.1. Anatomical structure of ovaries

The ovaries are paired organs situated close to the wall on each side of the minor pelvis. The uterus, fallopian tubes and the ovaries, which are almost completely covered with peritoneum, are suspended by the peritoneal ligaments in the lower space of pelvis that is also occupied by the ileum and sigmoid colon and fill the cavity of pelvis completely. The suspensory ligament of the ovaries, which is called mesovarium, consists of two layers of peritoneum, the posterosuperior layer of the broad ligament, in addition the second layer continuous coated epithelium of both the ovaries. The blood supply of the ovaries is provided through the ovarian arteries that originate as branches of the abdominal aorta. The ovaries have two surfaces, lateral and medial; two borders, anterior and posterior; and two poles, tubal and uterine. Each ovary weighs from 2 to 5 grams and is about 4 cm in length, 1.5-3 cm in diameter, 2 cm wide, and 1 cm thick. The main functions of the ovaries are the production of ova and steroid hormones, (oestrogen and progesterone) [2].

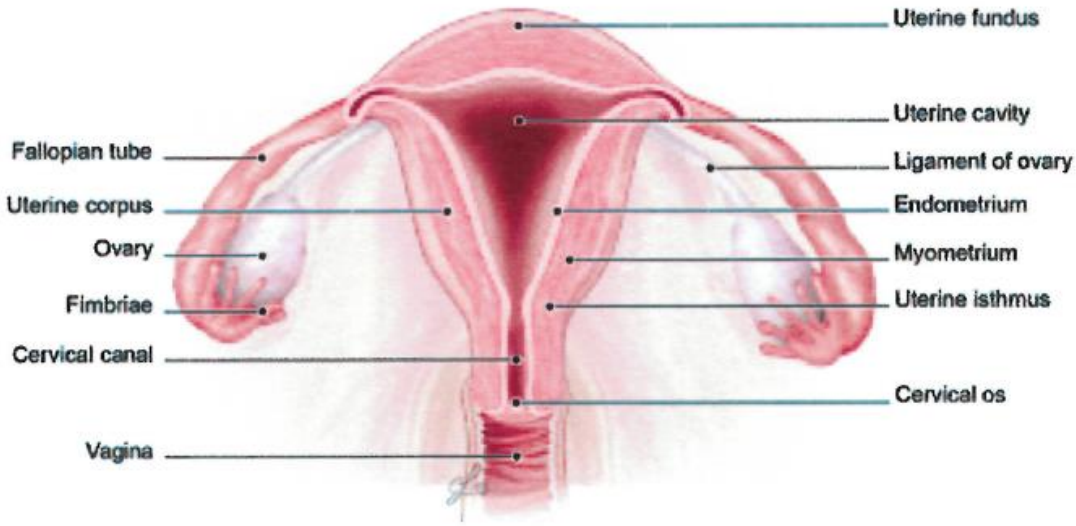


Figure (1-1): The major organs of the female genital system. The female reproductive system is made up of ovaries, fallopian tubes, uterus, cervix and vagina (vaginal canal)[3].

1.1.2. Hormonal control of female's gonads

The reproductive system is governed by a complex neuroendocrine system. The hypothalamic area located in the forebrain controls reproduction by releasing Gonadotropin-releasing hormone (GnRH) from the anterior hypothalamic, preoptic and ventromedial hypothalamic areas as well as in the supra-optic nucleus, arcuate nucleus, medial eminence and medial basal hypothalamus. The collection of dispersed GnRH neurons is called the "GnRH pulse generator", and is activated by endogenous stimuli including neurotransmitters, hormones and growth factors [4]. GnRH regulates gonadal activity by controlling the production and release of the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Secretion of GnRH induces increased synthesis and secretion of FSH, stimulating gametogenesis, steroidogenesis and secondary sexual behaviour. In addition, hypothalamic/pituitary function is regulated by feedback mechanisms by gonadal steroid or peptide hormones (Fig 1-2) [5].

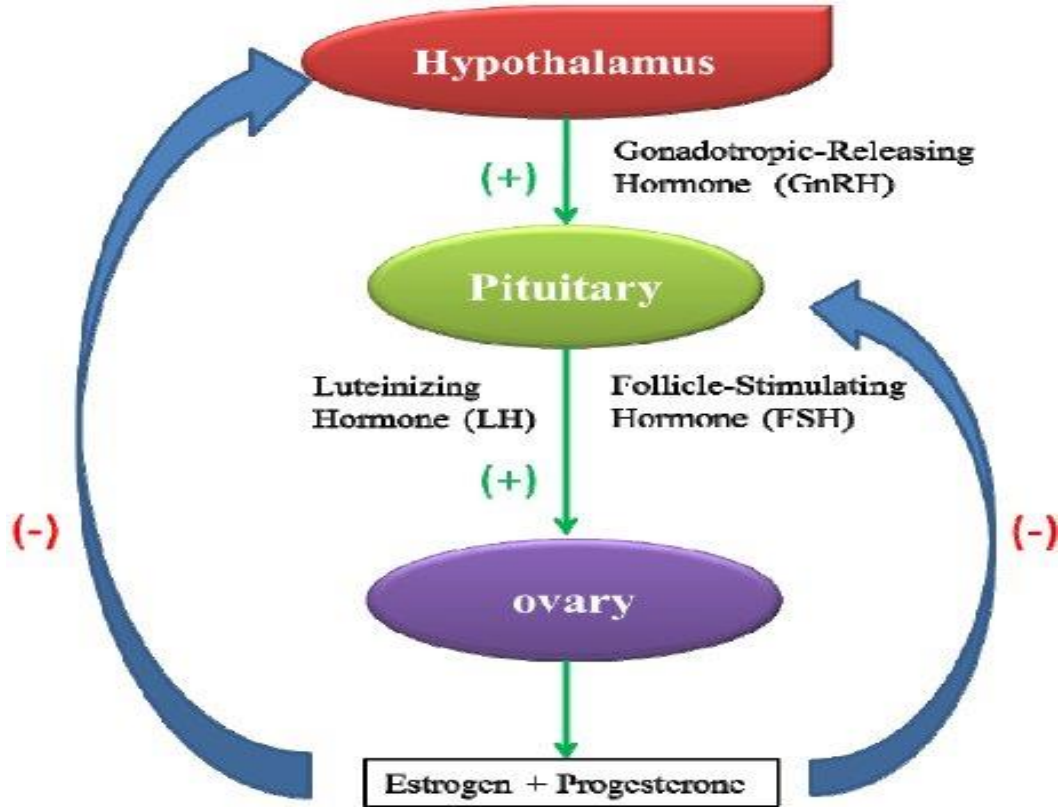


Figure (1-2): The hormonal control of female reproductive system. Gonadotropin releasing hormone (GnRH), follicle-stimulating hormone (FSH) and leutenizing hormone (LH) are the hormones regulating the female reproductive system[6].

1.2. Etiology

Ovarian cancer ranks fifth in cancer deaths among women and is associated with an overall 5-year survival rate of less than 45%. Ovarian cancer is the principal cause of death from gynaecological cancer in the UK[7]. Several factors have been identified which can increase the probability of developing ovarian cancer.

1.2.1. Risk factors and preventive factors

1.2.1.1. Hormonal and reproductive risk factors

Epidemiological research has clearly shown that pathogenesis of ovarian cancer is associated with hormonal and reproductive variables. There have emerged two predominant hypotheses that fit the data. The hypothesis of' incessant ovulation' suggests that the number of ovulatory

cycles rises is linked to surface epithelium repair and that this can act as driver of spontaneous mutation [8]. There is a correlation between the number of lifetime ovulations and a greater risk of ovarian cancer.

The gonadotropin theory postulates that the ovarian surface epithelial cells are also activated by the bursts of pituitary gonadotropins that cause each ovulation and remain at high levels for years after menopause and induce cell transformation of the ovaries [9].

1.2.1.2. Age at Menarche and menopause age

Early age at menarche and late age at menopause raises the risk of developing ovarian cancer potentially as a result of raising the number of ovulatory cycles (Fig. (1-6)). This is understandable when considering the incessant ovulation hypothesis.

In comparison, according to the gonadotropin hypothesis, a late menopause age slows the production of postmenopausal gonadotropin hormones, potentially reducing the risk. Results from research that examined age at menstrual initiation are not terribly consistent [10]. One study of Chinese women revealed lower risk of ovarian cancer in women with menarche at a late age (after 18 years) [11]. In contrast, other research found a slightly higher risk in patient's menarche at an earlier age [12]. Further study has failed to resolve the discrepancies in the literature[13], although a meta-analysis of a total of 27 observational studies were included in their study, consisting of 22 case-control and five cohort studies which reported a reciprocal association between risk and age at menarche (RR=0.85, 95% CI: 0.75–0.97) [14], [15]. In the European Cohort of Prospective Research on Cancer and Nutrition (EPIC), menopause age (>52 *vs*. ≤45 years) was correlated with enhanced risk (HR=1.57, 95 percent CI: 1.16–2.13). However, the risk was slightly reduced and marginally statistically significant after women were diagnosed with OC within the first two years of follow-up. (HR=1.40, 95 percent CI:

0,98–2.00) [16]. The writers speculated that elderly females may mistake bleeding for menstration in the sub-clinical phase of ovarian cancer. Other[17] case-control studies and several cohort studies discovered no connection.

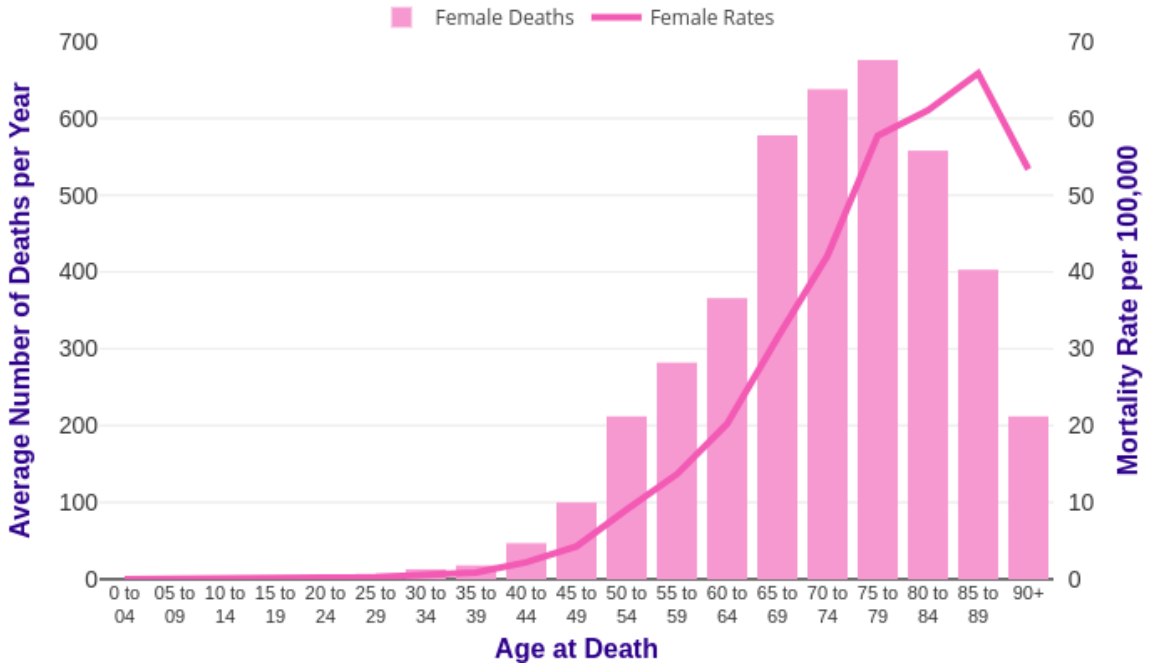


Figure (1-3): Average ovarian cancer death rate per year and age-specific mortality rate per 100,000 female population, UK, 2015-2017 From (Cancer Research UK, 2017).

1.2.1.3. Parity and infertility

There has been extensive study of the connection between pregnancy and ovarian cancer risk. Pregnancy triggers an ovulation and suppresses pituitary gonadotropin secretion and is therefore compatible with both the incessant ovulation and gonadotropin hypotheses. In fact, the risk of parous women is 30%-60% smaller than that of nulliparous women [18]. And each extra full-term pregnancy reduces the danger by around 15% [19]. Similar outcomes have been observed in African American[20] and Asian communities [21]. The protective effect associated with parity is evident across major histotypes, although perhaps slightly weaker in the case of serous carcinomas, with approximately 20% lower risk in parous women compared to other subtypes, particularly clear cell and endometroid [22].

Recent data also show that the risk of OC does not differ between the first and last birth periods[23]. Whether spontaneous or induced abortions affect the risk of ovarian cancer is uncertain. Approximately half of the published research discovered that an enhanced amount of incomplete pregnancies could mildly reduce risk [24], whereas others have been increased [25], while other not affected [26]. In several studies, induced abortions were correlated with lower risk [27], but again this was not found in other studies [26].

1.2.1.4. Lactation

Lactation suppresses pituitary gonadotropin secretion and leads to anovulation, especially in the first months following delivery [28]. This is consistent with both the incessant ovulation and gonadotropin hypothesis as contributing to ovarian cancer. Indeed, most trials show a slight protective impact from breastfeeding, with odds ratios approximating 0.6–0.7 [29] although this has not been observed in other studies [26]. Few trials investigated a link to tumour subtype, with one study showing that lactation conferred the biggest decrease in decrease for endometrioid tumours [29], although another reported the biggest decrease in risk for mucinous cancers[22]. A recent meta-analysis shows a substantial protective impact for breastfeeding (summary RR=0.68, 95 percent CI: 0.61–0.76) that increased with the duration of breastfeeding (the RR was0.85, 0.73, and 0.64 for < 6 months, 6–12 months, and > 12 months of complete breastfeeding, respectively) [30]. Thus, particularly for long-term duration, lactation protects against epithelial ovarian cancer.

1.2.1.5. Benign gynecologic conditions and gynecologic surgery

Several gynecological conditions, including polycystic ovary syndrome (PCOS), endometriosis, and pelvic inflammatory disease (PID), were examined as risk variables for ovarian cancer. PCOS is a multi-factor disease that often results in obesity, hirsutism, menstrual abnormalities and infertility. Women with PCOS have an increased risk of endometrial cancer

due to unopposed endogenous estrogen and/or elevated androgens. Data from the Cancer and Steroid Hormone Study, a population-based case-control study [10], were used to investigate the connection between PCOS and ovarian cancer. The limited data was inadequate to create a consensus that PCOS is a risk factor for ovarian cancer [31].

Endometriosis is one of the most common gynaecological disorders in women, affecting about 10%–15% of reproductive years[32]. Endometriosis has been linked to ovarian cancer in the medical literature since 1925, despite being deemed a benign condition. Ovarian cancers with endometriosis and clear cell histologies[22], [32], show the strongest connections with endometriosis, consistent with molecular information supporting endometrial epithelium as the origin of these subtypes[33]. In addition, Pearce and colleagues [34], recognized an enhanced threat of low-grade serous ovarian cancer ovarian cancer (OR=2.11, 95 percent CI: 1.39–3.20) and clear cell cancer (OR=3.05, 95 percent CI: 2.43–3.84) among women with endometriosis and endometriosis (OR=2.04, 95 percent CI: 1.67–2.48). The writers suggested that endometriosis and endosalpingiosis procedures may result from a comparable underlying host susceptibility to both endometrium and fallopian tube implantation of exfoliated Müllerian epithelial neurons. The association of endometriosis and endometrioid with apparent ovarian cell carcinomas may constitute mutual risk factors[32], genetic susceptibility[35], and/or pathogenesis rather than a causal association[36].

1.2.1.6. Oral contraceptives and other forms of contraception

Over the previous several decades, epidemiological literature has continuously revealed that the use of oral contraceptives is inversely linked to ovarian cancer danger. With longer use, the protective effect increases[17], [37] with about 20 percent reduced danger per 5 years of use persisting decades after use reduced [38]. In addition, risk decrease does not seem to be specific to any particular oral contraceptive formulation or ovarian cancer histotype [38], although in some studies oral contraceptive use appears to be less efficient for mucinous cancer [22]. Oral

contraceptive use amounts to the avoidance of around 30,000 instances of ovarian cancer each year and is estimated to have already avoided 200,000 instances of ovarian cancer and 100,000 fatalities over the past 50 years [39]. Mostly owing to the low incidence of use, progestin-only contraceptives were less researched, but the available information indicate that they may also lower the risk of ovarian cancer [40].

Other than oral contraceptives, relatively few studies have examined techniques of contraception. In several studies [41], the use of an intrauterine device (IUD) was associated with decreased danger of ovarian cancer, although a separate study noted enhanced risk [42]. However, a small incidence of intrauterine device (IUD) use happened in that population prior to the latest IUD formulations. Any protective impact connected with IUD use may depend on the length of use, similar to oral contraceptives. In the Shanghai Women's Health Study cohort, Huang and colleagues, 2015 assessed IUD use and ovarian cancer risk and discovered long-term use of at least 20 years of IUD was associated with a 38% decrease in danger. IUD use is the most prevalent contraceptive technique in China, with a prevalence rate of approximately 50 percent among year-old females[43]. The writers suggest that the elevated prevalence of long-term use of IUD and the related powerful protective impact may contribute to China's low incidence of ovarian cancer[41]. Vasectomy was assessed in conjunction with ovarian cancer danger and results were inconclusive [42], although Reid and colleagues[11] indicated that vasectomy could result in a tiny risk decrease, possibly owing to decreased sperm exposure. Because contraceptive methods can be modified, further study is required to replicate these results. Additionally, a study is required to clarify how distinct kinds of contraception affect ovarian cancer risk, particularly by histotype.

1.2.1.7. Hormone replacement therapy (HRT)

The connection with HRT is less evident than oral contraceptive use. HRT decreases gonadotropin secretion and is therefore expected to reduce risk, but the decreased

concentrations are still greater than premenopausal females[44]. Postmenopausal HRT, on the other hand, may improve ovarian cell proliferation induced by oestrogen and thus boost risk[45]. Initial studies on the subject have concentrated among postmenopausal females on restricted oestrogen therapy (ET). Several case-control studies[9], [17], cohort [46] and meta-analysis [47] discovered no link with length of use, although a substantial or suggestive trend in enhanced danger was noted by two[11]. More recent studies show that the risk of OC in HRT users is increasing[34], [48]. For instance, in one study present and previous HRT users of five years or more had a considerably greater risk than patients who had never used HRT (RR=1.41, 95 percent CI: 1.07–1.86 and RR=1.52, respectively, 95 percent CI: 1.01–2.27). However, there was no change in risk if HRT was used for less than five years (RR=1.01, 95% CI: 0.70–1.44 and RR=0.88, 95% CI: 0.64–1.19, respectively)[49]. The writers found that the increased danger appeared to be mainly driven by length rather than usage status. In comparison, a collective re-analysis of 52 epidemiological studies found increased the risk of OC in existing HRT users, even those with less than 5 years of use[39]. In addition, risk reduced over time after discontinuation of use, although a tiny excess of risk was still noted even 10 years after discontinuation of long-term HRT. Only recently has research with adequate statistical power examined the effect of the combined oestrogen and progestin use. It was assumed that progestin promotes apoptosis while oestrogen encourages ovarian epithelial cell proliferation [50].

1.2.1.8. Obesity

In postmenopausal females, aromatization of androgens in adipose tissue is the predominant source of circulating oestrogens [51]. The convincing role of obesity in hormone-related cancers pathogenesis, such as endometrial and post-menopausal breast cancers [52], has spurred research into the prospective connection with ovarian cancer [53]. Body mass index (BMI) is one metric of significant concern. A meta-analysis of 28 population surveys revealed an enhanced danger of ovarian cancer in overweight females (25–29.9 kg / m^2 BMI) and obese

females (30 kg / m^2 BMI) compared to normal weight (18.5–24.9 kg / m^2 BMI), RR=1.2 and 1.3 pooled, respectively [54]. In a 2008 evaluation of premenopausal obese women, there were an increased risk of incidence in compared to normal weight females (RR=1.72; 95 percent CI: 1.02–2.89); however, this enhanced risk was not evident among postmenopausal females (RR=1.07; 95 percent CI: 0.87–1.33) [55]. A more recent assessment by the Ovarian Cancer Association Consortium (OCAC) of 12 case-controlled studies also discovered that the positive association with BMI was greater among premenopausal females[56]. The EPIC cohort study, on the other hand, found the greatest risk connections for adiposity measurements (BMI and weight) among postmenopausal females[57]. In the NHS, a measure of fat distribution, higher hip circumference was a risk factor among post-menopausal females, but there was no link to waist-to-hip ratio, waist circumference, and BMI [57].

1.2.1.9. Diet and nutrition

Despite countless epidemiological analytical research, it is mainly unresolved whether diet impacts ovarian cancer risk. The noteworthy exception is the intake of vegetables, for which there is proof that greater intakes are connected with reduced risk and also to some extent for the consumption of whole grain products and low fat milk[58]. The risk associated with specific fats and oils, fish and meats and certain dairy products are inconsistent and there can be no firm conclusions. Recently, the EPIC cohort study and the Netherlands Cohort Study conducted a nutrient-wide association assessment assessing 28 food groups and 29 nutrients from 430,476 females, including 1,522 incident ovarian cancer instances, using nutritional questionnaires. Meta-analysis of the two cohort research discovered that females with high saturated fat consumption had increased hazards (HR=1.21, 95 percent CI: 1.04–1.41). There are no consistent studies of meat consumption[59], [60]. A major prospective study found that women had an increased risk of OC in the highest intake quartile of dietary nitrate (HR=1.31, 95 percent CI: 1.01–1.68, and P=0.02). Similarly, there is an inconclusive connection between coffee and

tea consumption [61]–[63]. While most vitamin D is manufactured from UV-B exposure in the skin[64], it is also partially derived from our diet or dietary supplements. Vitamin D is transformed in the liver to 25- hydroxyvitamine D (25(OH)D) and metabolized in the kidney to the active form. 1, 25-dihydroxyvitamin D (1, 25(OH)2D3) controls bone metabolism, immune reaction modulation, and cell proliferation and differentiation regulation [64], [65]. Experimental studies showed that 1, 25$(OH)_2$D3 inhibits the proliferation of cells in ovarian cancer cell lines and induces apoptosis [66]. However, there is inconsistent epidemiological evidence that vitamin D affects the risk of ovarian cancer. A systemic evaluation found that there is no evidence that vitamin D reduces risk [67]. A comparable conclusion was reached by a meta-analysis of ten longitudinal studies[68] and other cohort studies[69]. However, a recent Mendelian randomization study of nearly 32,000 European women was conducted to address conflicting findings from observational studies and found single nucleotide polymorphisms (SNPs) associated with low circulating vitamin D levels were associated with increased risk of ovarian cancer (OR=1.27; 95% CI: 1.06–1.54)[70].

1.2.1.10. Exercise and physical activity

The overall health advantages of exercise are well known and a particular impact on ovarian cancer could be anticipated, at least indirectly, by reducing adipose tissue (and thus oestrogen concentrations), lower ovulation frequency and decreased chronic inflammation [71]. To date, 29 epidemiological studies have explored physical activity and risk of ovarian cancers, including fourteen prospective cohort studies [72]–[74] two historical cohort studies [75], ten population-based case-control studies [76]–[79], and three case-control studies based on hospital[80]. Results are not completely coherent, but a 2007 meta-analysis estimated an approximately 20 percent reduced risk compared to the least active (pooled relative risk=0.81, 95 percent CI: 0.72–0.92) for the most active females [54]. Most lifelong physical activity measurement surveys revealed consistent null finding[54] or reduction of risk in each age span

[72]. Similarly, prolonged sedentary behaviour [73], elevated rates of complete sitting duration [73], [74], and chronic physical recreational inactivity were all associated with increased risk[78]. It does not seem that the advantage of physical activity varies by histological type [74], [78], but there is inadequate information to draw a convincing conclusion [77]. While further study may refine this situation, the promotion of periodic activity should be encouraged when considering the extra advantages of exercise on weight control, bone density, and heart disease.

1.2.2. Other lifestyle and environmental factors

1.2.2.1. Cigarette smoking

Many early reports have concluded that smoking is not a risk factor[81]. Results from more recent research indicate that this is most probably because analyses for the effect on different histological subtypes were not performed. Indeed, smoking appears to boost the risk of mucinous ovarian cancer, but not other subtypes[22]. A meta-analysis of 51 epidemiological surveys in 2012 found that present smokers have a 50 percent rise in the danger of invasive mucinous ovarian cancer and a more than double rise in the danger of borderline mucinous ovarian cancer (RR=2,25, 95 percent CI: 1,64–3,08) compared to none smokers, however, there is no increased risk of serous (0.96, 95% CI: 0.87–1.06) or clear cell (0.80, 95% CI: 0.63–1.01) cancer and a lower risk of endometrioid cancer (0.82, 95% CI: 0.71–0.95) [82]. In another meta-analysis, the risk of mucinous disease increased with the quantity smoked in a dose-response relationship, but returned to the risk observed those who had never smoked within 20–30 years of stopping smoking [83]. Histologically, mucinous ovarian tumours are similar to mucinous gastrointestinal cancers, some of which have also been associated with smoking (pancreatic gastric and colorectal cancers) [83]. These results collectively indicate that ovarian cancer danger is another reason to avoid smoking cigarettes.

1.2.2.2. Alcohol consumption

Consumption of alcohol raises the circulating levels of androgens, oestrogens and other sex hormones in serum and urine and is associated with enhanced risk of breast cancer [84], [85]. Studies examining the effect of alcohol use on the risk of ovarian cancer studies are inconsistent with reports of no associations [86], [87], proof of enhanced risk[88], and of reduced risk[88]. Efforts have been made to address the observed inconsistency by quantifying the danger by the type of alcohol consumed (wine, beer or alcohol)[88], [89]. In a large population-based case-controlled study, intake of beer (not liquor or wine) during early adulthood (20–30 years of age) was correlated with a mildly enhanced danger of invasive ovarian cancer, with the association being restricted to serous tumours (OR=1,52, 95 percent CI: 1,01–2,30), although outcomes for other histological subtypes were based on sparse information. Regular intake (1 or more beverages per day) was connected with this risk, and there was no proof of a dose response relationship [90]. Data from the Netherlands Cohort Study on Diet and Cancer discovered no risk connection with wine, beer or liquor consumption[91]. A pooled assessment of 10 cohort surveys involving more than 500,000 females instances also found no risk connection with overall alcohol consumption (pooled multivariate RR=1,12, 95 percent CI: 0,86–1,44 comparing more than> 30g to 0 g alcohol per day) or alcohol consumption from wine, beer or spirits [92]. In a recent meta-analysis of 10 studies (3 cohort studies and 7 case-control surveys) with 135,871 females, including 65,578 wine drinkers [93], there was no connection (OR=1,13, 95% CI: 0,92–1,38) between wine intake and ovarian cancer risk. Based on this information, it seems reasonable to conclude that alcohol consumption minimally influences the risk of ovarian cancer and is potentially restricted to specific histological subtypes.

1.2.2.3. Asbestos and talcum powder

Asbestos fibres have been discovered in the ovaries of both human [94] and animal studies[95]. However, a connection between asbestos exposure and ovarian cancer was not evident, partially

owing to tiny numbers of exposed female subjects and misclassification of illness (i.e., asbestos-related peritoneal mesothelioma is often misdiagnosed as ovarian cancer on death certificates). A systematic review and meta-analysis of fourteen cohorts and two case-control studies observed a statistically important 75% increased risk of ovarian cancer in asbestos-exposed females (impact size = 1.75, 95% CI: 1.45–2.10) [96]. The association, however, was attenuated (impact size=1,29, 95 percent CI: 0,97–1,73) in a study when the cancer diagnosis was restricted to pathologically confirmed instances [97]. Despite the absence of consistency, the International Cancer Research Agency (IARC) has stated that human proof that exposure to asbestos causes ovarian cancer is 'adequate'. Talcum powder, similar to asbestos, is a silicate that has been widely researched with inconsistent outcomes in relation to cancer risk. While mechanistic, pathological, and animal studies do not provide evidence of carcinogenicity of talc on the ovarian epithelium [98], epidemiological studies have shown that talc use is associated with enhanced risk of ovarian cancer. A meta-analysis of 21 studies recorded an increase in the risk of genital exposure to talc of approximately 35 percent in 2006, and a previous meta-analysis had similar findings [94], and a previous meta-analysis had comparable results [99]. Newer studies, however, have continued to report conflicting outcomes. In 2014, among a cohort of 61,576 post-menopausal females, the Women's Health Initiative recorded no connection. Cramer and colleagues conducted a case-control retrospective study that found increased risk among talc users similar to those previously reported (OR=1.3, 95 percent CI: 1.16–1.52) [100], especially among serous and endometrioid cancers. The research also discovered that risk among premenopausal females and in postmenopausal females using hormonal therapy was highest, indicating that oestrogen plays a part in the connection. Furthermore, genetic trials indicate that females with certain variations in glutathione S-tansfase M1 (GSTM 1) a n d / o r Glutathione-transferase T1 (GSTT1) may have a greater risk of ovarian cancer connected with talc use [101]. Based on the evidence available, genital talc use was classified as potentially carcinogenic to humans by the IARC in 2006[102].

1.2.2.4. Drug use

Epidemiological proof that links Pelvic Inflammatory Disease PID and endometriosis to increased risk of ovarian cancer indicates that inflammation plays a major role in ovarian carcinogenesis. Furthermore, studies in animals and *in vitro* indicate that aspirin inhibits development ovarian cancer[103]. Several prospective studies [104][105] and case-control [106]–[108] found an inverse connection between aspirin or non-steroidal anti-inflammatory drugs (NSAIDS) and ovarian cancer incidence, although no association was recorded in other trials [109], [110]. Prizment and peers studied these drugs using information from the Iowa Women's Health Study's prospective cohort of about 20,000 women [111]. Compared to females who did not report aspirin use, the relative risks of ovarian cancer for those who used aspirin < 2, 2-5 times and 6 times a week were 0.83, 0.77 and 0.61, respectively (P=0.04), but there was no connection between NSAID use and risk. In contrast, frequent use of NSAIDS was protective (HR=0.81, 95% CI: 0.64–1.01) [112], but aspirin use was not (HR=1.11, 95% CI: 0.92–1.33). No dose-response relationship was noted with enhanced frequency or length of use and outcomes were not different when tumour histology stratification was performed [112]. A later pooled assessment of 12 case-control studies evaluating aspirin in the Ovarian Cancer Association Consortium OCAC [106] showed aspirin use was correlated with a decreased risk of ovarian cancer (OR=0.91, 95 percent CI: 0.84–0.99), particularly among daily users of low-dose aspirin (OR=0.66, 95 percent CI: 0.53–0.83). Thus, the same aspirin regimen prescribed to safeguard against cardiovascular occurrences and other diseases (e.g. colorectal cancer) could decrease ovarian cancer danger by 20%-34%. An increasing body of proof promotes the role of metformin, the antidiabetic agent, in various cancer prevention and therapy [113]. A case-control study was conducted using the UK-based General Practice Research Database [112], including 1,611 incident ovarian cancer instances. Long-term use (about 30 prescriptions) of metformin (not sulfonylureas or insulin) was potentially connected with a risk

reduction trend (OR=0.61, 95% CI: 0.30–1.25), but the findings were not being statistically significant. Additional trials have shown reduced incidence and mortality among groups treated with metformin [113]. Due to the lack of robust studies, further exploration of the potential for the use of metformin as a chemopreventive agent are needed.

1.3. Origin of ovarian cancer

1.3.1. Type I Tumours

One group of ovarian cancers are termed type I tumours. Generally, these tumours behave in an indolent fashion and are relatively stable genetically. They lack mutations in *TP53* but exhibits a distinctive molecular genetic profile for each histologic type[114].

1.3.1.1. Endometrioid and clear cell ovarian cancers

Endometriod tumours morphologically resemble cells that line the internal part of the uterus (endometrium) and may result from the aberrant overgrowth (hyperplasia) of endometrium outside the uterus. Endometrioid tumours are occasionally benign; it is unilateral and occurs in association with cysts[115]. Malignant endometrioid ovarian tumours are either cystic or predominantly solid. The prognosis of malignant ovarian endometrioid carcinomas is better than either mucinous or serous carcinomas. The inflammatory processes that occur during endometriosis may have important role in the malignant transformation and growth of the surface epithelium of ovaries. This may be triggered by tumour necrosis factor-alpha (TNF-α) and growth factors that promote cellular growth and proliferation such as insulin-like growth factor (IGF)[116].

1.3.1.2. Mucinous ovarian cancer

Mucinous tumours are epithelial ovarian tumours containing cells that are similar to the endocervical epithelium. Benign mucinous tumours occur most frequently rarely with bilateral incidence.

Borderline mucinous tumours resemble benign mucinous tumours but the difference is that the cyst chambers have papillae projecting into them and exhibit solid regions. These accounts for about 10– 14% of all ovarian mucinous tumours[117].

1.3.2. Type II Tumours

Type II Tumours are considered highly aggressive tumours and are often detected at advanced stages because they rapidly metastasize to the adjacent tissues to form many small nodules on the peritoneal surface. Recent studies have provided cogent evidence that ovarian tumours originate in other pelvic organs and metastasize to the ovary secondarily.

Type II tumours are characterized by inactivation of *TP53*[118]. *TP53* functions as a tumour suppressor and thus has a critical role to prevent cancer. Missense mutations of *TP53* in many tumours lead to single amino acid substitution in full-length p53[119]. The tumour progression may be driven by accumulation of the mutant p53 protein in cancer cells, resulting from its prolonged half-life [119]**.**

1.3.2.1. Serous ovarian cancer

Serous ovarian cancer is an epithelial – stromal tumour and it is now thought to gradually developed from the cells that are lining the Fallopian tube. Benign serous tumours are formed with a single chamber with a flat internal lining and filled with straw-coloured watery fluid. Up to 20 % of benign serous tumour are bilateral, simultaneously occurring in both ovaries[120]. Benign serous tumours have more exuberant fine papillary projections in the lining side of cyst cavity. Borderline serous tumour accounts from 10% to 15% of all serous tumour. Malignant serous tumour usually contains multiple chambers or locations in the same cyst with solid consistency. Malignant serous tumour account for approximately half of all malignant ovarian neoplasm[120].

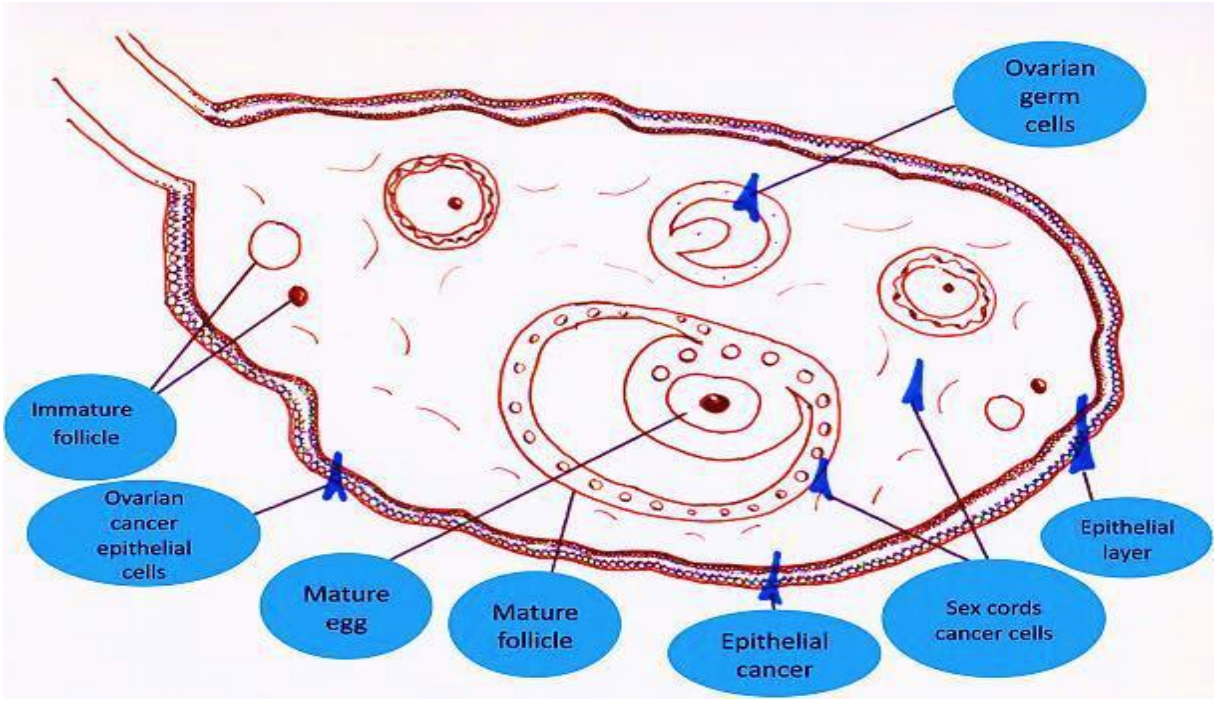


Figure (1-4): Shows the origin of ovarian cancer. Types of cancer of the ovaries according to cell origin. Different cell types may develop into different types of cancer cells in the ovaries. BRCA1's abnormal presence is associated with more probability of ovarian cancer predisposition that arises from these cells in women. Adapted from [121].

1.4. Neoplasms of the ovary

Ovarian cancer is a broad and wide term used for a vast range of neoplasms which apparently arise in the ovaries. Based on their putative tissue of origin, ovarian neoplasms are broadly classified for three groups: surface epithelial–stromal tumours, germ cell, sex cord–stromal cell. Histologically, each group contains many subtypes of ovarian tumour. Since the tumours of ovarian epithelium are the most prevalent and the most lethal this discussion will focus primarily on cancers of the ovarian surface epithelium[122].

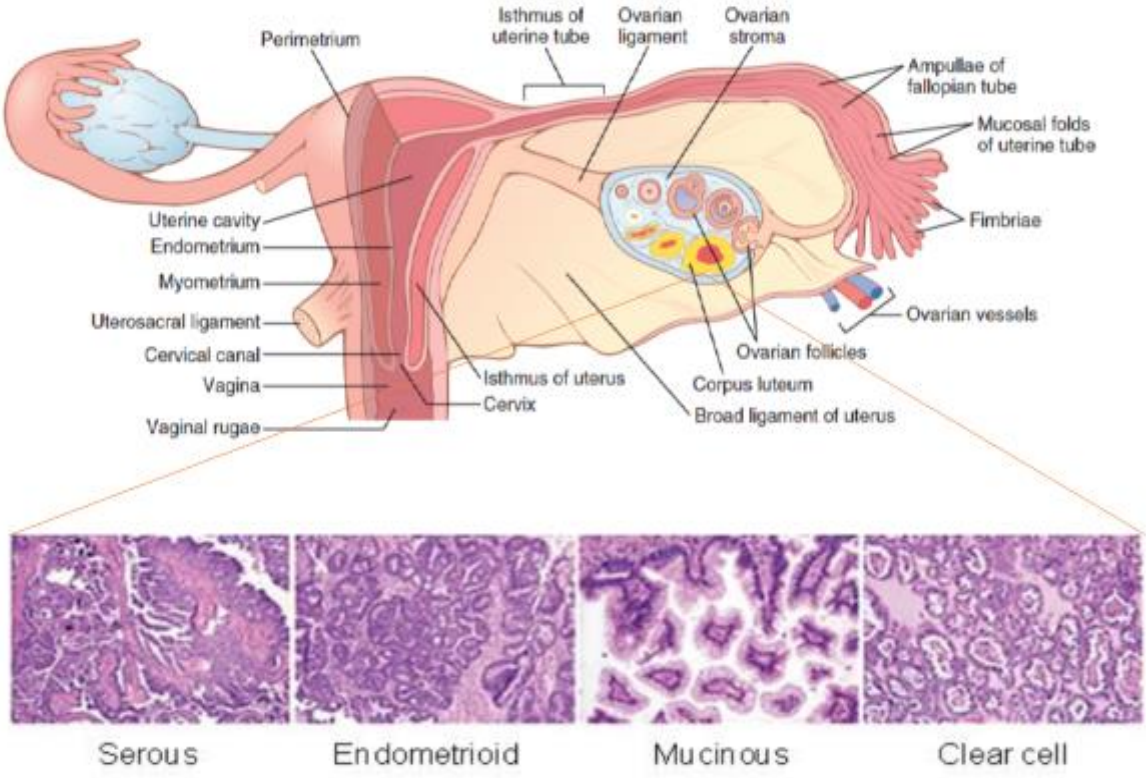


Figure (1-5): Classification of ovarian cancer based on cell origin, There are four main histological subtypes of ovarian cancer: serous (the most common), endometrioid, mucinous, and clear cell [122].

1.4.1. Class I: Epithelial Ovarian Tumours

Epithelial ovarian tumours are derived from the epithelial cells on the surface of the ovary. It is the most common form of ovarian cancer, starting from the cells that cover the outer surface of the ovary. Most ovarian tumours are epithelial cell tumours, accounting for approximately 85-90% of ovarian cancers [123]. Some studies have suggested that serous ovarian carcinoma actually originate in the Fallopian tube. This is reinforced by the observation that analysis of gene expression showed a high similarity between tubal carcinoma gene expression profiles and those of high grade serous ovarian cancer (HGSC). About 50-60% from women who reportedly have been tubal carcinomas reported in with pelvic HGSC [124]. The ovaries tumours can be further subdivided into three categories.

1.4.1.1. Benign epithelial Ovarian Tumours

Benign epithelia ovarian tumours are a non-cancerous tumour, with no cytologic atypia in the surface epithelium of ovaries, occasionally treated by surgical removal of the tumour with preservation of the ovarian tissue[125].

1.4.1.2. Borderline epithelial Ovarian Tumours

Borderline low-malignant potential (LMP) tumours are a borderline type of cancer that is growing slowly, and which is less life threatening than most ovarian cancer, although it may eventually invade other tissues. This kind is different from typical ovarian cancers. It does not grow into the stroma of ovary (the supporting tissue of the ovary). This kind of cancer occurs in younger women and can be effectively treated if it is correctly diagnosed [126].

1.4.1.3. Invasive carcinoma epithelial Ovarian Tumours

Invasive carcinoma has typically invaded other organs by invading and metastasizing from the ovaries[127]. Invasive epithelial ovarian cancers tend to invade the lining and organs of the pelvis and abdomen, results in accumulation of fluid in the abdominal cavity (ascites). These tumour cells can be classified into different histological types according to their morphology. Serous ovarian cancer is the most common and characterized by chromosomal anomalies and high genomic instability that include intrachromosomal breaks and aneuploidy. This type of ovarian cancer is almost invariably associated with mutations in tumour suppressor gene TP53 [127], [128].

1.4.2. Class II Germ cell tumour

These tumours start from the cells that produce the eggs (ova) and accounts for approximately 10-15% of ovarian tumours. Germ cell tumours are divided into two types.

1.4.2.1. Benign germ cell tumours

Benign germ cell tumours often occur as non-cancerous matures cystic teratomas. Chemotherapy treatment is not necessary because these tumours can usually be successfully treated by surgical removal with preservation of the uninvolved ovarian tissue[129].

1.4.2.2. Malignant germ cell tumours

Ovarian germ cell tumours are relatively rare, accounting for less than 10% of total ovarian cancers, about 2% to 3% of germ cell tumours are malignant [130], [131]. Such tumours start from the primitive germ cell and then slowly separate to resemble embryonic developmental tissues (ectoderm, mesoderm, endoderm) and extraembryonic tissues (yolk sac and trophoblast). Tumours of germ cells originating in the ovary have homologous equivalents in the testis [132].

1.4.3. Class III: Stromal Ovarian Tumours

Sex cord stromal ovarian tumours are the least common type of ovarian tumous, accounting for approximately 5-10% of ovarian tumours. They originate from the structural tissue that hold the ovaries together, cells that produce the female reproductive hormones oestrogen and progesterone. Most of these tumours are benign and never spread beyond the ovary[133].

1.4.3.1. Benign tumours of stroma cell

Benign tumours can be treated by removing the ovary partially or completely[134].

1.4.3.2. Malignant tumours of stroma cell

Stromal tumours arise from cells in the connective tissue of the ovaries and those containing estrogen and progesterone in the female hormones. These are considered low-grade cancers,

which means that the cells grow slowly and appear almost healthy. These cancers have a survival rate of 95% when diagnosed at stage I[135], [136].

1.4.4. Other cancers that are similar to epithelial ovarian cancer

1.4.4.1. Primary peritoneal carcinoma

Primary peritoneal carcinoma (PPC) is a rare form of cancer. At surgery, this form of cancer looks the same as epithelial ovarian cancer due to spread through the abdominal cavity[137]. Under the microscope, its histology suggests that it develops from the cells of the peritoneum lining of the pelvis and abdomen; these lining cells closely resemble the epithelial ovarian cells. As a result, this type of cancer may be referred to by different names including *serous surface papillary carcinoma, extra-ovarian* and *primary peritoneal carcinoma* (EOPPC)[138].

Primary peritoneal carcinoma occurs when women still have their ovaries, while it is more of a concern for women have had removed their ovaries to prevent ovarian cancer. The symptoms are similar to those of ovarian cancer, including increase in the blood level of the tumour marker CA-125 as well as changing in bowel habits, bloating, abdominal pain, vomiting and indigestion. In addition, patients with PPC usually receive the same treatment as those with ovarian cancer, including surgery to remove as much as possible of the cancer then followed by chemotherapy similar to that given for ovarian cancer [139].

1.4.4.2. Fallopian tube cancer

This type of cancer is also rare and similar to epithelial ovarian cancer. It begins in the fallopian canal that carries an ovum from the ovary to the uterus. Fallopian tube cancer has similar symptoms of the PPC and ovarian cancer. Its treatment is similar to that for ovarian cancer, while the prognosis is slightly better [140].

1.4.4.3. Ovarian germ cell tumours

Ovarian germ cell tumours are principally diagnosed in young women but sometimes occur in infants and older women. Germ cell tumours account for 15-20% of all ovarian neoplasms and this type of cancer could be the life threating. There are many subtypes of this tumour, the most important one being teratomas, and more than one subtype can occur at the same time [141]. Teratomas may be a cancerous form called *immature,* which occurs usually in young women and girls younger than 18 years, and a benign form called *mature,* which is the most common type of germ cell tumour and usually affects women of reproductive age[142].

1.4.4.4. Dysgerminoma

This type of ovarian germ cell cancer is relatively rare. It usually affects women less than 20 years old, it is considered malignant, but it does not spread very quickly. The disease is limited to the ovaries in more than 75% of patients. Additionally, more than 75% of patients who are affected by this type of tumour are treated by surgical removal, without any further treatment[143].

1.4.4.5. Endodermal sinus tumour (yolk sac tumour) and choriocarcinoma

This type of tumour typically affects girls and young women. It grows and spreads quickly but in most cases, it is very sensitive to chemotherapy[11], [144].

1.5. Theories for cancer of the ovaries

The clear link between the history of ovulation in patients and the risk of developing ovarian cancer in clinical aspects of ovarian cancer pathogenesis theories has prompted many hypotheses to explain the causes of ovarian cancer.

1.5.1. Theory 1: The incessant theory of ovulation

Fathallah proposed this first theory to explain the aetiology of ovarian cancer in 1971. This speculates that repeated damage to the ovarian surface epithelium (OSE) during ovulation accompanied by cell proliferation results in successive accumulation of DNA mutations, resulting in cancer of the ovaries. Based on this hypothesis, serial rounds of programmed cell death and OSE repair at the ovulation site cause genetic instability, rendering this cell layer more vulnerable to neoplasm growth [145], [146]. Murdoch and colleagues presented evidence to support this hypothesis where the OSE cells in the ovulation site experience oxidative DNA damage, p53 expression and apoptosis. A genetically altered progenitor cell with defective DNA that is not committed to death can result in a transformed phenotype that is then propagated after ovulatory wound healing. Then, with each ovulation cycle, there is an increased likelihood of generating genetically modified and carcinogenic cells in the OSE and this increases the risk of development of ovarian tumours[147].

1.5.2. Theory 2: gonadotropin of pituitary theory

Gonadotropins, FSH and LH, are important hormones for ovarian cell control and indicated a potential role in ovarian carcinoma pathogenesis. Specific gonadotropin receptors have been identified for ovarian cancer. The role of endocrine factors in regulating normal ovarian growth can also provide appropriate conditions for malignant transformation. In addition, the presence of high levels of FSH and LH in ovarian cancer fluids indicates that these hormones play a role in ovarian cancer development and progression [148], [149]. Gonadotropins activate mitogenic pathways, including the extracellular signal-regulated kinase (Erk) pathway, promoting ovarian cancer cell proliferation and invasion [150]. Gonadotropins stimulate proteolytic enzymes and cytokines during ovulation to produce an inflammatory process that causes the release of the ovum to rupture the OSE surface. The oestrogen-producing follicle is transformed to a progesterone-producing corpus luteum after breakup of the follicle and release of the ovum at

the OSE, which then provides feedback inhibition which lowers gonadotropin levels [151]. There is total depletion of the germ cells in the ovary during menopause, followed by loss of the follicular structure which protects the germ cells, resulting in the absence of corpus luteal input, resulting in high levels of serum gonadotropin and proinflammatory cytokines. High levels of gonadotropin in postmenopausal women may therefore promote an inflammatory environment that may not contribute to ovulation but may lead to a high risk of developing ovarian cancer by inducing morphological changes in OSE and promoting the transformation of genetically damaged cells into ovarian cancer [151]. Apoptosis failure in granulosa and theca cells after the ovulatory period can promote carcinogenesis because these cells retain the ability to produce steroid hormones [152]. Epidemiological evidence supports this theory and has shown that in women with multiple pregnancies, breast feeding, and those who use oral contraceptives, the risk of developing ovarian cancer has decreased as a result of suppressing pituitary gonadotropin secretion in these circumstances[150] .

1.5.3. Theory 3: The theory of androgen / progesterone

The theory of hormonal enhancement, also known as the theory of androgen / progesterone, indicates that androgens (elevated after menopause) can induce ovarian cancer generation. On the other hand, progestins, protect against disease growth through increasing ovarian testosterone levels, progesterone contraception may reduce the risk of ovarian cancer [153]. In addition, androgen receptors can drive the proliferation of cells in the ovarian surface epithelium. The follicles are supposed to produce androgen and create a rich androgen environment around the epithelial cells. It has been shown that documented hyperandrogenic disorders such as acne, hirsutism and polycystic ovarian syndrome are associated with increased risk of ovarian tumour development [154].

1.5.4. Theory 4: The theory of inflammation

The inflammatory theory suggests that inflammation may work in conjunction with steroid hormones and ovulation to increase the possibility of ovarian epithelium carcinoma as a cause of ovarian cancer growth [155]. According to this theory, repair damaged DNA is impaired, particularly in those patients with defective BRCA1 and BRCA2 function, resulting in a high risk of carcinogenesis. Furthermore, inflammatory stimulation as a result of exposure to endometriosis, talc or asbestos, or as a result of ovulation itself, may work to support ovarian cancer development[156]. Inhibition of inflammation induced a decrease in cancer incidence in a study using anti-inflammatory drugs, where a statistically significant inverse correlation between the use of anti-inflammatory drugs and the risk of cancer was reported [157]. Several mechanisms have been anticipated to explain the beneficial effects of anti-inflammatory drugs in reducing the risk of this disease, including apoptosis induction, cyclo-oxygenase activation of prostaglandin synthesis, or cellular immune response enhancement [157]. Inflammation biomarkers can be used as a way of tracking ovarian cancer progression. These biomarkers can also help prevent and treat cancer by developing a new anti-inflammatory medication that can also be used as an adjuvant to radiation therapy or chemotherapy, activating NF-kB and mediating resistance on its own [158].

1.5.5. Theory 5: The theory of tubal origin

This is the new theory which indicates that the vast majority of ovarian cancers occur in the fallopian tube, both in women at high risk and in the general population, and that there is no obstacle to peritoneal spread [159]. Most of the ovarian cancers found in women with BRCA mutations in the fallopian tube in their early stages [160]. Pre-invasive changes in the fimbriated tube end are usually associated with early HGSOC[161]. In addition, disabling PTEN, a crucial negative regulator of the PI3K pathway, and Dicer, an important gene for microRNA synthesis, with Amhr2-Cre in mice, resulted in the subsequent spread of fallopian tube cancer to the ovary

and then rapidly metastasized to the abdominal cavity, leading to ascites and death[162]. Research is currently underway to investigate the molecular and genetic basis essential to the production of ovarian cancer from fallopian tube[163].

1.6. Epidemiology

1.6.1. Descriptive epidemiology

Ovarian cancer incidence shows broad geographic variation [164]. In advanced areas of the globe, including North America and Central and Eastern Europe, the largest age-adjusted incidence rates are found, with levels usually exceeding 8 per 100,000. Percentages are intermediate in South America (5.8 per 100,000) and lowest in Asia and Africa (3 per 100,000). Migration from low rates countries to high rates countries leads to increased risk underlining the value of non-genetic risk factors [165]. Race differences in incidence and mortality within the United States mimic the observed international variation with the highest rates among whites, intermediate for Hispanics, and the lowest among Blacks and Asians, in big nations such as China, variation also mimics global variation with greater incidence and mortality in developed, urban, versus less advanced, rural areas[166]. Ovarian cancer incidence and mortality have gradually decreased in most developed countries, including North America and Europe, since the 1990s [167]. In contrast, the rates of incidence and mortality have increased in historically less developed countries with latest economic growth and lifestyle modifications. In China, the rise is only evident among rural females, rather than in more advanced, urban areas [168], [169].

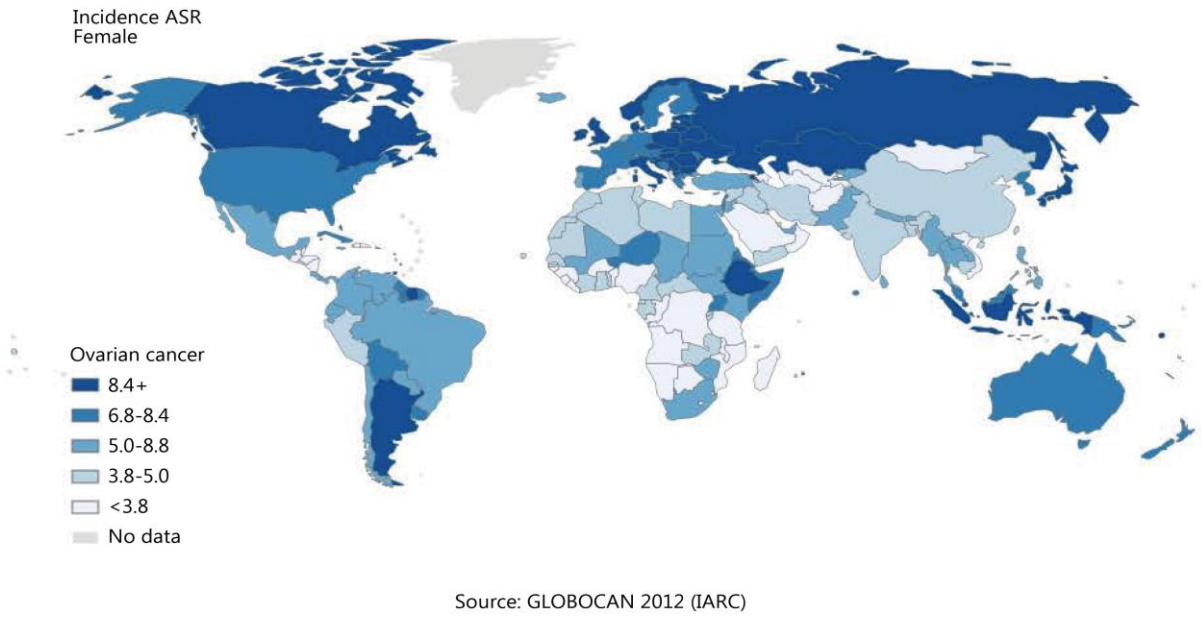


Figure (1-6): The prevalence of ovarian cancer has wide variation in geography[11].

1.6.2. Genetic epidemiology

A family history of the disease is one of the most important risk variables for ovarian cancer [170]. First-degree probands relatives have a 3- to 7-fold increased risk, especially when multiple relatives are affected and at an early age [171], [172]. Rare elevated penetrating mutations in the genes *BRCA1* and *BRCA2* significantly boost lifetime risk [173] and account for the majority of inherited instances and 10%-15% of all instances [174], [175]. Data from the Breast Cancer Linkage Consortium indicate that the risk of ovarian cancer in BRCA1 mutation carriers over the age of 70 years is as high as 44% and in BRCA2 mutation carriers it is close to 27%[176]. Hereditary non-polyposis colorectal cancer syndrome (HNPCC) can account for at least 2% of instances and confer a lifetime risk of up to 20% [173]. Women with mutations in DNA repair genes like BRIP1, RAD51C, and RAD51D have estimated lifetime hazards of 5.8%, 5.2%, and 12%, respectively, [177], [178]. Deleterious mutations in BRCA1/2 and other double-strand DNA break genes are more closely correlated with susceptibility to HGS ovarian cancer, although they happen in other tumour subtypes [178]. Ovarian cancer associated with HNPCC typically occurs as endometrioid or clear cell tumours rather than the prevalent serous subtype[179], known syndromes collectively account for 36 percent of

comparative ovarian cancer family danger [180]. Genome-wide association studies [181] found 22 vulnerability alleles with weak to mild impacts in European populations for invasive ovarian cancer. Eighteen of these risk loci are associated with all and/or serous ovarian cancer, five are connected with mucinous ovarian carcinoma danger, one with ovarian cancer, and one with complete cytoreduction ovarian cancer, exemplifying histotype genetic heterogeneity[182]. Furthermore, a large-scale pooled assessment of ovarian, breast, and prostate cancer genome-wide association research recognized five novel loci [183].

1.7. Symptoms of ovarian cancer

Most patients who have ovarian cancer have symptoms prior to diagnosis. There is no symptom specific to ovarian cancer. Most women diagnosed with ovarian cancer do experience pelvic or abdominal pain, bloating, difficulty eating, and quickly feeling full after eating with increase abdominal size. The symptoms of ovarian cancer may be different for each woman. The symptoms may be caused by noncancerous conditions due to the ovaries being situated near the intestines and bladder. As a result, the gastrointestinal symptoms are common but sometimes women ignore these symptoms. Diagnosis is complicated by the fact that these symptoms also occur in irritable bowel syndrome, gastritis, stress and depression and has several months may elapse before the patients are correctly diagnosed with ovarian cancer[184], [185].

Table (1-1): the frequency of ovarian cancer symptoms [185].

No.	Symptoms	Frequency (%)
1	Increased abdominal size	61
2	Bloating	57
3	Fatigue	47
4	Abdominal pain	36
5	Indigestion	31
6	Urinary frequency	27
7	Pelvic pain	26
8	Constipation	25
9	Back pain	23
10	Pain with intercourse	17
11	Unable to eat normally	16
12	Palpable mass	14
13	Vaginal bleeding	13

14	Weight loss	11
15	Nausea	9
16	None	5
17	Bleeding with intercourse	3
18	Deep venous thrombosis	1
19	Diarrhoea	1

1.8. Diagnosis

The accurate diagnosis of ovarian cancer is complicated by non-cancerous and gastrointestinal cancers tumours which have similar symptoms to ovarian cancer. Physical examination is performed to look for signs of ovarian cancer and signs of fluid accumulation inside the abdomen. Further examinations will be done if ovarian cancer is suspected [186]. Of these, ultrasound imaging is preferred to detect whether the ovaries and abdominal cavity possess abnormal masses. Additionally, the ultrasound is able to detect whether the masses are solid or cyst filled with fluid [187]. Other imaging modalities may be conducted such as positron emission tomography (PET), computed tomography (CT) and magnetic resonance imaging (MRI), these imaging may be used to detect the type and stage of tumour [188]. CA-125, a biomarker of ovarian cancer, may also be measured in blood samples. Laparoscopy, and biopsy are used to detect precisely the type and stage of cancer so the treatment plan can be devised that is most appropriate to the patient's ovarian cancer [189], [190].

1.9. Treatment

Currently, several options are available for treatment of ovarian cancer. These often involve surgical removal of the tumour followed by chemotherapy which is used as adjuvant to reduce the residual number of surviving cancer cells. Radiation therapy is used rarely due to the adverse effects of it on the adjacent abdominal organs [191]. The chemotherapy regimen has advanced over the last 50 years. In the late 1970's, the use of platinum-based therapy in combination with other chemotherapeutic agents became popular[192]. Currently it is considered the best standard of care.

1.9.1. Surgery

The main treatment of ovarian cancer is surgery and it can be used for all types and stages of ovarian cancer. There is clinical evidence that debulking large masses of tumour is beneficial to the patient, even if all gross tumour cannot be removed. The main aim of surgery to remove all of the palpable tumour and in advanced stage diseases this may be accompanied by removal of the ovaries with Fallopian tubes, omentum, womb or a fatty layer that covers the abdominal organs. Surgery alone may be necessary only in patients diagnosed with early disease, chemotherapy is generally used in late-stage disease after debulking surgery [193], [194].

1.9.2. Chemotherapy

Chemotherapy is used after surgery to treat the remaining of cancer or delay relapse. The guidelines of chemotherapy in the UK are that must be started no more than 8 weeks after surgery [195]. Several factors govern the prescribing of chemotherapy, including the extent of surgical treatment that is required, the stage of disease and side effects of chemotherapy [196]. The adverse outcomes associated with chemotherapy outweigh the beneficial effects patients in early stage disease, so chemotherapy is not advised to be used with stage I disease [196]. Ovarian cancer typically is treated with a combination of two or more chemotherapeutics that are given intravenously or directly into the abdominal cavity. The chemotherapy used depends on the type and stage of ovarian cancer. Malignant germ cell tumours require removal of the whole ovary by surgery and intensive multi-agent adjuvant chemotherapy. The chemotherapy of malignant germ cell tumours is completely different from the chemotherapy administered after surgical treatment of surface epithelial tumours, these tumours consist of embryonic mature cells and embryonic cells carcinoma, and metastasize frequently[197].

The current typical chemotherapy for ovarian cancer is a combination of carboplatin with paclitaxel. Survival rates of patients have been increased as a result of using combination

chemotherapy [198]. Initially, most patients respond to chemotherapy treatment, but patients may relapse with tumours resistant to the chemotherapy. This leads to a poor prognosis in the majority of these cases [199]. In advanced cases of ovarian cancer, the platinum-based chemotherapy is considered as essential treatment and has a better prognosis.

1.9.2.1. Platinum chemotherapy

Cisplatin and carboplatin are platinum-based drugs which cause inter- or intrastrand linking in DNA, there are also newer platinum-based drugs including satraplatin, oxaliplatin and picoplatin. These compounds lose chloride ions after entry into the cell, and then subsequently react with DNA. This process causes DNA damage, cells cycle arrest and apoptosis. Platinum-based drugs are used for treating the cancer of ovaries, lung, breast, testicular, head, colorectal, neck, and bladder cancers. Cisplatin received approval for use in 1978 for testicular and ovarian cancer, while carboplatin (second generation platinum drug) was approved for the treatment of ovarian cancer in 1989. Carboplatin has fewer toxic side effects than cisplatin which causes nephrotoxicity, nausea, vomiting, bone marrow suppression, anemia and neutropenia. In addition, carboplatin and cisplatin have similar activity but carboplatin is more stable than cisplatin [200]. The third-generation platinum-based drug (oxaliplatin) received approval use from the FDA for treatment of colorectal cancer but is not widely used to treat ovarian cancer[201].

1.9.2.2. Taxanes

Taxanes are an important family of chemotherapeutic drugs identified in the early 1990s and which are derived from the bark of the yew tree. They are used for treatment different types of cancers [202]. Taxanes cause cycle arrest and apoptosis by inhibiting cell division in G_2/M phase by stabilising microtubules in a polymerised state. They inhibit mitosis by interfering with microtubule function during mitosis, thus they are also called as mitotic poisons [200],

[203]. Paclitaxel and docetaxel are widely used taxanes that are very active chemotherapeutic agents for earlier stages of cancer with an established beneficial effect. In addition, they are also used to treat other types of cancers including breast, head, oesophagus, lung and bladder [204]. Additionally, taxanes decrease cell migration and angiogenesis of tumour and stimulate the immune system also by increase the effect of tumour necrosis factor against cancers [205]. Patients who receive taxanes may suffer adverse effect such as erythema and desquamation, which primarily appeared on the hands and nail pigmentation due to induced cutaneous toxicity and skin reactions [206]. Unfortunately, these side effects may be exacerbated by impaired metabolism and excretion that results in increased level of drugs in the serum [207].

1.10. Mechanisms of Chemotherapy Resistance

Chemotherapy resistance prevents effective therapy and can eventually lead to death of the patient; thus, it is important to understand drug resistance mechanisms [208]. There are several mechanisms of resistance to the drugs that are used to treat ovarian cancer.

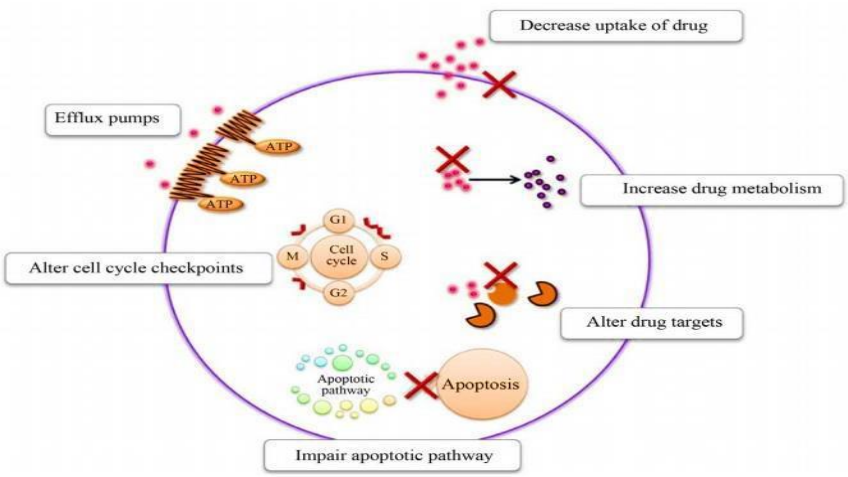


Figure (1-7): Cellular Chemotherapy resistance mechanisms. Cells may develop resistance to chemotherapy through various pathways, such as increasing the activity of efflux pumps (such as ATP-dependent transporters) to reduce drug influx, resulting in drug removal, triggering detoxifying proteins (such as cytochrome P-450), repairing their damaged DNA, disrupting and preventing apoptotic signalling processes as well as changing cell cycle control points[209].

1.10.1. Reduce intracellular Drug Accumulation

One of the most important mechanisms that contributes to the resistance of cancer cells to chemotherapy is to reduce the accumulation of anticancer drugs inside the cells [210]. This may be due to decreased uptake of drug into cancer cells and/or increased efflux of drugs. The copper transporter-1 (CTR1) regulates the entry of cisplatin and carboplatin, and downregulation of the transporter competitively inhibits the transport resulting in decrease entry of the drugs into ovarian cancer cells[208].

1.10.2. Drug Inactivation

In vivo drug activation requires complex mechanisms in which drugs interact with different proteins, which can modify, partially degrade or confuse the drug with other molecules or proteins, ultimately leading to its activation. To achieve therapeutic effectiveness, most anticancer drugs must undergo metabolic activation. Nevertheless, cancer cells can also resist these treatments by reducing the activation of the drug. Furthermore, cancer cells can develop resistance through decreased drug activation due to many of anticancer drugs require metabolic activation. Resistance may also result from detoxification of platinum-based compounds via conjugation to glutathione S-transferase. This leads to inactivation of chemotherapeutic drugs[210].

1.10.3. Apoptosis Deregulation

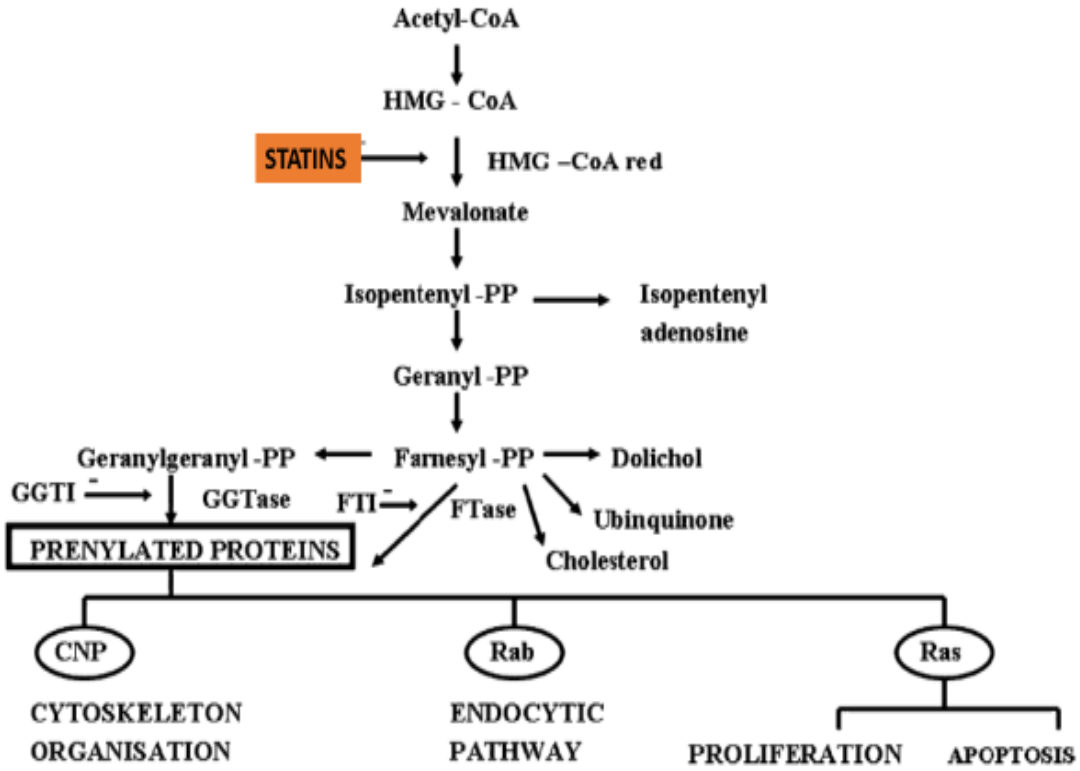
Inhibition of cell death by apoptosis in ovarian cancer cells can also result in chemotherapeutic drugs resistance. Inhibition of cell death may be caused by expression of the Bcl-2 family of apoptosis regulators such as Bcl-X_L and Bcl-2. [211].

1.11. The Mevalonate Pathway

The Mevalonate Pathway is considered as the most important and fundamental metabolic network for cholesterol synthesis in the cell[212]. It produces some essential constituents of the cells, including cholesterol, geranylgeranyl pyrophosphate and farnesyl pyrophosphate which tightly regulates proliferations and growth of cells. Therefore, blocking this pathway interfers with protein prenylation [213].

Statins inhibit HMG-CoA-reductase enzyme, the rate limiting step in lipid synthesis pathway . There are several commercially available statins. Type 1 derivatives of statins include lovastatin, simvastatin, and pravastatin. Full synthetic analogue (type 2 statins) include fluvastatin, cerivastatin, atorvastatin, rosuvastatin and pitavastatin. These synthetic products show better pharmacodynamics activities due to the size and polarity of the groups attached to the HMG-mimetic moiety and result in improved binding to HMGCR.

These drugs are currently widely used to reduce plasma cholesterol. Unfortunately, statin-induced myopathy is one of the most common adverse effects associated with statins and in extreme cases can result in rhabdomyolysis [214].



Figure(1-8): The mevalonate pathway, Prenylated proteins play critical roles in cytoskeletal, cell growth and apoptotic regulation [215].

1.11.1. Protein Prenylation and the Role in Cancer

1.12.1.1. Protein Prenylation

The pathway for the biosynthesis of mevalonate, isoprenoid and cholesterol plays a crucial role in human health and disease. The importance of this pathway is highlighted by the discovery that two major isopreneids, farnesyl and geranylgeranyl pyrophosphate, are needed to alter a variety of proteins via a process called protein prenylation[216]. Farnesyl diphosphate and geranylgeranyl diphosphate are used to post-translationally prenylate several families of proteins[217], [218]. These proteins have a motif which identifies them for prenylation. A carboxy-terminal CAAX motif is recognised by the prenylation enzymes farnesyltransferase (FT) or geranylgeranyltransferase-I (GGT-1). CAAX represents cysteine (C), two aliphatic moiety (AA) and any amino acid(X), respectively [219]. The enzyme CAAX prenyltransferase

transfers either a 15-carbon farnesyl or 20-carbon geranylgeranyl to the cysteine residue. The protein is either a substrate of FT or GGT1 depending on the terminal X amino acid. FT catalyses the interaction with CAAX motifs in which X is methionine, serine, glutamine or cysteine, while GGT1 interact preferably with CAAX motifs in which X is leucine or isoleucine. A subsequent step taken place in the endoplasmic reticulum where the three terminal amino acids (AAX) of the prenylated protein are removed by enzyme Ras-convertase-1 protease resulting in a C-terminus prenylcysteine [220]. However, Rab family proteins possess a CC or CXC sequence instead of CAAX structure. These motifs are the binding site for Rab escort protein and through which they present the protein to geranylgeranyltransferase-II (Rab geranylgeranyltransferase, GGT2) [221]. The enzyme transfers two geranylgeranyl groups to the cysteine residues at CC and CXC motifs of the Rab protein and this phenomenon has been called a "dualistic prenylation".

Prenylation of small GTPases is essential for their localisation to the cell membranes[222]. However, the small GTPases are further regulated by the binding of GTP and its hydrolysis to GDP. The cycling of the prenylated proteins between activated and deactivated states is regulated by guanine nucleotide exchange factors (GEFs) and GTPase proteins, resulting in downstream signal propagation. The transport and/or dissociation from cell membrane compartments is regulated by binding of guanine nucleotide dissociation inhibitors (GDI) to the prenylated inactive proteins[223]. Upon arrival at their final destination, the prenylated proteins are detached from the GDIs resulting in liberation of the protein to the membrane in a reaction catalysed by is catalysed by GDI displacement factors (GDFs)[223]. Many prenylated proteins are part of cancer pathogenesis involved in cancer cell replication, apoptosis, dissemination and blood vessel formation [224].

1.12.1.2. Prenylated Proteins Involved in Cancer Development

Prenylated protein including small GTPase play an important role in tumourigenesis.

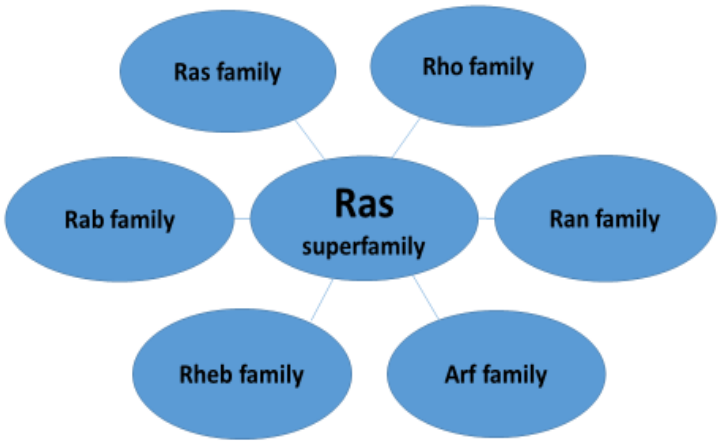


Figure (1-9): Ras-superfamily the subfamilies of Ras-superfamily are shown

1.12.1.2.1. Ras GTPases

GTPases are a large group of proteins including Ras subfamily including, Harvey rat sarcoma viral oncogene homolog (H-Ras), K-Ras and N-Ras, all of which are confirmed to be mutated in human cancers [225]. Both K-Ras and N-Ras are farnesylated and geranylgeranylated while H-Ras is farnesylated, and this is essential for localisation to the cell membrane and interactions with downstream signalling molecules (e.g. Raf-1) [226]. It has been reported that ovarian cancer shows overexpression of both K-Ras and N-Ras [227]. Activation of oncogenic Ras pathway activates subsequent signalling pathways (e.g. Raf/Mek/Erk and PI3K pathways) resulting in the overgrowth, replication and survival of cancer cells[225].

1.12.1.2.2. Rho GTPases

The Ras superfamily includes Rho family of GTPases. This subfamily comprises around 20 GTP-binding proteins such as RhoA, RhoB, RhoC, Rac1 and Cdc42. Some of these proteins are solely geranylgeranylated, for example, RhoA, RhoC, Rac1 and Cdc42 [228], [229], while RhoB is either geranylgeranylated or farnesylated [229]. Rho GTPases have been shown to enhance cancer cell survival in some cell types [230]. Similarly, RhoA, Cdc42 and Rac1

promote cell cycle progression and Ras-dependent alteration. Rho GTPases have also been demonstrated to control processes during angiogenesis and cell dissemination, including the formation of membrane profusions [231].

1.12.1.2.3. Rab GTPases

The Rab subfamily has more than 60 members [232]. Rab GTPase's primary role is to regulate the trafficking of vesicles between organelles regulating protein secretion, endocytosis, recycling and degradation [232], [233] In some tumour types, Rab GTPases have been shown to contribute to tumour-stromal cell communication and advancement of the cell cycle [234]. Rab proteins were also involved in the development and metastasis of cancer[232]. Rab25 was discovered to encourage migration and cancer cell invasion, and its over-expression is also associated with poor survival of ovarian cancer patients[235]. Finally, a number of this family's proteins also participate in drug resistance. Rab4a and Rab6, for instance, are under-expressed in multidrug resistant cells, whereas their overexpression is linked with enhanced sensitivity of cancer cells to cytotoxic medications due to enhanced intracellular accumulation. By comparison, Rab8 overexpression increases their resistance to cisplatin in delicate cancer cells [234].

1.12.1.2.4. The Arf family

The Arf family consists of about 27 proteins and there are three Arf protein classes, Class I (Arfs1–3), Class II (Arfs 4–5), and Class III (Arf6). They were involved in many cellular procedures, including vesicle membrane transport, morphology, metabolism, actin cytoskeleton, endocytosis, and exocytosis[236]. Despite being part of the Ras superfamily, Arfs are not prenylated, but instead are located on the membrane by adding a myristate fatty acid moetiy [223]. The Arf family plays a key role in the development of cancer and could be a prognostic factor for cancer patients. The aberrant activity or expression of Arf family proteins

has been demonstrated as a function in migration, invasion and proliferation[237]. Some Arf protein expression in breast cancer cell lines has been discovered to be upregulated [238]. Finally, Arf has lately been revealed to be negatively correlated with miR-221-3p, whose greater expression is connected with improved general survival in patients [239].

1.12.1.2.5. Ran GTPase

Nucleocytoplasmic transport is the major cellular function of Ran GTPase. The Ran protein consists of the assembly of mitotic spindles, the nucleation and dynamics of microtubules and the post-mitotic atomic assembly [240]. Numerous studies have shown that Ran GTPase is involved in cancer cell development, tumour transformation, apoptosis resistance, tumour aggression and increased metastasis in several cancer types [241]. Ran is overexpressed cancer tissue relative to ordinary tissue and its expression is linked with tumour development. In cancer cells, acute Ran silencing induces mitochondrial dysfunction and leads to cell death. Thus, this evidence suggests that the Ran pathway may be a useful target for cancer therapy [242].

1.13. Molecular Mechanisms of Tumourigenesis and the Cytotoxic Activity of Statins

1.13.1. The Cell Cycle and Cancer Progression

In normal cells proliferation, many hundreds of genes normally control the cell division process. Many of these genes are oncogenes or tumour suppressors. Cyclin dependent kinases (CDK) and cyclins form complexes which regulate the cell cycle intracellular machinery. The growth-dependent cyclin-dependent kinase2 (CDK2) activity promotes DNA replication during the G_1 phase and initiates the G1-to-S step transition [243]. Hypophosphorylated Rb proteins exerts an inhibitory activity on G_1 phase of the cell cycle through binding to transcription factors including E2F and downregulating the expression of genes whose synthesis is required for cell cycle propagation. Moreover, proteins like p15, p16, p17, p19, p21 and p27 are CDK inhibitors (CDKI) and they exert their activity through inhibition of activation of cyclin/CDK complexes

(M) [244]. This controlsthe transcription of the genes regulating G1–S transition including the gene encoding cyclin E. Cyclin E forms an complex with CDK2 proteins[245]. Cyclin D/CDK4/6 phosphorylate Rb, releasing E2F and allowing the cell enters S-phase. The cell then enters a second gap phase (G_2) which functions to prevent the cell from undergoing mitosis with DNA damage. During this stage, the cyclin B activates CDK1, and further activated by the phosphatase Cdc25, thereby allowing G_2 phase transition to mitosis (M). However, upon DNA damage or stalled replication, the phosphatase Cdc25 is hyperphosphorylated which results in the ubiquitination of Cdc25 and inhibition of cell cycle [246]. Aberrations in oncogenes (e.g. cyclins or CDKs) or tumour suppressor genes (e.g. *Rb1* or CDKIs) have been associated with tumourigenesis. In high grade serous ovarian cancer, the genes encoding p16 and Rb are inactivated in 32% and 10% of cases respectively. Furthermore, genes encoding cyclin D_1, cyclin D_2 and cyclin E_1 are l amplified in 4-20% of high grade serous ovarian cancer cases, likely participating in the induction of some ovarian cancers [243].

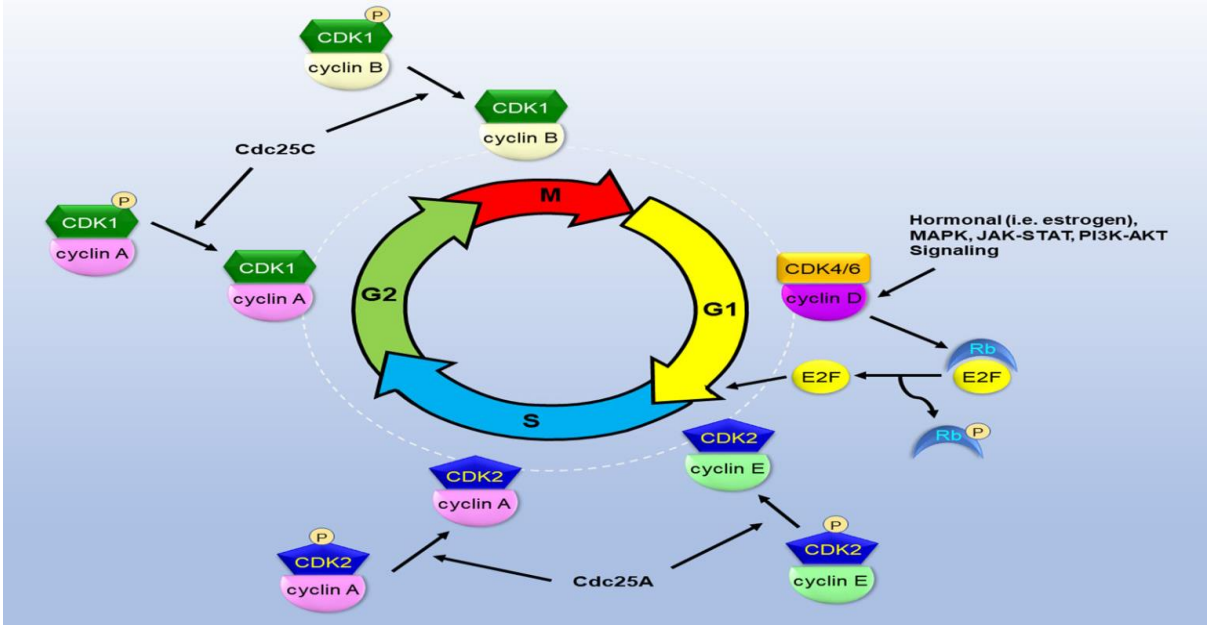


Figure (1-10): The role of Cdc25s which involve a Cellular Interactions [247].

1.13.2. Statins & apoptosis

In several and different kinds of cancers including breast [248]–[251], lung [252]–[254], prostate [254], [255], liver [256], [257], colon [258], [259], ovarian [260]–[262], bladder [252],

thyroid [263], [264], glioma [265], [266] lymphoma [259], [267], myeloma [268], osteosarcoma [269], head and neck squamous cell [270], and medulloblastoma [271] statins have been shown to induce apoptosis, mainly via the mitochondrial pathway; such pro-apoptotic effects have been induced on the mevalonate pathway via the degradation of geranylgeranyl diphosphate [252], [262]–[267], [269]. A variety of studies have shown that statins inhibit geranylgeranylation of RhoA, Rac1 and Cdc42, and subsequent translocation to the plasma membrane [264], [272].

Several preclinical studies have shown that incubation of cells with statins leads not only cell-cycle arrest but also cause cell death, via apoptosis, especially when prolonged exposure of cells to statins. Statins arrest cancer cells in G_1 or S phase [273]. The arrested cancer cells in the S phase have shown increased phosphorylation and increased activation of Chk1 kinase. Chk1 kinase promotes the activation of p53 which in turn is responsible for the transcription of proteins that arrest the cell cycle. Cdc25 phosphate activity and level were also observed to decrease after exposure to statins, along with reduced CDK2 cycline A, caspase cleavage 9, downregulation of Bcl-2 and subsequent apoptosis [274]. Another study showed obvious increase in the activation of caspase-8 [275] implicating the extrinsic pathway in the anti-cancer activity of statins. Reducing cholesterol-rich membrane rafts that can lead to Fas (CD95) induction, the development of Fas-FADD complexes, and the activation of extrinsic apoptosis and activation of caspase-8, the exclusion of Fas from lipid rafts in some cell types leads to spontaneous, ligand-independent activation of this death receptor [276]. Furthermore, there is a proteolytic activation after treatment cholangiocarcinoma cell lines with statin that caused activation of executioner caspase 7 and their finding shows that simvastatin induces death of cholangiocarcinoma cells by disrupting Rac1/lipid raft colocalization and Rac1 depression [277]. Treatment of lymphoma cells with fluvastatin caused chromatin condensation and DNA fragmentation [278], confirming the induction of apoptosis. In summary, these studies indicate

that statins can cause apoptosis in cancer cells possibly as a result of the inhibition of the synthesis of cholesterol or inhibition of small GTPase prenylation.

1.14. The Potential of Statins for Cancer Prevention or Treatment

1.14.1. Statins and Cancer Risk Reduction

1.14.1.1. Clinical Trials of statins for Cancer Treatment

A limited number of phase I / II clinical trials have been performed to date investigating the use of statins in treating a variety of different cancers. This research evaluated statins both as single agents as well as in combination with chemotherapy. In addition, a small number of studies evaluated statins for up to 7 days at the maximum tolerated dose. Multiple clinical trials of statins evaluated their toxicity profile in combination with several chemotherapeutic regimens Simvastatin (40 mg / day) plus irinotecan,5-fluorouracil, and leucovorin (FOLFIRI) led to a 21.8-month median survival in colorectal metastatic patients, with no further adverse effects from simvastatin supplement [279]. This was accompanied by a phase I study in patients with acute myeloid leukaemia where pravastatin (40-1680 mg / day) did not increase neutropenia or thrombocytopenia in combination with idarubicin and cytarabine, and the overall toxicity profile was unchanged compared to cancer treatment with chemotherapy alone[280]. Stage I / II clinical trials incorporating cyclosporine A, pravastatin, etoposide and mitoxantrone in an effort to circumvent drug resistance in acute myeloid leukaemia were aborted early due to undesirable toxicity[281]. This highlights the value of performing a small phase I trial and carefully evaluating the risk-benefit ratio while considering new drug combinations. For non-small lung cancer (NSCLC), Han and colleagues' studies showed that adding a normal dose of simvastatin (40 mg / day) to chemotherapy (irinotecan and cisplatin or gefitinib) did not significantly increase progression period or the one-year rate of survival[282], [283]. Nonetheless, in a subgroup of non-adenocarcinoma patients with wild-type epidermal growth factor receptor (EGFR) [282], patients receiving gefitinib and simvastatin had a longer PFS

compared to those receiving gefitinib as a single drug (3.6 months versus 1.7 months, P= 0.027). Nevertheless, the previous findings were confirmed by a study published in the journal in advanced patients with pancreatic cancer, where simvastatin (40mg / day) in combination with gefitinib had no additional clinical significance compared with gemcitabine alone [284]. Normal doses of pravastatin (20-40 mg / day) in hepatocellular cancer patients greatly increased PFS in transarterial chemoembolization (TACE) patients (18 months versus 9 months, P= 0.006 [285]; 20.9 months versus 12 months, P=0.003[286]). In comparison, pravastatin (40-80mg / day) alone did not boost the median OS relative to TACE-treated patients [287]. In addition, there has been no change in outcome in advanced patients with stomach cancer treated with pravastatin 40 mg / day in combination with epirubicin, cisplatin, and capecitabine[288]. For patients with squamous cell carcinoma of the head and neck or cervix, high-dose lovastatin has been tested. During this Phase I trial, patients tolerated doses of up to 7.5 mg / kg / day and had stabilisation of disease approximately one quarter [289]. These findings were contrary to a previous study in which high lovastatin doses of 35 mg / kg / day were administered for 7 consecutive days (repeated every 28 days) to patients with advanced gastric cancer and no respond was observed [290]. In addition, simvastatin (15 mg / kg / day) in patients with myeloma in combination with vincristine, adriamycin, and dexamethasone was discontinued due to poor response, because only one patient had a partial response [291], whereas the high dose was quite well tolerated for 7 days [292]. Nonetheless, a study in patients with relapsed and refractory multiple myeloma found that thalidomide, dexamethasone, and lovastatin (0.5 mg / kg / day) substantially enhanced PFS compared with patients receiving just thalidomide and dexamethasone (33 months versus 16 months, P= 0.048), with comparable side effect profiles of both arms [293]. It has been shown that high-dose fluvastatin (80 mg / day) decreases tumour proliferation and induces apoptosis in high-grade breast cancer in stage 0/1[294]. In addition, fluvastatin (8 mg / kg / day) substantially improved survival between days 1-14 in conjunction with chemotherapy, decreased tumour volume and boosted quality of life in

children with brain stem tumours [295]. However, there was no significant PFS change in standard doses of fluvastatin (40 mg / day) or atorvastatin (20 mg / day) in combination with zoledronate relative to the findings of previous studies in patients with renal-cell carcinoma and bone metastasis [296]. Overall, clinical studies testing statins at normal doses used to treat high cholesterol showed minimal beneficial effects, and only two studies in patients with hepatocellular cancer indicated a survival advantage. This may represent the transporter liver-selective uptake of pravastatin like OATP1B1, thus potentially raising the concentration of pravastatin in contact with the tumour [297]. Despite this weak positive story, the majority of the current clinical trials test statins for cancer care at doses which give rise to low drug concentrations in plasma [298]. Trials using higher doses of statins have shown some promising outcomes, and some clinical trials are currently testing lower doses of statins in combination with standard chemotherapy agents for leukaemia, glioma and lung cancer treatment [298].

1.14.1.2. Xenografts of Statins as anticancer activity

Human ovarian cancer xenografts are commonly used to assess the activity of preclinical drugs. Xenograft studies with ovarian cancer cell lines have been used to evaluate drug cytotoxic activity and model biomarker responses. These studies make us of immunodeficient mice although this eliminates any activity of a healthy immune system on tumour growth or the interaction between tumour cells [299]. In addition, there may be significant variations in drug pharmacokinetics in mice xenografts compared to human pharmacokinetics which must be taken into account when conducting a research on xenograft[1].

A wealth of evidence has shown how important the MP is for health and disease. In some animal models the inactivation of the MP seems fatal. Mice with HMGCR deficiency stop growing at the blastocyst stage while mice with squalene synthase deficiency exhibit growth

retardation and neural tube defects[300]. Furthermore, gunmetal mice, a GGT-II deficient mouse, usually mature but show a defect in platelet function and prolonged bleeding time [301]. Statin have shown anti-tumour effects uses cell lines origination from thyroid cancer [302]. Rat lymphoma [303], rat fibrosarcoma [303], mouse mammary tumour [304], murine colon tumour [305] and mouse melanoma [306]. Statins have also increased the antitumour effect of the tumour necrosis factor by inhibiting angiogenesis caused by tumour in a murine tumour model[307]. Previous studies in our laboratory have shown that pitavastatin causes regression of ovarian cancer xenografts in mice [308]. In vitro studies have shown that cerivastatin, pitavastatin, and fluvastatin are the most potent anti-proliferative, autophagous inducing agents in human cancer cells including primary glioblastoma cell lines of stem cells[309]. The combination of vorinostat and fluvastatin induced robust apoptosis and effectively inhibited both *in vitro* and *in vivo* development of renal cancer[310].

1.14.1.3. Preclinical study

Preclinical in vitro studies in various cell lines have demonstrated statins' ability to inhibit cell growth. Statins exhibit antiproliferative, pro-apoptotic and anti-invasive activity in various cell lines of cancer [311]. Numerous in vitro studies have shown that statins have important tumour-suppressive effects on various leukaemia and solid tumour cells[312]. Experimental studies have drawn the following conclusions. First of all, various statins have clear anti-proliferative and proapoptotic effects on different lineages of cancer cells. Secondly, the anti-neoplasty results only display lipophilic statins. Fluvastatin, simvastatin, and lovastatin is cytotoxic to adenocarcinoma breast cells [313]; Atorvastatin, simvastatin, lovastatin and cerivastatin were cytotoxic to cancer cells in myeloma [272], and simvastatin and lovastatin to cancer cells in ovaries [314]; Third, the statins vary in their ability for anti-neoplastics. Four cell lines of acute myeloid leukaemia were analysed using specific statins. Cell lines were most sensitive to cerivastatin, tenfold less sensitive to lovastatin and fluvastatin, and just slightly atorvastatin-

sensitive [315]. Finally, cytotoxicity of statin can depend on the type of target tumour. Dimitroulakos et al. identified a subset of tumours (young monomyelocytic leukaemia, medulloblastoma, rhabdomyosarcoma, choriocarcinoma, and cervical, head and neck squamous cell carcinomas) that are prone to lovastatin-induced apoptosis and display HMG-CoA reductase as a possible therapeutic target for these cancers [316]. Statin antimyeloma activity in humans was first recorded in refractory multiple myeloma (MM) with simvastatin administration [317]. A preoperative research in primary invasive breast cancer patients explored atorvastatin-induced effects on tumour proliferation and expression of HMGCR while evaluating HMGCR as a predictive marker for statin response, the findings of this study indicate that HMGCR is inhibited by statins in *in vivo* breast cancer cells and that statins in HMGCR-positive tumours may have an antiproliferative effect[318].

1.14.1.4. Retrospective studies

Meta-analyses evaluating the likelihood of cancer-related death in patients using statins to control plasma cholesterol have also suggested an anti-cancer effect of statins. However, mixed results have been obtained. For example, there was not a significant correlation between statin therapy and breast[319], lung [320], colorectal, melanoma or non-melanoma [321] bladder [322], renal[323], and pancreatic cancers[324] in several studies. In contrast another 4 meta-analysis studies in Asian and Western population reported a reduction in the risk of liver cancer in patients treated with statins (RR: 0.58 – 0.64) [322], regardless of the type of statin (hydrophobic versus hydrophilic) or duration of the therapy[325]. Furthermore, statin therapy was associated with a reduction in the risk of gastric cancer [326] and haematological malignancies (RR: 0.74 – 0.81) [327] while it has no effect on the risk of prostate cancer [328]. Meta-analyses have reported that statin treatment reduced the risk of advanced prostate cancer by 20% (RR: 0.8, $P<0.001$ [329], and improved survival in prostate cancer in post-radiotherapy patients [330].

Regarding the activity of statins against ovarian cancer, Elmore and colleagues reported that patients with end stage or invasive epithelial ovarian cancer that used statins showed significant improvement in PFS and OS in comparison to non-statin users [331]. Additionally, a case-control study showed that statins significantly reduce the risk of ovarian cancer and mortality [332]. In another study, despite a non-significant improvement in PFS or disease-specific survival (DSS) in statin-based patients with ovarian cancer using statins in comparison to non-statin user, patients with non-serous papillary subtypes showed improvement in both PFS and DSS [333]. Collectively, a recent meta-analysis combining different studies reported that statin therapy induces acceptable protection against ovarian cancer (RR: 0.79), and prolonged therapy (>5 years) induced reduction in the risk of ovarian cancer (RR: 0.48) [334]. However, a meta-analysis of patients using statins to reduced plasma cholesterol showed discrepant results regarding cancer risk association with statin therapy. Some of these studies reported that generally there is no correlation between statin use and cancer risk [214].

1.14.2. Foods deregulate a mevalonate cancer pathway

MP de-regulation was first confirmed in mouse hepatomas about 50 years ago [335]. Some studies indicate that HMG-CoA reductase plays a major role in human cancer. Most cancers displayed either increased expression and HMGCR activity or lack of feedback regulation of HMG-CoA reductase activity [336]. Synthesis of cholesterol that rely on the availability of MP precursors like Acetyl-Co-A [337]. For development, cancer cells need synthesis of de novo lipids. Increased lipid synthesis was found to be involved in the pathogenesis of cancer including ovarian neoplasms [338] . Evaluation of the MP in cancer by Fumagalli et al. (1964) [339] confirmed that large amounts of cholesterol are synthesised by human glioblastoma cells. In addition, exogenous mevalonate administration has been shown to encourage growth of

tumours in xenograft-bearing mice [340]. Furthermore, mevalonate induces the growth of human cancer cell-tumours *in vivo* and induces proliferation *in vitro* [340].

There are many dietary components known to inhibit HMG-CoA reductase activity and the synthesis of mevalonate. In comparison to statins, these compounds frequently have no recorded adverse effects. Evidence that inhibition of mevalonate synthesis by such components can mediate protective effects on experimental breast cancer has been obtained using cholesterol, plant isoprenoids, genistein, and long-chain n-3 polyunsaturated fatty acids (PUFAs)[341].

Importantly, however, diet may alter the activity of statins as anti-cancer agents. Mevalonate is a precursor to cholesterol farnesol and geranylgeraniol isoprenoids[342]. If statins exert their anti-cancer activity by blocking the synthesis of these, the presence of these compounds in food may bypass the anti-cancer activity of statins in patients. In support of this, solvent extracts from human and mouse food have also been able to block pitavastatin-induced apoptosis *in vitro* and dietary geranylgeraniol blocks the growth of ovarian xenografts in mice, This suggests that a patient's diet could influence the result of clinical trials[308] Specifically, a diet containing geranylgeraniol may restrict pitavastatin's anti-tumour activity and consequently diet should be controlled in clinical trials of statins as anti-cancer agents. Although the complete human food supplement Ensure does not contain geranylgeraniol it may be monotonous for patients to consume only this during a clinical trial. This makes it important to understand which foods contain geranylgeraniol, or any other isoprenoid that interferes with the anti-cancer activity of statins. Geranylgeraniol diterpenes (GGOH), has been identified in rice, sunflower oil, linseed oil, Cedrela to on a wood oil, sucupira branca fruit oil, and annatto seed oil[343]. However, the abundance of geranylgeraniol in other foods is unknown. The identification of foods which lack significant quantities of geranylgeraniol would facilitate a clinical trial of

statins in cancer because it would provide patients the opportunity to eat a more varied diet while taking statins.

Chapter 2

Aims and objectives

2. Aims and Objectives

Three different aims were set for this project. Firstly, to support clinical trials of pitavastatin in ovarian cancer patients by evaluating which foods patients do not interfere with the activity of pitavastatin. Secondly, identify genes whose expression correlates with sensitivity of cancer cells to pitavastatin to help further understand the mechanism of action of statins as anti-cancer agents. Finally, to identify additional drugs that are synergistic with pitavastatin's anticancer activity in the expectation that this would allow lower doses of pitavastatin to be used and consequently minimize the likelihood of adverse drug reactions.

The following targets were set to accomplish this.

1- Prepare extracts from a range of human foods and evaluate whether these inhibit the cytotoxic activity of statins in cell growth assays using ovarian cancer cell lines.

2- Make use of publically available database reporting gene expression profiles and drug sensitivity data to identify candidate genes whose expression correlates with activity of statins in cancer cells. Test the expression of these candidate genes in ovarian cancer cell lines and compare with the sensitivity of these cells pitavastatin. Use this information to develop and test hypotheses around the mechanism of action of pitavastatin in cancer.

3- Conduct drug repurposing studies to evaluate whether the anti-parasitic agent ivermectin is synergistic with pitavastatin in cell growth assays and then confirm these results in further assays measuring apoptosis.

Chapter 3

Materials and Methods

3. Materials and Methods

3.1. Cell culture

3.1.1. Ovarian cell lines

The ovarian cancer cell lines used in these studies which were initially selected from the "NCI-60" panel of cells. Subsequent information [344] identified cells lines that are more representative of high grade serous ovarian cancer (HGSOC) and these were then used instead (Table 3-1).

Table (3-1): Description of the cell lines used in this study.

Cell line	Description
COV-362	This cell line was formed by a pleural effusion of female carcinoma with epithelial endometroid1 [345]
Ovcar-4	This cell line was developed by a woman with high-grade ovarian serous adenocarcinoma resistant to cisplatin1 [346].
COV-318	This cell line was developed by a female with ovarian epithelial-serous carcinoma peritoneal ascites1[345] .
OVSAHO	This cell line was derived from serous papillary adenocarcinoma of high ovarian grade1 [347]
OVCAR-8	This cell line was derived from a high-grade ovarian serous adenocarcinoma from carboplatin-resistant ovarian carcinoma1 [348].
FUOV-1	This cell line was obtained after hysterectomy from a woman's tumour tissue with high-grade serous ovarian cancer1[346], [348].

3.1.2. Mediums and conditions for cell growth

Human ovarian cell lines (Ovcar4, Ovsaho and Ovcar-8) were grown in Roswell Park Memorial Institute (RPMI 1640; Lonza) medium supplemented with 10 % fetal bovine serum (FBS; Lonza), 2 mM glutamine (Lonza) and 50 μg/mL penicillin/streptomycin (Lonza). Fuov-1, Cov318 and Cov362 were grown in DMEM medium supplemented with 10 % fetal bovine serum (FBS; Lonza), 2 mM glutamine (Lonza) and 50 μg/mL penicillin/streptomycin (Lonza).

Cells were incubated in a NAPCO water-jacketed incubator (Precision Scientific) at 37°C and in a humidified 5% CO2 atmosphere.

3.1.3. Reviving Cryopreserved Cells

Frozen cells in liquid nitrogen were rapidly thawed in a Grant JB Series water bath (Grant Instruments) at 37°C and then added to 2 mL pre-warmed growth medium in a 15 mL polypropylene tube. Cells were then centrifuged at 150 *g* for 3 minutes at room temperature and the pellet was re-suspended in 8 mL growth medium. The resulting cell suspension was transferred into a T25 tissue culture flask and incubated for 24 hours at 37°C before checking the viability under a phase-contrast light microscope. Subsequently, the growth medium was replaced to remove residual DMSO and dead cells. The adherent cells were grown to an appropriate density for experimentation or sub-culture.

3.1.4. Trypsinisation of Adherent Cells

All cells more than 80% confluent as determined by using an Olympus CKX41 light microscope were sub-cultured. The cells were washed with phosphate buffered saline (PBS; Lonza, 3 mL for 25 flasks and 6 mL for T75 flask) and subsequently exposed to trypsin (Lonza) (1700 U/L) EDTA (0.002 g/L) diluted in PBS, incubated at 37°C and gently agitated to encourage detachment. The trypsin was neutralized by the addition of 1 mL growth medium. Cells were transferred into a 15 mL polypropylene sterile tube, centrifuged at 150 *g* for 3 minutes in a Thermo Scientific Heraeus Megafuge 8 centrifuge at room temperature. The supernatant was aspirated carefully and the pellet was re-suspended in fresh cell culture medium. At least 100 cells were counted by using a Neubauer haemocytometer to determine the appropriate number of cells for experiments (see below) or for routine passage 25% of the cells were transferred to fresh T25 or T75 tissue culture sterile flasks (Sarstedt).

3.2. Chemical agents

Pitavastatin (Adooq) & Ivermectin (Sigma-Aldrich), geranylgeranyl (Sigma-Aldrich), mevalonate (Enzo Life Sciences), isopentenol (Sigma-Aldrich), Farnesol (Sigma-Aldrich) dolichol (Avanti) and Coezyme Q10 were dissolved in DMSO (20 mM). siRNAs (Dharmacon) were dissolved (20 μM) in siRNA buffer (Dharmacon).

3.3. Extraction of Lipids from Foodstuffs

Thirty foodstuffs were obtained for the purposes of extracting isoprenoids. These comprised oils, food and fruits and were obtained in the United Kingdom from commercial suppliers. These included kiwi, pears, pomegranate, lettuce, cherry, tomato, squash, potato, passion fruits, gooseberry fruit, butter and milk (Aldi), strawberry jam, cheese, spaghetti, oat, beans, nuts, eggs, dolmio (spaghetti sauce), black bean, oats and bread (Tesco), cheddar, corn oil, pure Ripe seed oil, Sunflower oil, ground nut oil, grape seed oil and sesame oil(Sainsbury's),

Extracts were prepared by the method reported by[349] , with some modification of the "gold standard" methods by Folch [350], Bligh, and Dyer [351]. Each solid foodstuff (50 g) was homogenized in a food electric blender and then transfer to a mortar and homogenised manually with a pestle in 60 mL methanol. 30 mL of chloroform/methanol (50%/50%) were added and the extract homogenized briefly again. The mixture of extracts and solvents were filtered through fluted filter paper. Oils (50 g) were directly mixed with methanol and chloroform methanol without homogenization. Both types of mixture extracts (oils & foods) were transferred to a separating funnel and the upper layer collected and evaporated by using an RE100 rotary evaporator (Fisher Scientific) to dryness in a round bottom flask. The dried residues were dissolved in 25 mL 99% ethanol and 25 mL 5M potassium hydroxide were added and the solution incubated at 56°C for 1 hour in a water bath. After cooling and neutralisation with 25 mL 5 M hydrochloric acid (Sigma-Aldrich) and addition of 30 mL water, the resulting solution was partitioned with 120 mL n-hexane (Fisher Scientific) The upper phase was

collected in a pre-weighed round bottom flask and evaporated using an RE100 rotary evaporator to dryness. Residual solvent was removed under vacuum in a freeze dryer overnight and the weight of the extract determined. The residue was dissolved in 1ml DMSO and stored at -20°C for later analysis.

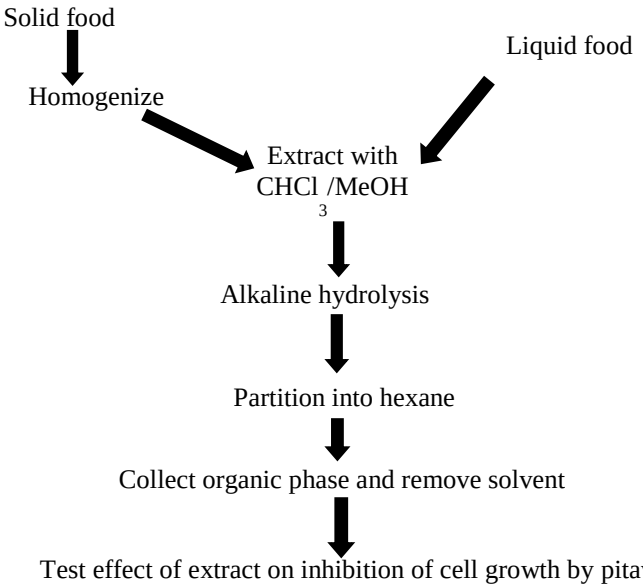


Figure (3-1): the method of isoprenoid extraction[350]–[352].

3.4 Cell Growth Assays (sulforhodamine B assay)

Cells were collected by trypsinization and suspended in the culture medium (62,500 cells / mL, with the exception of OVCAR-8 cells which were suspended at a concentration of 25,000 cells / mL). 80 µL of the suspension was seeded into each well of a 96 well plate and incubated for 24 hours at 37 ºC in 5% CO_2. The following day, cells are treated with pitavastatin and/or geranylgeraniol at the indicated concentrations in 20 µL of growth media alone or in combination with 20 µL food extracts. Each experimental condition was measured in triplicate wells. The cells were incubated for 72 hours, the growth medium removed and the cells in each well were fixed in 100 µL cold 10% trichloroacetic acid (TCA, Sigma-Aldrich) for 30 minutes on ice. The TCA was removed and the cells washed in water, before being left to dry. They were then stained with 0.4% sulforhodamine B (SRB, Sigma-Aldrich) in 1% acetic acid (Sigma-Aldrich) for 30 minutes. Excess SRB was removed by washing the wells three times in

1 % acetic acid and the plates dried. Lastly, the dye was solubilised in 100 µL 10 mM Tris (pH 10, Sigma-Aldrich) and the absorbance at 570 nm (A570) was determined using a BioTek Synergy 2 multi-mode microplate reader.

3.5. GC/MS experiments

One—two mg of bioactive extract fractions were dissolved in 200 µl ethylacetate and sonicated for 5 minutes at 40 °C. Subsequently, 1-2 µl of the solution was injected into the gas chromatography mass spectrometer (GC-MS), an Agilent 7890 coupled with Agilent MS type 5975 C MSD (Agilent Technologies, USA). The gas chromatography started for two minutes with an initial oven temperature of 60 °C and increased to 300 °C at a rate of 10 °C / min, followed by 4 minutes at 300 °C to produce a total run of 30 minutes at a steady helium pressure (10 psi). Mass spectral data were acquired in scanning mode within the 40-1000 m/z range.

3.6. Trypan blue assay

To estimate the cell viability 100,000 cells / well were seeded in 12 well plates and incubated overnight and the cells exposed to the drug for 24-72 hours as indicated. To assess viability, the supernatant was removed from the cells and the cells collected by trypsinization. The detached cells were added to their corresponding supernatants, centrifuged at 150 *g* for 3 minutes and the pellet re-suspended in 0.5 mL of medium. The cells were mixed with an equal volume of 0.4 % (v/v) trypan blue (Sigma-Aldrich) and viable and non-viable cells counted by light microscopy by using Neubauer haemocytometer. The viability percentage was determined on the basis of the following formula.

Viability = 100 x number of viable cells / (number of dead cells + number of live cells).

3.7. Measurement of annexin V/PI labelling

Cells were plated and exposed to drugs accordingas described in section 3.5. Cells were stained with Annexin V/Propidium iodide using a commercial kit (Miltenyi Biotech). Cells were

washed with 1 mL of 1X annexin V (Miltenyi Biotec) binding buffer and the cells collected by centrifugation (300 *g*, 10 minutes). The supernatant was aspirated and the cells were re-suspended in 100 µL of 1X binding buffer. 10 µL of Annexin V were added and the suspension gently mixed and stored in the dark at room temperature for 15 minutes. After incubation the cells were washed with 1 mL of 1 × annexin V binding buffer again and collected by centrifugation (300 g, 10 minutes). The supernatant was removed and the cells re-suspended in 500 µL of 1X of binding buffer. To detect dead cells, 5 µL PI solution was added immediately before flow cytometry analysis.

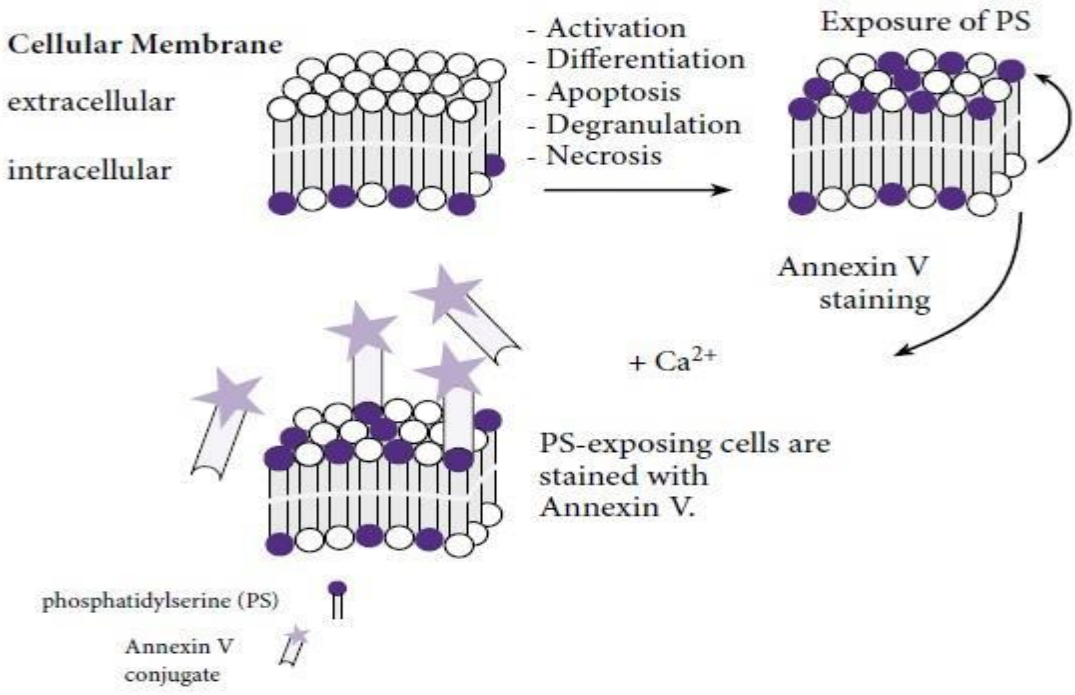


Figure (3-2): Annexin V staining mechanism.

3.8. Caspase 3/7 activity assay

Caspase activity was measured using the Caspase-Glo assay (Promega; Madison, USA). Cells were collected by trypsinization as described above and resuspended (62,500 cells / mL) and 80 µL seeded per well in 96 well plates and incubated at 37 ºC overnight. The next day, 20 µL of pitavastatin and/or 20 µL of ivermectin added to the indicated concentration. The cells were

incubated for 72 hours at 37 ºC. To test caspase 3/7 activity assay, Caspase 3/7 reagent was prepared in compliance with the manufacturer's instructions and then stored at -80 ºC. 25 µL of Caspase-Glo 3/7 reagent was added directly to each well. The plate was covered with foil to shield it from light and gently agitated on a rocker for 30 minutes at room temperature. Subsequently, 100 µL of each well was transferred to a white 96 well plate. The luminescence was being measured using a BioTek Synergy 2 multi-mode microplate reader.

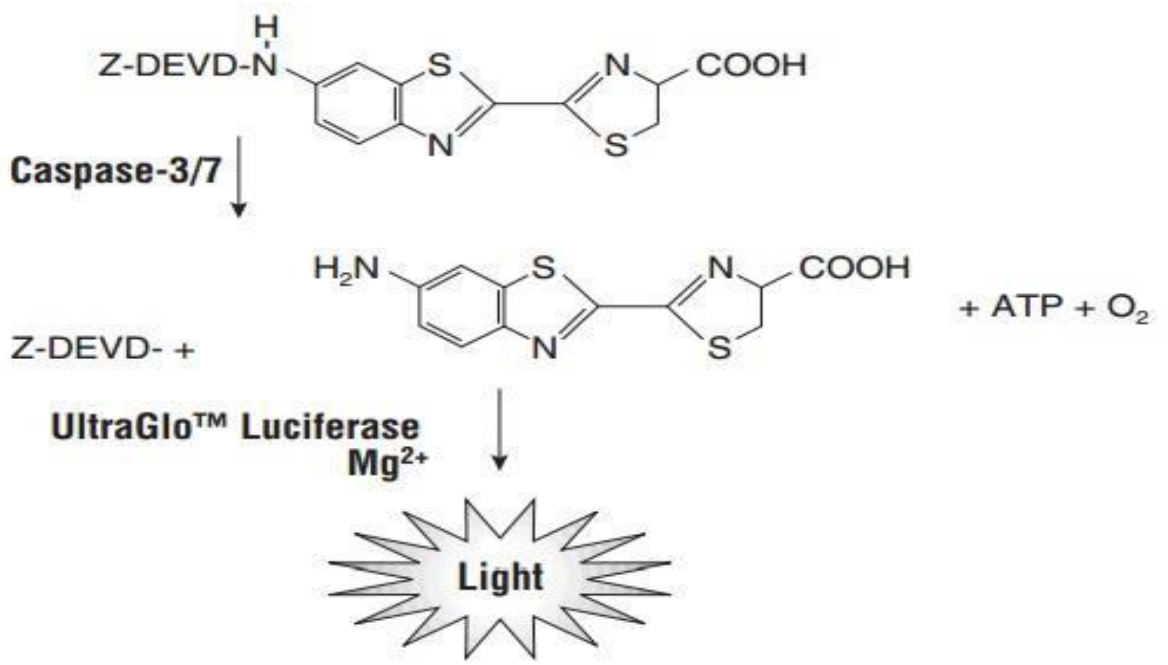


Figure (3-3): Caspase 3/7 cleavage of the luminogenic substrate containing the DEVD sequence.

3.9. Cytoplasmic and membrane protein fractionation

Ovcar-8 and Ovsaho were seeded in 6 well plates (2×10^5 cells in 2 mL) medium and incubated overnight. The next day, 20 µL of medium containing geranylgeraniol and farnesol with or without pitavastatin were added to the indicated final concentration. Both cytoplasm and membrane proteins were separated using a Mem-PER™ Plus Membrane Protein Extraction kit (Thermofisher) according to the manufacturer's instructions. After harvesting the cells by trypsinization, cells were washed twice with 2 mL of ice cold washing buffer, then re-suspended in 0.75 mL cell permeabilization buffer and incubated for 30 minutes at 4 °C with constant

mixing. The cytoplasmic fraction (the supernatant) was collected after centrifugation at 16, 000 x *g* for 15 minutes at 4 °C. The membrane fraction was obtained by re-suspending the pellets in 0.5 mL of solubilisation buffer and incubating for a further 10 minutes at 4 °C with constant shaking. The supernatant was centrifuged at 16000 x *g* for 15 minutes at 4 °C and the supernatant collected to yield the membrane fraction. Both cytoplasmic and membrane fraction were stored at -80°C.

3.10. Western blot analysis

3.10.1. Whole cell lysate

Ovarian cancer cell lines, Fuov1, Cov318, Ovsaho, Ovcar-4, Cov-362 and Ovcar8, were seeded in T75 flasks. The cells were incubated until the cells reach 80% of confluence. After trypsinization, the cells were washed with ice-cold PBS and lysed in a modified Radioimmunoprecipitation assay (RIPA) buffer (20 mM Hepes (CalbioChem) pH 7.0 , 150 mM sodium chloride (NaCl, Sigma-Aldrich), 2 mM ethylene-diamino-tetraacetic acid (EDTA, Sigma-Aldrich), 0.5% sodium deoxycholate (Sigma-Aldrich), 1% NP40 (Sigma-Aldrich)) 120 μM leupeptin (Sigma-Aldrich), 10 μM pepstatin (Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich).The lysate was centrifuged at 14,000 RPM for 15 minutes at 4°C, then the supernatant collected into a new tube and stored at -80°C.

3.10.2. Bicinchoninic acid protein assay

To determine total protein concentration, protein standards were used for calibration in each assay were prepared using Bovine serum albumin (BSA, Sigma-Aldrich) at eight different concentrations varying from 0.1 to 2 mg/mL The bicinchoninic acid protein assay reagent was prepared by adding Copper (II) sulphate solution (4%) (Sigma-Aldrich) to BCA solution at a ratio of 1:50. 10 μL of the standard or the sample was mixed with 100 μL BCA reagent per well. The plate was incubated for 30-minutes at 37 °C then the absorbance was measured at

570 nm. The concentration of protein was determined from a calibration curve using linear regression analysis of the results obtained with the standards.

3.10.3. Gel electrophoresis and immunodetection of proteins using SDS-page and western transfer

10-20 μg of protein was mixed with 5 μL of NuPAGE sample buffer (Invitrogen) containing 5 % (v/v) β-mercaptoethanol (Sigma-Aldrich) and denatured by heating for 15 minutes at 80°C. Proteins were separated using 4-20% Tris-Glycine/SDS gels (Serva) using Tris-Glycine/SDS electrophoresis buffer (Serva) and Blue Plus® II Protein Markers (14 to 120 kDa) (BIO Corp) were included on each gel. Electrophoresis was performed at 240 V, for 40 minutes. Next, the proteins were transferred to PVDF Hybond membrane (Hybond-ECL, GE Healthcare) using ice-cold transfer buffer (200 mM glycine ,25 mM Tris, 0.075 % (w/v) SDS (Sigma- Aldrich) and 10 % (v/v) methanol (Sigma-Aldrich)) at 70 V, 250 mA for 1 hour and fifteen minutes. Subsequently, the PVDF membrane was blocked by incubation in skimmed milk blocking solution (5% (w/v) skimmed milk powder in Tris-Buffered Saline with Tween (TBST)) for 1 hour with shaking at room temperature. The membrane was washed with Tris-Buffered Saline with 0.1% Tween (TBST) three times then incubated with primary antibody overnight with continuous shaking at 4 °C. The antibodies used are described in Table 3-2. The following morning, the membrane was washed five times (5 minutes each) in TBST buffer and the membrane was incubated with the secondary antibody for two hours. Then, the membrane was again washed with TBST buffer three times for five minutes each and the proteins visualized by using a chemiluminescent substrate (UptiLight HRP US chemiluminescent substrate (Interchim)) and imaged using a FluorChem M Imager. Each experiment was repeated at least three times, and the total signal of protein bands was quantified with AlphaView SA software (Protein Simple) after subtracting the product of the area of the region of interest and the mean background per pixel. Finally, the results were normalised to the GAPDH loading control.

Table (3-2): The list of primary and secondary antibodies used in protein immunodetection assay

Antibody	Dilution	Product code	Supplier
Anti-Frizzled-2	1:500-1:2000		St Johns Laboratory
Anti-FGF	1:500-1:2000	STJ96555	St Johns Laboratory
Anti-Axl	1:500-1:2000	STJ97643	St Johns Laboratory
Anti-VIM	1:500-1:2000	STJ96243	St Johns Laboratory
Anti-Rab11-FIP4	1:500-1:2000	STJ95310	St Johns Laboratory
Anti-GRHL2	1:500-1:2000	STJ112192	St Johns Laboratory
Anti-CFL2	1:500-1:2000	STJ110316	St Johns Laboratory
Anti-CYR61	1:500-1:2000	STJ23329	St Johns Laboratory
Anti-HMGCS1	1:1000-1:2000	STJ24047	St Johns Laboratory
Anti-PTRF	1:1000-1:2000	STJ27803	St Johns Laboratory
Anti-BSPRY	1:500-1:1000	ab92920	abcam
Anti-MAP7D1	1 µg/ml	ab98274	abcam
Anti-PRR15L	1:100-1:500	ab171614	abcam
Anti-RBM35A	1:1000	ab106585	abcam
Anti-MARVELD3	0.25 µg/ml	Ab118916	abcam
Anti-CCDC64B	1 µg/ml	ab177564	abcam
Anti-FXYD3	1:1000	ab205534	abcam
MAP7 Antibody	1:500-1:3000	PA5-31782	ThermoFisher
Anti-KIF5B	1:1000-1:10000	Ab167429	abcam
Anti-Tubulin antibody [YL1/2] - Loading Control	1:1000	ab6160	abcam

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488	1:1000	# A32723	ThermoFisher, Invitrogen
Goat Anti-Rat IgG H&L (Alexa Fluor® 488) preadsorbed (ab150165)	1:1000	ab150165	abcam
Actin Polyclonal Antibody	1:1000	PA5-11570	ThermoFisher
Anti-CNPase antibody [11-5B]	1:1000	ab6319	abcam
GAPDH Polyclonal Antibody	1:1000	TAB1001	ThermoFisher
Anti-mouse IgG, HRP-linked Antibody	1:1000	7076S	Cell signalling
Goat anti-Mouse IgG Alexa Fluor Plus 488	1-10 µg/mL	A32723	ThermoFisher

3.11. RNA extraction from culture cells

Ovarian cancer cell lines Fuov1, Cov318, Ovsaho, Ovcar-4, Cov-362 and Ovcar8 were seeded in T75 flasks. Cells were harvested using an RNeasy mini kit (Qiagen) following the manufacturer instruction. Lysates were centrifuged for 3 min at 16000 x *g*. The supernatant was transferred to a new 1.5 mL tube and mixed with 350 µL of 70% ethanol by pipetting up and down several times. Next, 700 µL of each sample was transferred to mini spin column placed in 2 mL tube and centrifuged for 15 second at 8000 x *g* and the flow-through discarded. The

centrifugation process was repeated to wash with 700 µL of RW1 buffer and subsequently twice more with 500 µL RPE buffer. The column was dried by centrifugation for 2 minutes. Lastly, the spin column was placed in new 1.5 mL tube and 50 µL of RNase free water added, incubated for 2 minutes and then centrifuged for 1 minute at 8000 x *g* in order to elute the RNA. The purity and concentration of the extracted RNA were measured using a Nano-Drop2000 spectrophotometer (Thermo fisher scientific). The extracted RNA stored at -80 °C.

3.11.1. Synthesis of cDNA by reverse transcriptase

SuperScript™ III Reverse Transcriptase was used to generate the complementary DNA strand (cDNA). The reaction mixtures were maintained on ice during the procedure. A mixture of 11.5 µL of RNA extracted from cells and 1.5 µL (0.5 µg) of Oligo (dT) 20mer (50 µM) were mixed and denatured at 65ºC for 5 minutes in thermal cycler (MJ Research PTC-200 Thermal Cycler, USA) and stored on ice. The reaction mixture for each sample was prepared by mixing 4 µL of 5X reverse transcriptase RT reaction buffer, 1 µL of dNTP mix (10 mM), 1 µL of reverse transcriptase and 1 µL of DTT (0.1 M). The reaction mixture (7 µL) was added to the previously prepared RNA mixture. The tubes were briefly centrifuged and transferred to thermal cycler again and incubated for 5 minutes at 65 ºC, 30 minutes at 50 ºC, 5 minutes at 85 ºC and lastly cooled to 4 ºC. The concentration and purity of the cDNA was measured using Nanodrop2000 spectrophotometer. The ratio of the absorbance measured at 260/280nm were used to assess the purity of DNA. The reaction product was diluted 5 times by adding 80 µL of water and the sample were stored in -80.

3.11.2. qRT-PCR

A master mix was prepared by adding 6.25 µL of Sybr green (Absolute SYBR Green ROX mix Thermo Scientific), 0.125 µL of forward and reverse primer (10 mM) (Table 3-4) and 1.125 µL of nuclease-free water for each sample and kept on ice. The master mix was mixed gently and

collected by brief centrifugation. 7.5 µL reaction mixture were added to each wells of 8-tube optical strips, then 5 µL of DNA sample or water were added to each tube in duplicate. The optical strips were briefly vortexed and centrifuged to collect the reaction mixture at the bottom of the wells. A Strata gene Mx3005P thermal cycler (Agilent Technologies) was used to conduct the analysis (Table3-4). To confirm that only one amplicon was detected the dissociation curves were analysed for each sample. In addition, the efficiency of genes was confirmed by measuring the C_T value of four 4-fold serial dilutions of the samples using qRT-PCR. The standard curve produced from the measured C_T data and the log of the dilution factors were analysed by liner regression using the following equation to measure the efficiency.

$$\text{Efficiency} = (10\text{-}1/\text{slope})^{-1}$$

A comparative cycle threshold (CT) method was used to analyses the data, which compares the CT value of target gene to the CT value of the reference gene.

ΔCT= CT (target gene) – CT (reference gene)

$\Delta\Delta CT=2^{(-\Delta CT)}$ Ratio (Fold changes) =ΔΔCT of treated sample ΔΔCT of control sample

Table (3-3): The list of Primer sequences (Forward and Reverse)

Primer Name	**Forward (5' → 3')**	**Reverse (5' → 3')**
BSPRY	TCAAAGAGCAGTGAGCATGG	TCTCGCAAAGACTCCTCACC
CCDC64B	GCAGGGGGAAAACCAGAT	ACCCTTCTCCACCTCCTCAC
MARVELD3	TACTTGTGCACTGGGAGAGC	GCACCATCAAAGCCACTGTA
PRR15L	TACAGAAAGCCCCATCCTTG	ACAGCACTTTGGGAGTGGAT
RAB11FIP4-211	CAAGTCCCTGGTCCACACTC	ATCGTCACCCTCCCCATAGT
RBM35A	GCCCTCCGACAGTTTAACCA	TTTGCCTGA CATGAAGCTGC
VIM#209	TGACATTGAGATTGCCACCT	TCCAGATTAGTTTCCCTCAGGT
Fzd2	TCGTGTACCTGTTCATCGGCAC	CTGTGTAGAGCACGGAGAAGAC

FGF-5	GGAATACGAGGAGTTTTCAGCAAC	CTCCCTGAACTTGCGTCAGTCATCTG
AXL	GAGGCTCATCGGTGTCTGTT	GAGCCGGGAATAGAGGAGGA
CFL2-202	TCTGGGCTCCTGAAAGTGC	CCAAGTGTCGAACGGTCCTT
CYR61-201	GAAGGGGATCTGCAGAGCTC	GCGCCATCAATACATGTGCA
FXYD3-207	TAAGAGGCCCGAGTTTCACC	GGGTCACCTTCTGCATGTCA
GRHL2-201	AAGAAACTGCCTTGGCACCA	GATGCTGTACTGTTCCCGCT
HMGCS1-202	ACCGGGGTCTGCTCTTGATA	CGAAGACATCTGGTGCCACA
MAP-7	AATAAACCAGACCCTCCGCC	GCTGTTTCTCCCGTTCCTCA
MAP7D1	GACATAAGACAATCGCAGCC	GAGAGAGTGGGCGTCATCAG
PTRF	GGCAGATCAAGAAGCTGGAGGT	CAGCGATTTGCTGATGCTCAGTT

Table (3-4): Thermal cycling profile for qRT-PCR

	First segment	**Second segment**	**Third segment**
	Denaturation of DNA strands at 95◦C for 15 minutes	Denaturation of the DNA at 95◦C for 30 second Primer annealing at 60◦C for 1minute DNA strand elongation at 72◦C for 30 second	For dissociation curve at 95◦C for 1 minutes, 55◦C for 30 second and 95◦C for 30 second
Cycle Number	1	40	1

3.12. Small Interfering RNAs (siRNA) gene Transfections

3.12.1. Experiment of small interfering ribonucleic acid (siRNA) transfections

Suspensions of OVCAR-8 or OVSAHO cells in antibiotic-free growth media were seeded in 96 well plates (5000 cells per well for OVSAHO and 2000 cells per well for OVCAR-8) and incubated overnight in 5 per cent CO2 at 37 ºC. The next day, 1 percent of Dharmafect-1 was prepared in the minimum critical media (Opti-MEM, Invitrogen) of a serum-free modified eagle and incubated at room temperature for 5 minutes before mixing with the same amount of siRNA prepared in serum-free Opti-MEM and incubated at room temperature for 20 minutes to facilitate complex formation between the siRNA and the liposomes. Consequently, the growth medium of the original seeding cells has been replaced by 80 µl of fresh and pre-warmed antibiotic-free medium and 20 µl of the siRNA / DharmaFECT 1 mixture has been applied to the 96 well plate. In all transfection experiments a non-targeting siRNA (NT-1siRNA) and on-targeting siRNA, which is designed not to target known genes, has been included as regulation (off-target gene silencing). The cells were incubated overnight after the transfection mixture was added, the growth medium was replaced by 100 µL of fresh antibiotic-free growth media and cells were incubated for an additional 48 hours, then the cells were either exposed to a range of different pitavastatin concentrations or left untreated. Finally, the number of cells was evaluated by staining with SRB to be calculated sensitivity of cells to pitavastatin. Similarly, western blotting was used to test proteins by following the same method as in whole cell lysate and immunoblotting.

Table (3-5): The list of siRNA s used for RNAi

Gene Name	Working Conc. (nM)	Target Sequence
ON-TARGETplusNon-targeting siRNA#1	100	UGGUUUACAUGUCGACUAA

ON-TARGETplusNon-targeting siRNA#3	100	UGGUUUACAUGUUUUCUGA
ON-TARGETplus HumanPTRP(5788)siRNA-Individual	100	GGCUUAAACUCUUGGCAUU
ON-TARGETplus Human Set of 4siRNAJ-008867-0**5**,KIF5B	100	GAACUGGCAUGAUGAUGA
ON-TARGETplus Human Set of 4siRNAJ-008867-0**6**,KIF5B	100	CAACAGACAUGUAGCAGUU
ON-TARGETplus Human Set of 4siRNAJ-008867-0**7**,KIF5B	100	GCAGGAACGUCUAAGAGUA
ON-TARGETplus Human Set of 4siRNAJ-008867-0**8**,KIF5B	100	CAAUUGGAGUUAUAGGAAA
ON-TARGETplus Human Set of 4siRNAJ-008867-0**5**,MAP-7	100	CCAUGAAUCUUUCGAAAUA
ON-TARGETplus Human Set of 4siRNAJ-008867-0**6**, MAP-7	100	CUACAAAGCUGCACACUCU
ON-TARGETplus Human Set of 4siRNAJ-008867-0**7**, MAP-7	100	GGACAAAGAACGCCACGAA
ON-TARGETplus Human Set of 4siRNAJ-008867-0**8**, MAP-7	100	UCAGAGAAACGGUGAUAUA

3.13. Immunocytochemistry

25,000 Ovcar-8, Ovcar-4 or Ovsaho cells were seeded in 400 µL of growth medium per slide chamber of an 8-well Chamber Slide with removable wells (Thermofisher) and incubated overnight. The cells were treated with a concentration of pitavastatin equal to the IC_{50} measured

in cell growth assay for three different periods (24, 48 and 72 hours). The medium was removed and the cells washed with PBS and fixed in 10% neutrally buffered formalin for 5-10 minutes. The cells were washed with PBS twice and permabilized using 0.01% Triton-X 100 for 15 minutes. After washing twice in PBS, cells were treated with 1% BSA in PBS for 1 hour at room temperature, before incubating the cells with diluted primary antibody at room temperature (or overnight 4 ^{o}C) for two hours. The cells were washed twice with PBS for 5 minutes and exposed to the secondary antibody (Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 from (Thermofisher) (2µg/ml (1/1000) in 0.1% BSA, 0.1% Tween-20 in PBS) for two hours protected from light. After washing with PBS three times cells were stained with DAPI (counterstain) for cell nuclei and F-actin (ActinRed™ 555 ReadyProbes™ Reagent) for cytoskeletal staining were performed and covered from light as well. Finally, the samples were placed in mounting medium then stored at 4 ^{o}C protected from light before viewing by confocal microscopy.

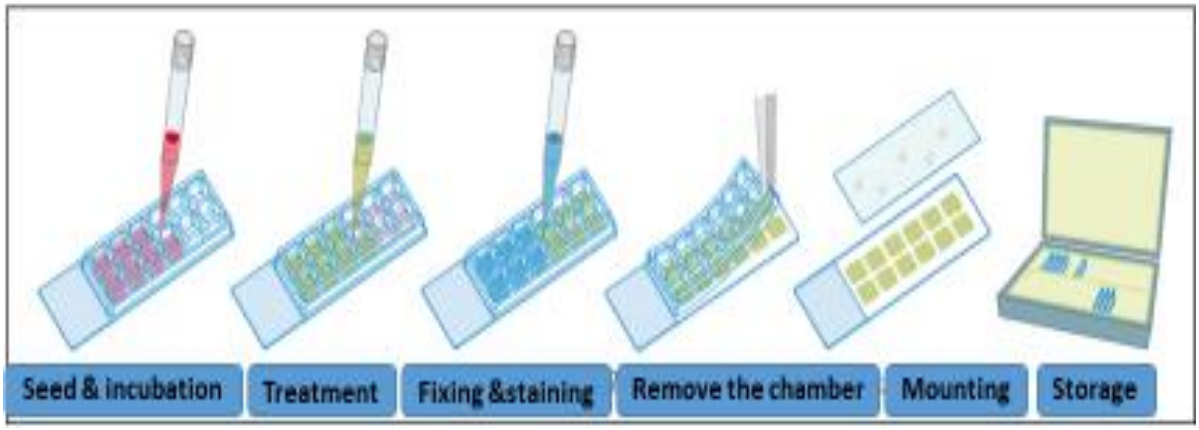


Figure (3-4): show the process of staining cells in slide chamber.

3.14. Analysis of drug combination

There are several quantitative methods for measurement the effects of combinations drug[353]. Drug combinations are wide strategy used for immune disease indication, cancer treatment and infection. The Bliss independence criterion is an effect-based strategy to calculate the expected effect of a drug combination if they interact additively. Alternatively ,the effect of a drug

combination can by assuming that a drug used at a fraction of its IC50 will have the same effect as another drug used at the same fraction of its IC50 and hence the effect of the combination can be calculated and the change in concentration needed to have a certain effect when the drugs are used in combination expressed by a combination index [354].

3.14.1. Combination index

To evaluate the efficacy of the drug combinations, a combination index (CI) was measured using the equation Chou and Talalay, which offers a quantitative measure of the synergistic, additive or antagonistic effects between two drugs. Synergy is demonstrated when a CI value is less than one (CI<1) between two drugs CI values, however, are equal to or greater than 1 and display additive and antagonistic effect respectively. The CI value for two compounds is described by the following equation.

$$\mathbf{CI} = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

(D)1, represents the concentration of drug 1 and (D)2 the concentration of drug 2 which when tested in combination inhibit cell growth by X%; (D**x**)1 represents the concentration of drug 1 which inhibits cell growth by X% when used on its own; (D**x**)2 represents the concentration of drug 2 which inhibits cell growth by X% when used on its own. The combination index was quoted at 50% of cells affected (fraction affected, $f_a = 0.5$) and compared for deviation from unity using a paired *t*-test. If the IC equals 1 is an additive interaction, whereas the IC value of the interaction greater than 2 is considered antagonistic, while the IC value is less than 1 is considered synergistic.

Table (3-6): Symbols and definition of synergism, additive and antagonism calculated by the combination index system in drug combination studies [355]**.**

Range of Combination Index	Description	Graded Symbols
<0.1	Very strong synergism	+++++
0.1–0.3	Strong synergism	++++
0.3–0.7	Synergism	+++
0.7–0.85	Moderate synergism	++
0.85–0.90	Slight synergism	+
0.90–1.10	Nearly additive	±
1.10–1.20	Slight antagonism	−
1.20–1.45	Moderate antagonism	− −
1.45–3.3	Antagonism	− − −
3.3–10	Strong antagonism	− − − −
>10	Very strong antagonism	− − − − −

3.15. Bliss independence criterion

The Bliss independence criterion was used for trypan blue and Annexin V / PI assays to assess the expected effect of drug combinations. This has been used instead of the combination index in studies where the full dose response relationship curves have not been determined, and so fractional effects could not be calculated. This approach still allows quantification of synergy between two drugs. The criterion allows the expected effect of a combination of drugs to be estimated if the two drugs work additively. This can be compared with a drug combination's observed effect to determine if there is synergy or antagonism between drugs[356] .

The Bliss independence principle is expressed in the following formula:

$$E(x,y) = E(x) + E(y) - E(x) \times E(y)$$

Where E (x, y) is the combination's predicted effect and where E(x) and E(y) are the individual drugs' effect. Paired t-tests were used to compare the expected effect determined by the Bliss independence criterion with the drug combination's experimentally observed effect.

3.16. Statistical Analysis to Determine IC_{50} Value

The data obtained from cell growth assays was analysed by using Graph Pad Prism software version 8. Non-linear regression was used to fit a four-parameter (Hill-equation) sigmoidal dose-response curve, and then subsequently, the concentration of drug which caused 50% inhibition of cell growth (IC_{50}) was determined. Statistical analyses were conducted by ANOVA or and *t*-test as indicated in the text.

Chapter 4

The Influence of Food extracts on Pitavastatin Anticancer Activity of Pitavastatin in Ovarian Cancer Cell line

4. Introduction

Ovarian cancer is a generic term used for all tumours of primary malignancy presenting in the ovaries [357]. It is considered the fifth leading cause of cancer death in women with more than 4000 deaths annually in UK [358]. The 5-year survival rate for patients diagnosed with advanced (stage II or greater) ovarian cancer is less than 46 percent [359]. Initially ovarian cancer responds to chemotherapy but most of ovarian cancer patients relapse, often the tumours develop drug resistance alternative effective cancer therapies are urgently required [360], [361]. The poorer prognoses and the low survival rates command attention and force researchers to focus on ovarian cancers to find alternative methods of treatment [360], [362].

Cancer cells utilise the mevalonate pathway to synthesize isoprenoids for the post-translational modification of proteins, some of which play important roles in tumour initiation, growth, and metastasis. Protein prenylation can be an essential step in protein recruitment to the cell membrane, protein-protein interaction and localisation [363]. In particular, farnesyl and geranyl-geranyl pyrophosphate are essential for post-translational modification of small GTPases, many of which are oncogenes. Consequently, statins have showed pro-apoptotic, anti-proliferative and anti-invasive effects of cancer cells in preclinical studies [274], [364], [365]. In addition to their pro-apoptotic effects, statins also induce cell cycle arrest at G1/S phase through up-regulation of the inhibitors of cell-cycle $p21^{WAF1/CIP1}$ and/or $p27^{KIP1,}$ and also by reducing the expression of the CDK2, CDK4, Cyclin D1 and Cyclin E [366], [367]. Therefore, the mevalonate pathway appears a reasonable target to treat malignant disease.

4.1 MVA-derived metabolites in cancer

The mevalonate pathway is responsible for the synthesis of several metabolites, in addition to isoprenoids and cholesterol synthesis. Although it is reasonable to propose that statins exert an

anticancer effect by blocking the production of mevalonate, it is possible that this is also due to the reduced supply of other mevalonate pathway products. [231], [368].

4.1.1 Cholesterol

Cholesterol is essential for the biosynthesis of cell membranes. As such, it is essential for rapid synthesis of cell membranes in cancer cells which are highly proliferative. In addition, cholesterol is an important precursor for steroid hormones, bile salts and vitamin D. Cholesterol and sterols have a direct effect on the mevalonate pathway by feedback inhibition [369]. Many epidemiological and experimental data indicate that ovarian cancer shares several pathways controlled by oestrogen analogous to other hormonal cancers. Local synthesis of oestrogen from circulating steroid hormone precursors by steroid-forming and steroid-inactivating enzymes could therefore be essential for the development of ovarian cancer in women after menopause. In addition, these enzymes and receptors have been identified in ovarian cancer cells and their expression has been shown to be linked to clinical outcome [370].

Cholesterol is generated primarily through the liver and transported through low-density lipoproteins to other tissues[231]. However, HMGCR activity is regulated not only by cholesterol concentration but by other intracellular factors (MP sterol and non-sterol products) and extracellular factors (insulin, tri-iodothyronin, glucagon and cortisol) (insulin, tri-iodothyronin, glucagon and cortisol)[369], [371].

4.1.2. Dolichol

Another candidate by which statins may exert an anti-cancer effect is by blocking the production of dolichol by the mevalonate pathway. Dolichols are polyisoprenoids with long chains that are unsaturated in all eukaryotic cells and consists of 18-20 isopentenyl units. Dolichols are considered to be essential components for the N-glycosylation of membrane

protein in the endoplasmic reticulum[372]. This molecule can contribute to tumourigenesis, proliferation and metastasis due to the frequent alteration of protein N-glycosylation in cancer. Dolichol can contribute to tumourigenesis, proliferation and metastasis due to the frequent alteration of protein N-glycosylation in cancer. In some cases of cancer, the complex branching of N-glycans has a tumour-suppressive property. Consequently, N-linked glycosylation may prevent intracellular protein aggregation in the ER and may also be necessary for proper folding and cleavage of the precursor proteins. The N-linked oligosaccharides may also be required to guide proteins through various compartments of the Golgi network in which they are further processed before subsequent translocation to the cell surface [373]. Therefore, deficiencies in N-linked glycosylation at certain glycosylation sites can lead to incorrect protein folding, suppressed efficiency and reduced intracellular glycoprotein transport or impaired protein function[374].

4.1.3. Coenzyme Q10

Inhibition of the mevalonate pathway also restricts the biosynthesis of coenzyme Q10 (also known as CoQ10 or ubiquinone (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone)). In humans, the major role of this molecule is in electron transfer during oxidative phosphorylation in mitochondria [375]. It transfers electrons in its quinone group from complex I or complex II to complex III and participates in oxidative phosphorylation and ATP production. In addition, Ql0 is considered a potent antioxidant and scavenger of free radicals, as well as being considered to be a membrane stabilizer and preserves cellular integrity[375], [376], considering its role in ATP production, it is plausible that reduced levels of CoQ10 could contribute to the cytotoxic activity of statins.

Statin-induced myopathy encompasses a wide range of statin-associated muscle symptoms (SAMs) and is distinguished by the presence of elevated creatine kinase (CK). Previous studies

showed a reduction in coenzyme Q10 after statin treatment, which could be associated with myopathy induced by statins [377]–[379]. Several studies have shown that Co-enzyme Q10 supplementation reduced statin-associated muscle symptoms, indicating that Co-enzyme Q10 supplementation may be a complementary solution to statin-induced myopathy treatment [380]–[384].

4.1.1 Farnesyl-diphosphate and gernaylgernayl-diphosphate

Farnesyl-diphosphate and geranylgeranyl-diphosphate, discussed above, are considered to be two keys products of the mevalonate pathway because they play important roles in protein prenylation, and consequently in differentiation and tumourigenesis. MP intermediate products (GGPP and FPP) are involved in post-translational modification, proliferation and differentiation of several important proteins involved in cell signals. This includes the GTPases Ras superfamily, a large protein family with over 150 members. In about 20 percent of human tumours, Ras itself is mutated. Mutated Ras can be stabilised in a constitutively active conformation and new therapies that inhibit Ras activity are being created [385].

Studies with cultured cancer cells have shown that supplementation of cell growth media with granylgeraniol can suppress apoptosis and inhibition of cell growth caused by statins[386]. Pitavastatin was found to be able to cause the regression of ovarian cancer xenografts in mice if the animals received a diet of “Ensure”. Ensure is a human complete liquid food used by many cancer patients and that lacks geranylgeraniol. However, when the Ensure was supplemented with geranylgeraniol, pitavastatin was no longer able to inhibit the growth of the xenografts in the mice. Importantly, geranylgeraniol is present in some human foods[387]. These observations raise the concern that the supply of one or more of these mevalonate pathway products in food, and in particular geranylgeraniol, may interfere with statins’ anticancer activity. It is possible that patients’ diet may contain sufficient quantities of geranylgeraniol to reverse the cytotoxic effects of the statins and limit their anti-cancer activity.

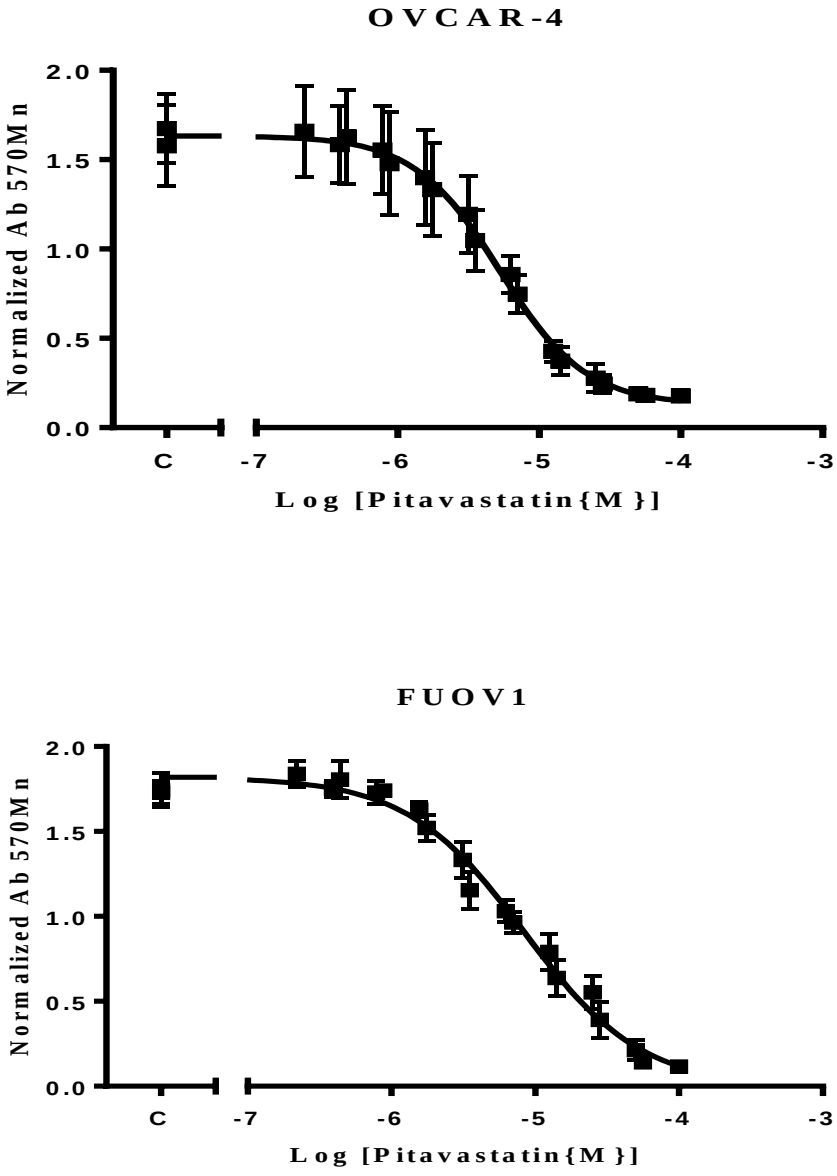
Therefore, clinical trials of statins in cancer may require patients to eat a diet limiting their exposure to geranylgeraniol. Consequently, it is important to identify foods that lack geranylgeraniol, or other metabolites that interfere with the anti-cancer activity of statins. Without this information, clinical trials of pitavastatin in cancer may fail.

Previous studies have reported the presence of geranylgeranyl diphosphate and or geranylgeraniol in foodstuffs including extract of sunflower oil, olive oil [387], Kinuhikari polished rice [349], and virgin olive oil [388]. Previous studies in our laboratory[308] have shown that solvent extracts of human and mouse food were able to block the anticancer activity of pitavastatin by inhibiting pitavastatin-induced apoptosis. Consequently, the main objective of the research described in this chapter was to test a selection of foods commonly eaten in the UK to determine if they block the effect of pitavastatin and thereby enable later clinical trials.

4.2 Results

4.2.1 Inhibition of the growth of Ovcar-4 and Fuov-1 cells by Pitavastatin

Cells were exposed to the indicated concentration of pitavastatin for 72 h and the relative number of surviving cells measured by staining with SRB. Pitavastatin inhibited the growth of Ovcar-4 (IC_{50} = 5.2 ± 1.20 μM) and Fuov1 (IC_{50} = 8.3 ± 0.9 μM) cells (Figure (4-1) & (4-2)).



Figures (4-1&4-2): Dose response curve of pitavastatin in ovarian cancer cell lines (Ovcar-4 and Fuov-1 cells). The activity of pitavastatin was evaluated in cell growth assays and assessed by staining with SRB. The results are expressed as a fraction of the cells measured in samples treated with solvent alone (mean ± SD; n = 3) "C" on the x-axis indicates a measured absorbance in the absence of the drug. The cells were exposed to the indicated concentration of pitavastatin for 72 hours and the relative number of surviving cells determined.

4.2.2 Food extraction

To determine which foods could potentially interfere with the cytotoxic activity of pitavastatin, solvent extracts were made from a range of foods and their ability to inhibit the cytotoxic activity of pitavastatin was measured.

4.2.2.1. Edible Oils

Extracts were also prepared from several edible oils. Organic solvent extracts were prepared from a range of oil (Fig (3-1)). Between 110 and 600 mg were extracted from 50 g of each oil (table (4-1)). The effect of food extracts on the growth-inhibitory activity of pitavastatin (10 μM) was assessed using six two-fold serial dilutions of each extract. Geranylgeraniol 5 μM in combination with pitavastatin in cell growth assays was used as a positive control. The extract from sunflower oil (fig 4-3 & 4-4) completely rescued the cells from the cytotoxic effect of pitavastatin while corn oil, ground nut oil and grape seed oil partially blocked the activity of pitavastatin (fig 4-5 & 4-6).

Table (4-1): the weight of oils extractions in 50 grams of foodstuff.

No	Extracts	Weigh of extract obtained from 50 g of oil
1	Sunflower oil	510 mg
2	Corn oil	370 mg
3	Grape seed oil	580 mg
4	Ground nut oil	347 mg
5	Rape seed oil	394 mg
6	Sesame oil	160 mg
7	Coconut oil	110 mg
8	Walnut oil	320 mg

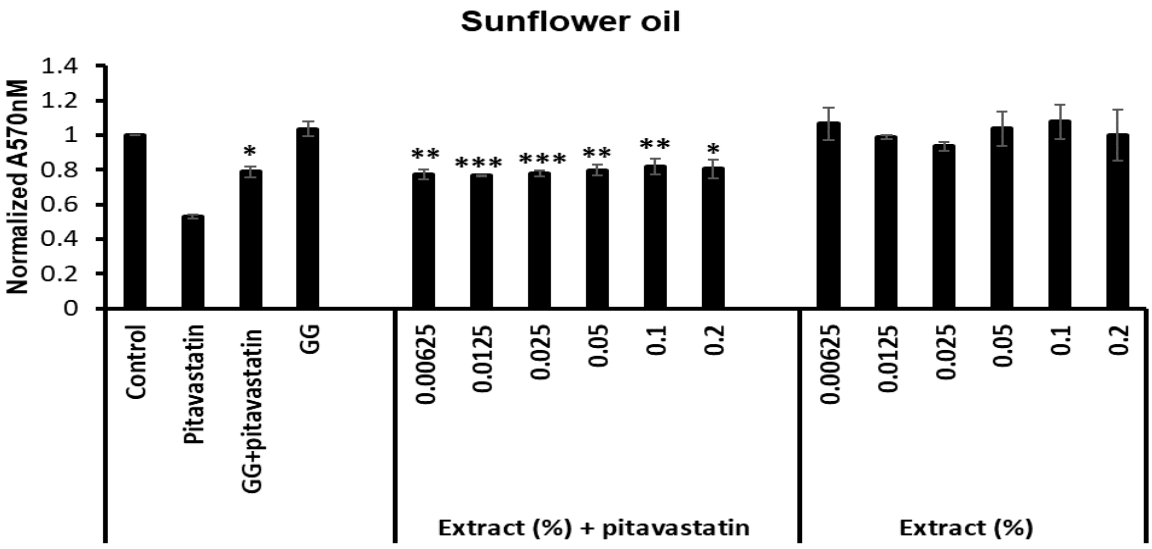


Figure (4-3): The effect of sunflower extract on pitavastatin activity against the Ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extract concentrations are expressed as a percentage of the final concentration (v/v). After 72 hours, the number of surviving cells was assessed by staining with SRB. The results (mean ± S.D., n=3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and the results were significantly different from this where indicated (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; One Way Anova).

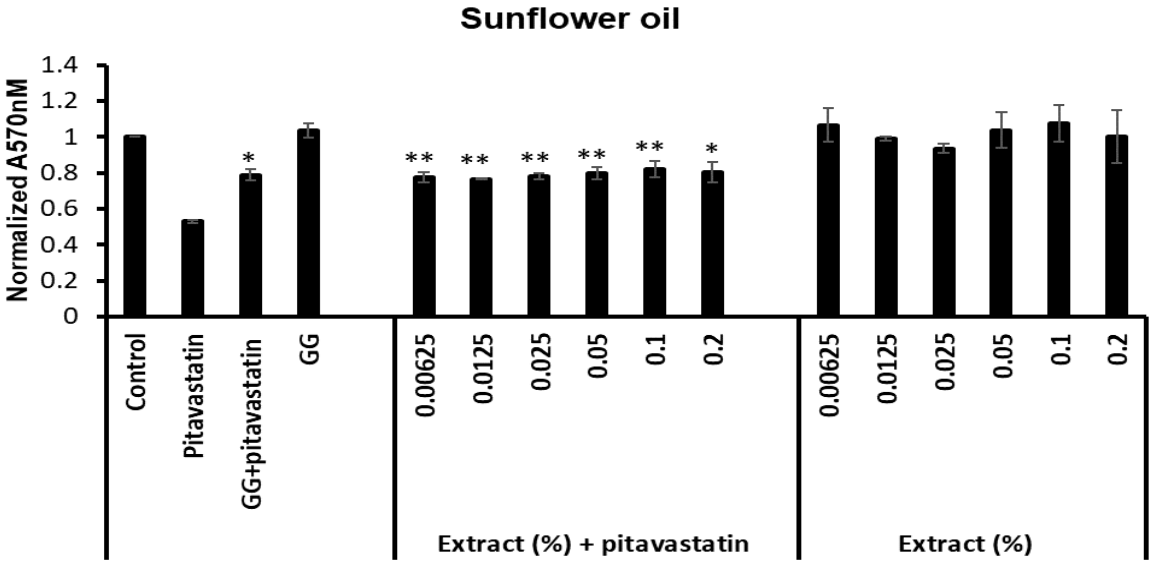


Figure (4-4): The effect of sunflower extract on pitavastatin activity against Fuov-1 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; One Way Anova).

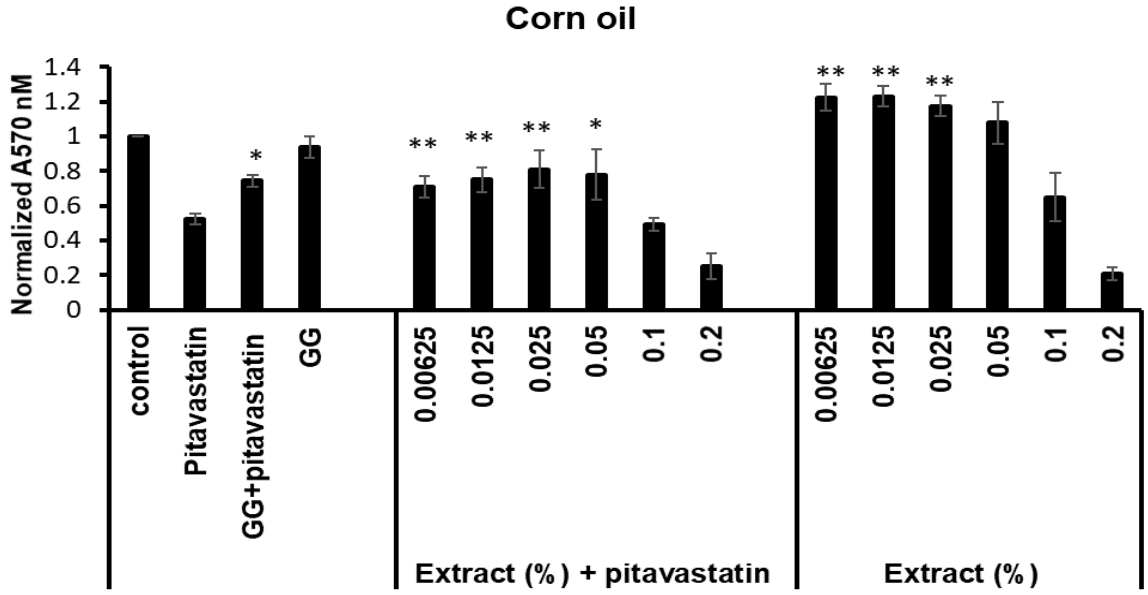


Figure (4-5): The effect of corn oil extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

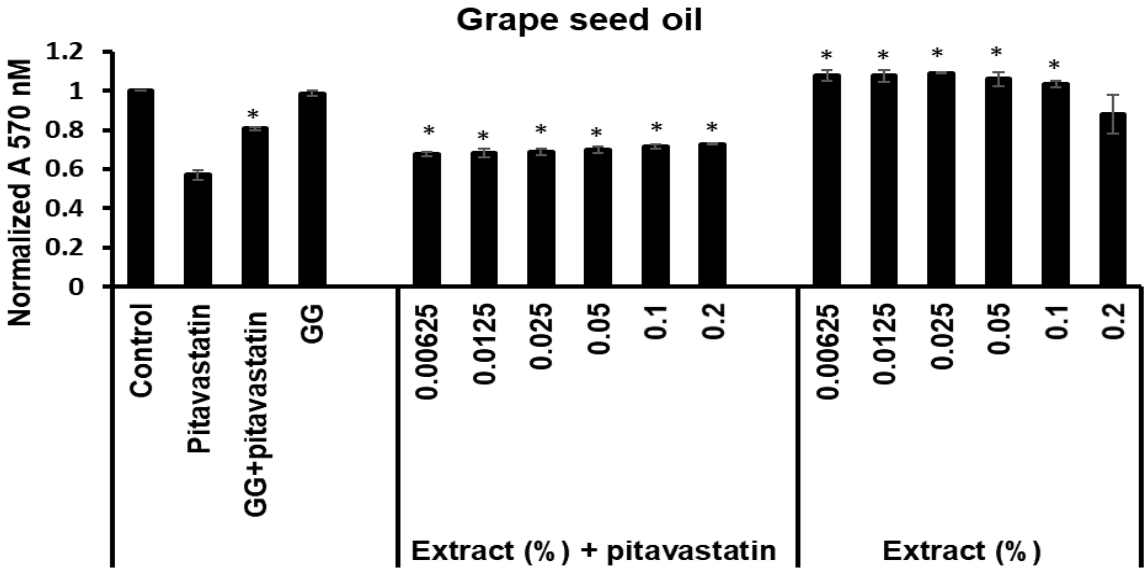


Figure (4-6): The effect of grape seed oil extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

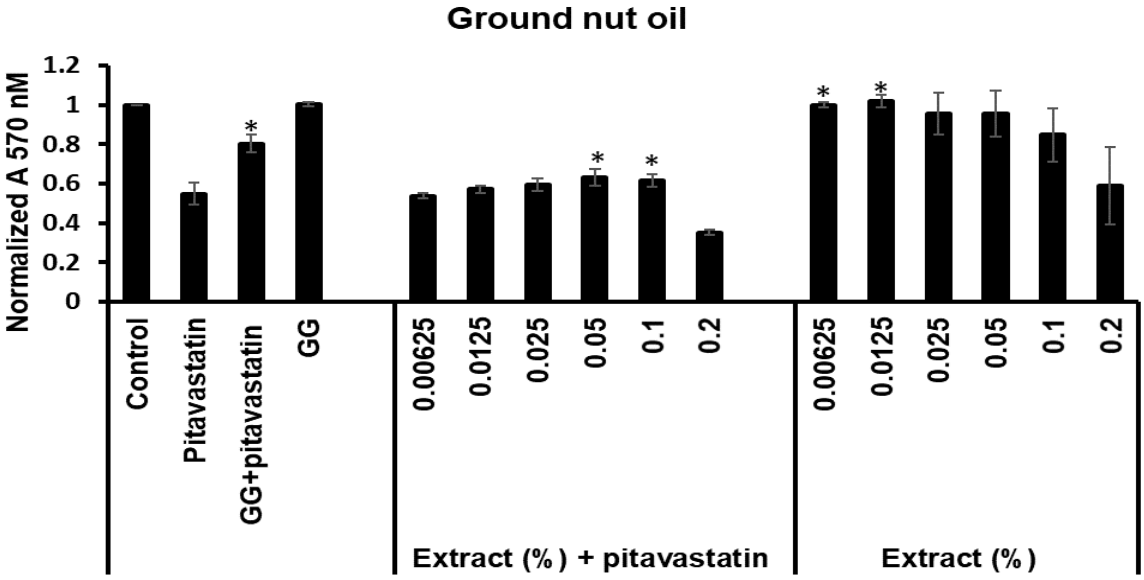


Figure (4-7): The effect of ground nut oil on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

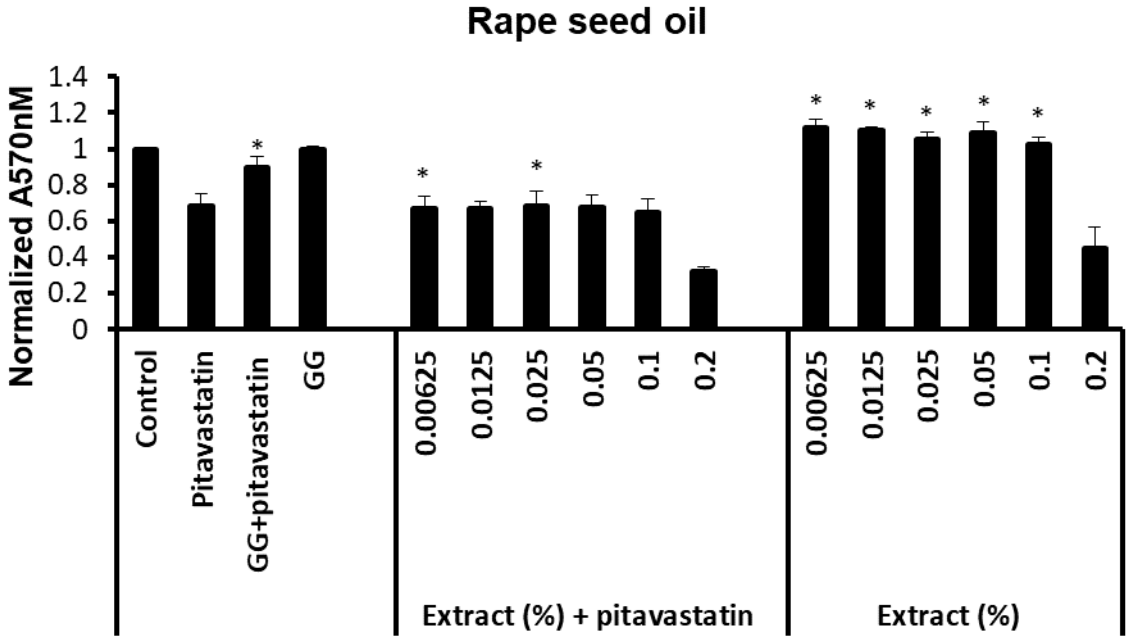


Figure (4-8): The effect of rape seed oil extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

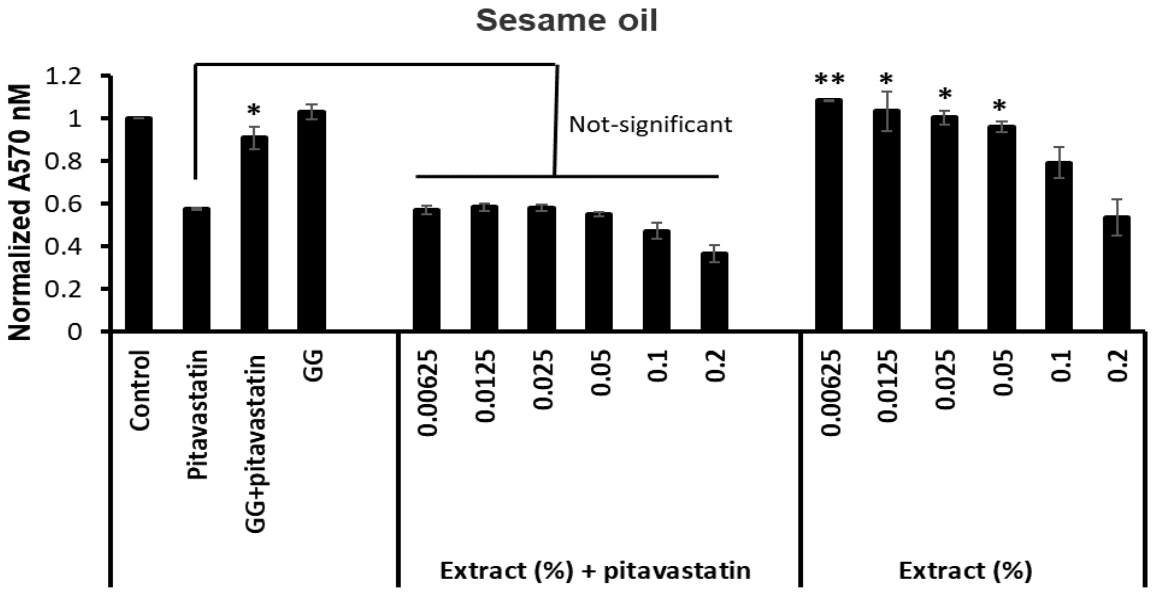


Figure (4-9): The effect of sesame oil extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; One Way Anova).

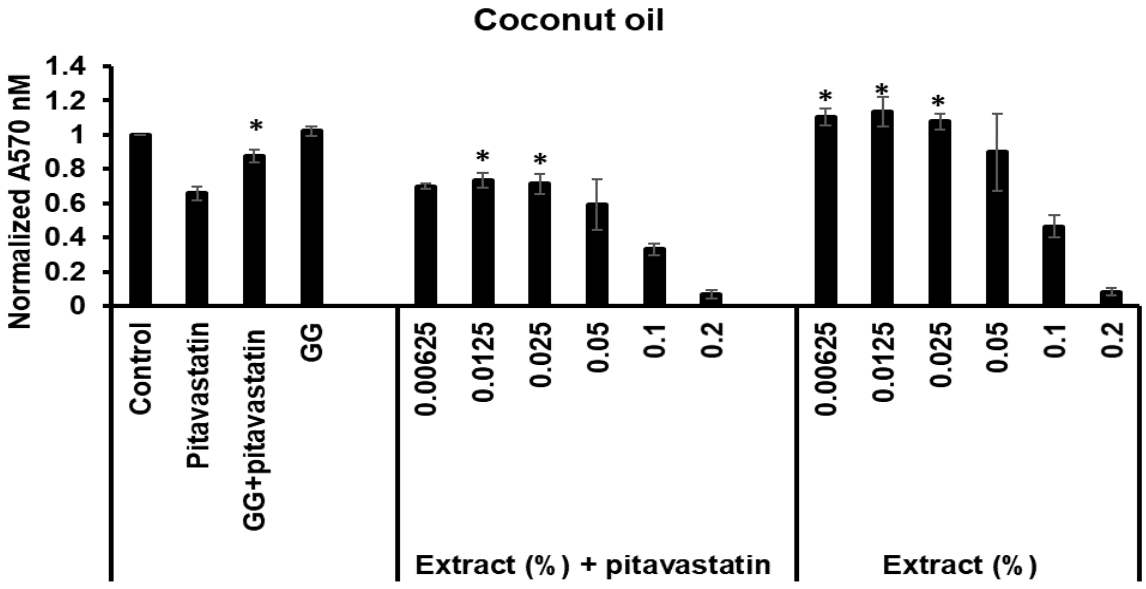


Figure (4-10): The effect of coconut oil extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; One Way Anova).

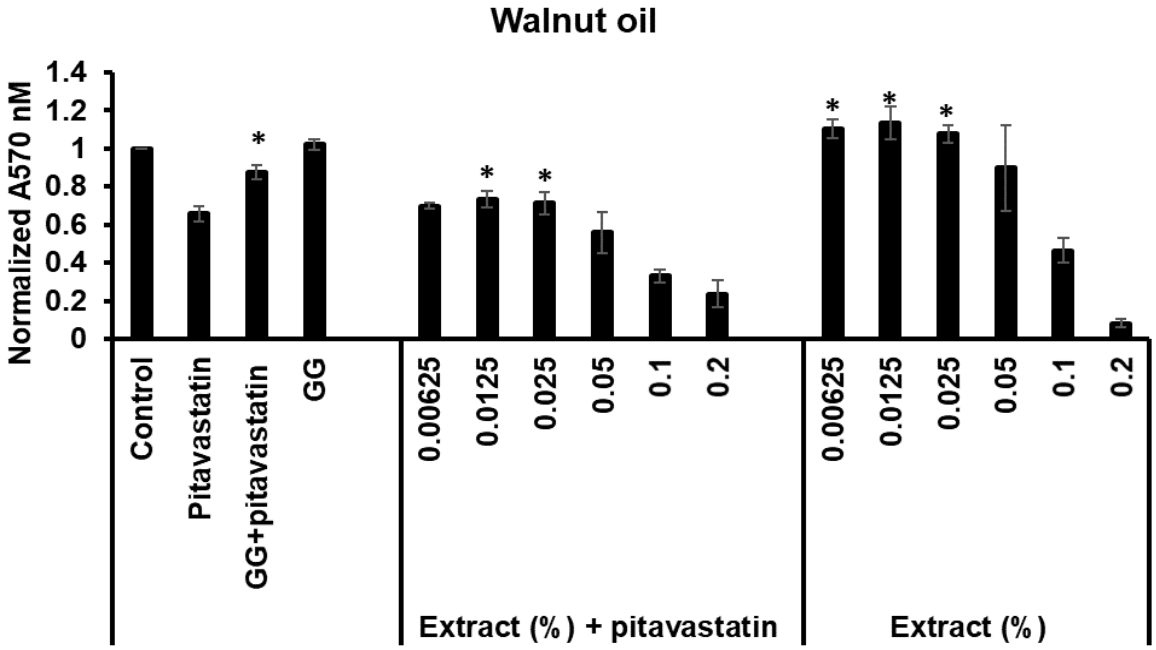


Figure (4-11): The effect of walnut oil extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

4.2.2.1 Solid foods

Extracts were also prepared from several solid foods many of which are primarily considered to be primarily carbohydrates (table 4.2). In general, a smaller mass of solid was recovered in the extract from the solid foods than from the oils. The extract from Dolmio (pasta sauce), oats, boiled egg, pecan nuts and boiled black beans partially reversed the effect of pitavastatin in cell growth assays while other food extracts (bread, tomato, squash, spaghetti, strawberry jam, cheese, butter, milk, and boiled potato) showed no or little effect on pitavastatin's cytotoxic activity.

Table (4-2): the weight of meals extractions in 50 grams of foodstuff

No	Extracts	Weigh of extract obtained from 50 g of solid food
1	Bread	140 mg
2	Cheese	60 mg
3	Butter	180 mg
4	Milk	50 mg
5	Spaghetti	30 mg
6	Pasta sauce	30 mg
7	Boiled potato	30 mg
8	Boiled black bean	70 mg
9	Boiled egg	120 mg
10	Oats	60 mg
11	Pecan nuts	110 mg

Figure (4-12): The effect of bread extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; One Way Anova).

Figure (4-13): The effect of bread extract on pitavastatin activity against fuov1 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P<$ 0.05 and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

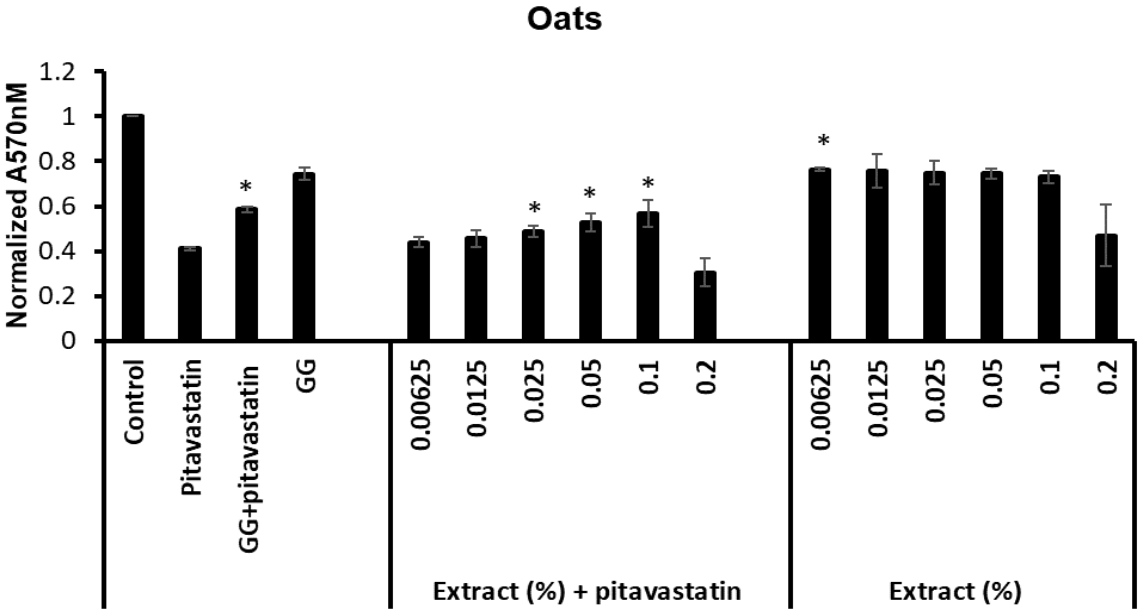


Figure (4-14): The effect of oats extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P<$ 0.05 and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

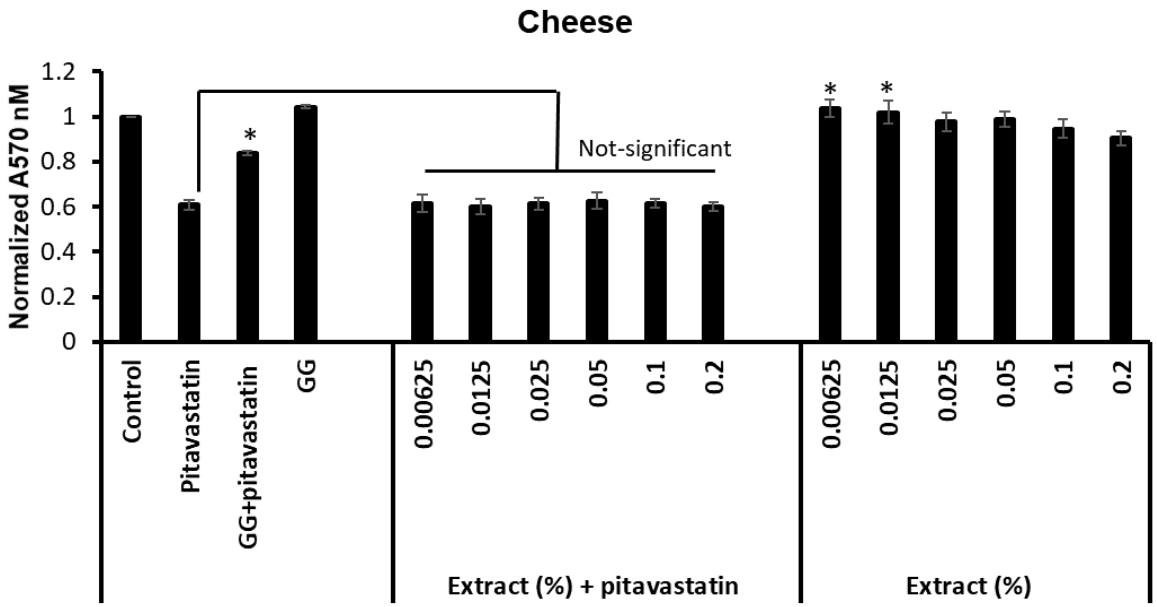


Figure (4-15): The effect of cheese extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

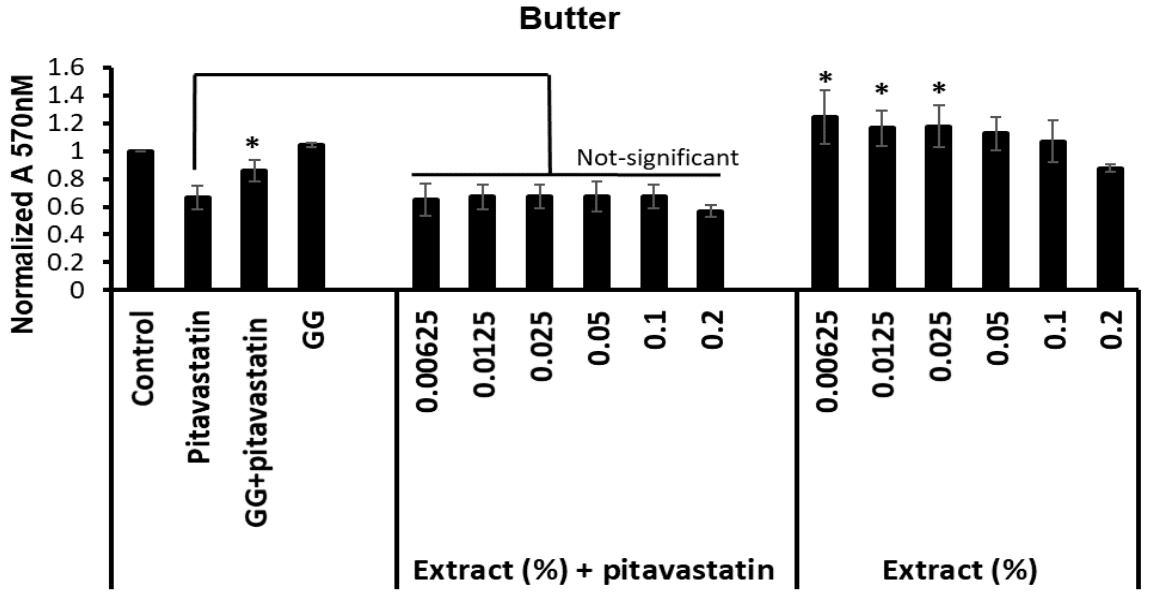


Figure (4-16): The effect of butter extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

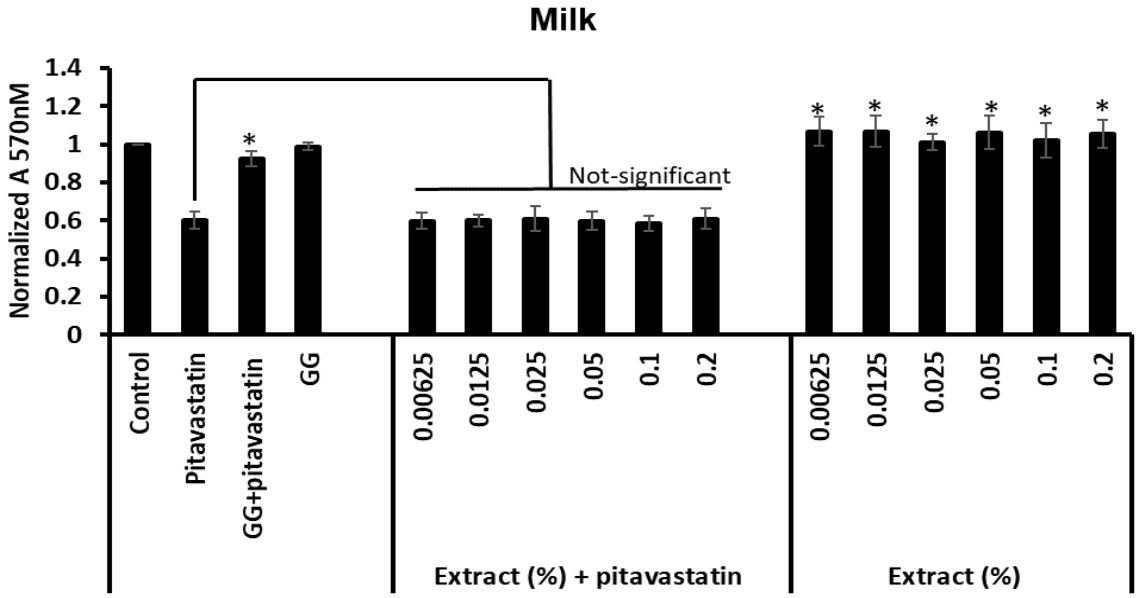


Figure (4-17): The effect of milk extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

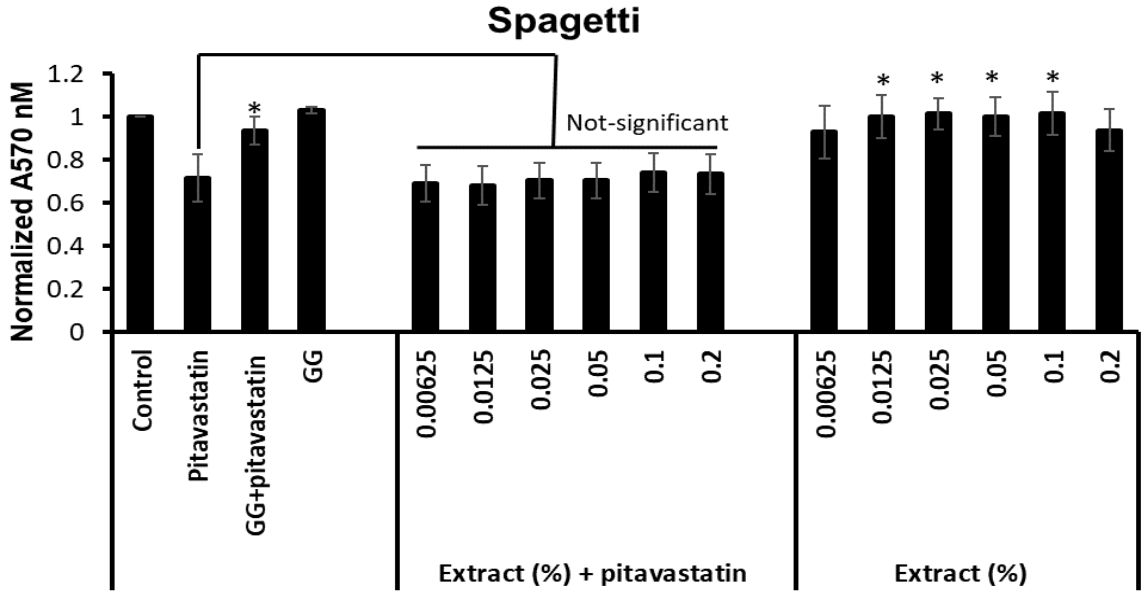


Figure (4-18): The effect of spaghetti extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

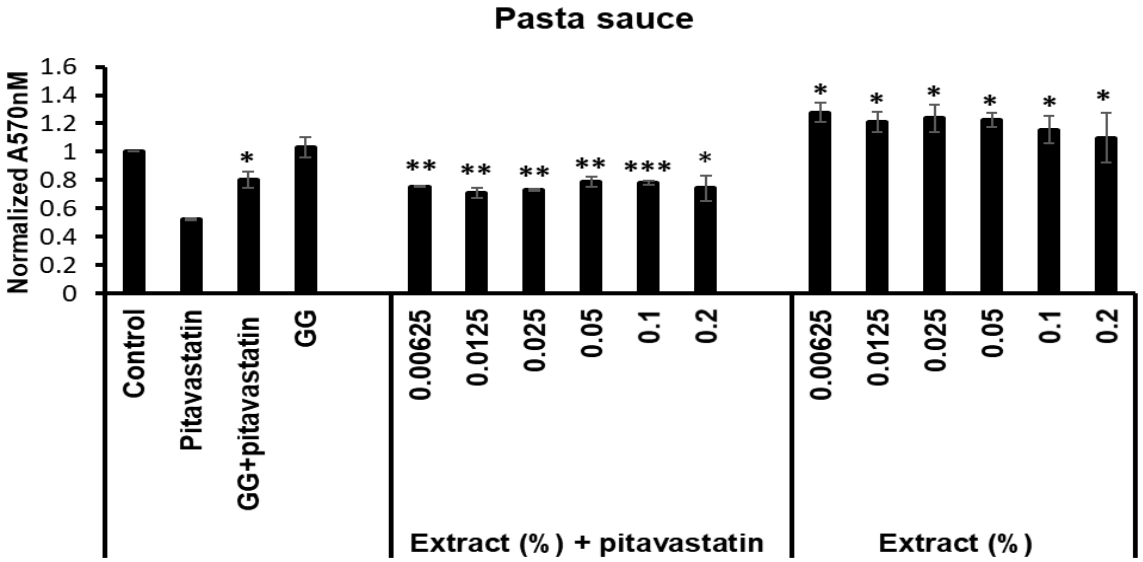


Figure (4-19): The effect of pasta sauce extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

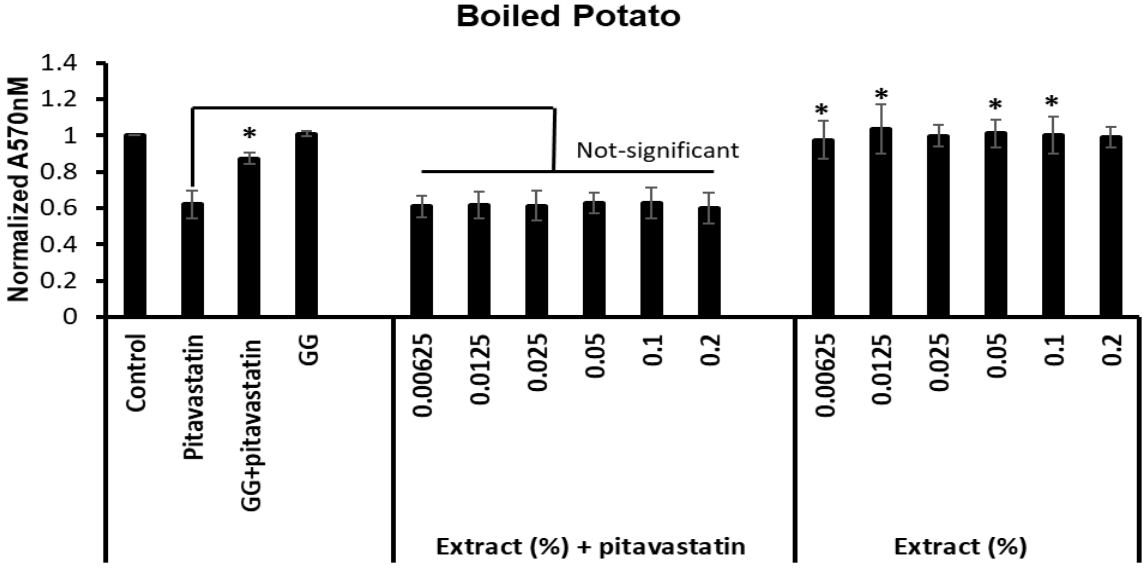


Figure (4-20): The effect of boiled potato extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

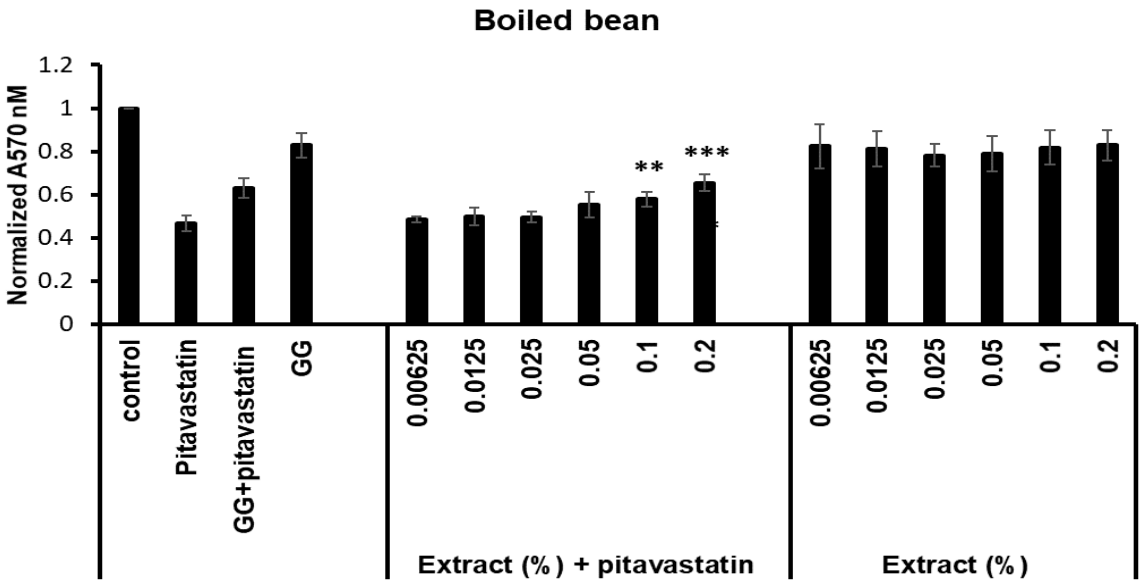


Figure (4-21): The effect of boiled black bean extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

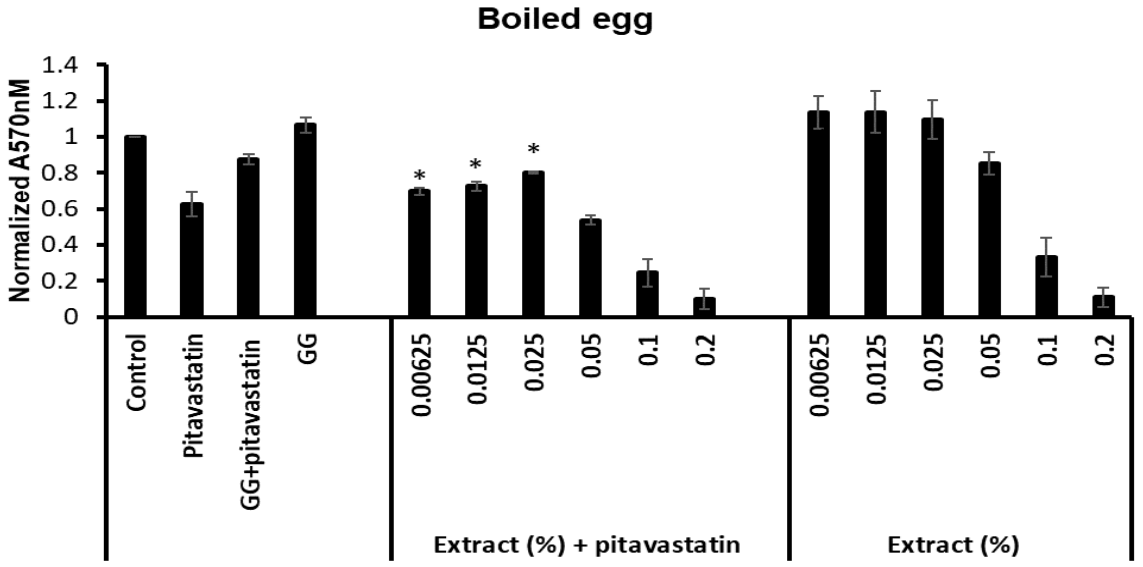


Figure (4-22): The effect of boiled egg extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

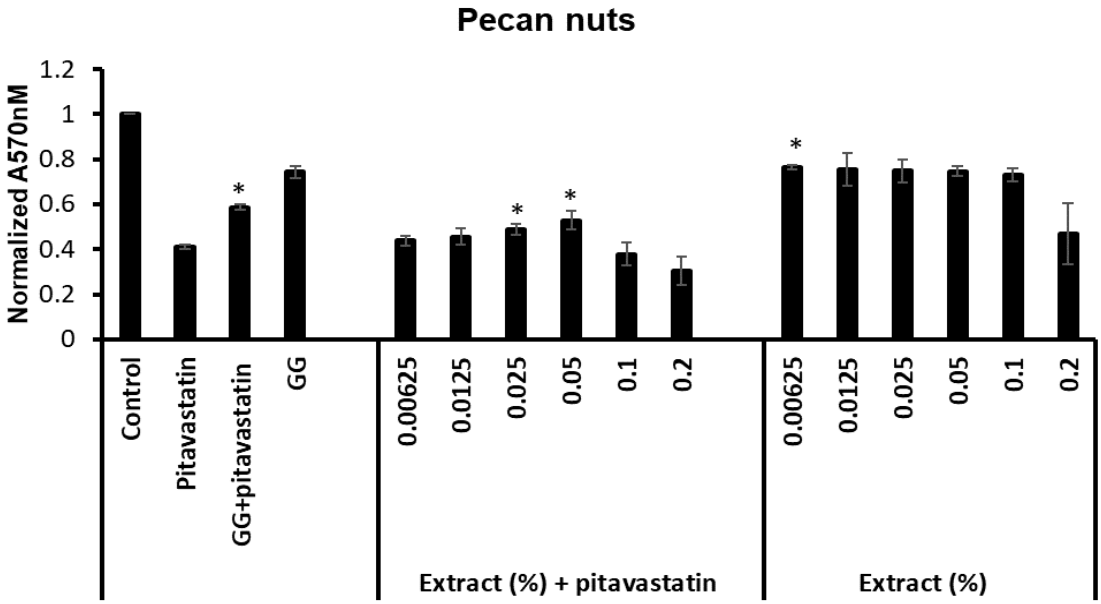


Figure (4-23): The effect of pecan nuts extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

4.2.2.2 Fruit & Salad

Extracts were also prepared from various fruits and salads. Again, relatively smaller amounts of solid were recovered compared to the oil extracts. These organic solvent extracts were also tested for their ability to suppress pitavastatin inhibitory activity in cell growth assays. The extracts from lettuce partially reversed the effect of pitavastatin while others (kiwi, pears, fig, passion fruit, pomegranate, gooseberry, tomato, squash, strawberry jam and cherry) showed no or little effect on pitavastatin cytotoxic activity.

Table (4-3): the weight of Fruits and vegetables extractions in 50 grams of foodstuff

No	Extraction	Weigh of extract obtained from 50 g of solid food)
1	Kiwi	10 mg
2	Lettuce	550 mg
3	Passion fruit	40 mg
4	Pomegranate	10 mg
5	Cherry	30 mg
6	Fig	40 mg
7	Gooseberry	10 mg
8	Pears	30 mg
9	Tomato	130 mg
10	Strawberry jam	80 mg
11	Squash	20 mg

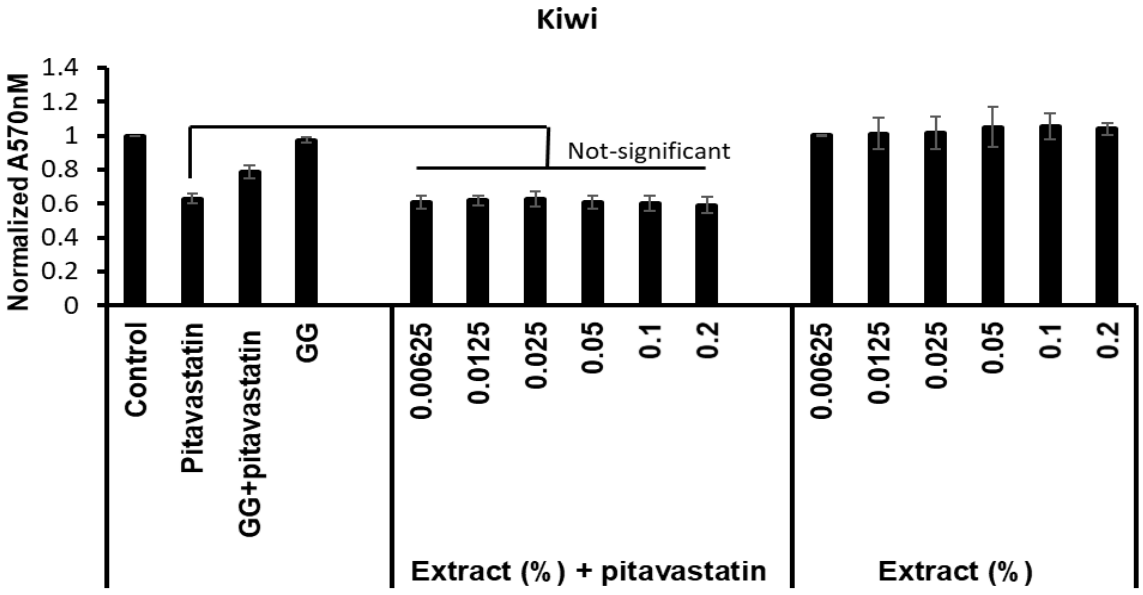


Figure (4-24): The effect of Kiwi extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

Figure (4-25): The effect of lettuce extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

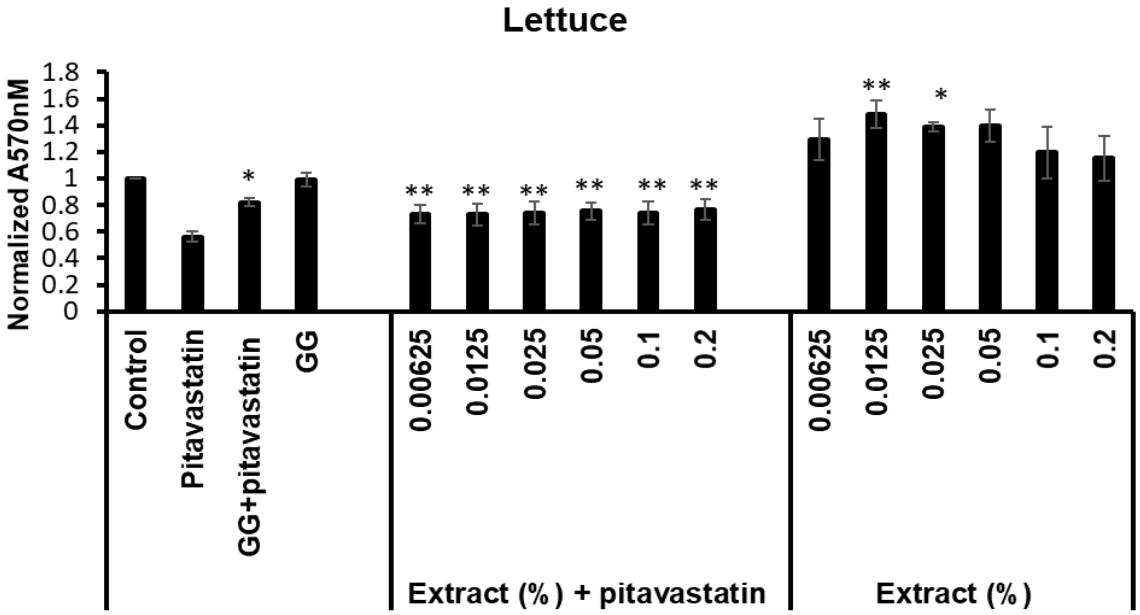


Figure (4-26): The effect of lettuce extract on pitavastatin activity against fuov1 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

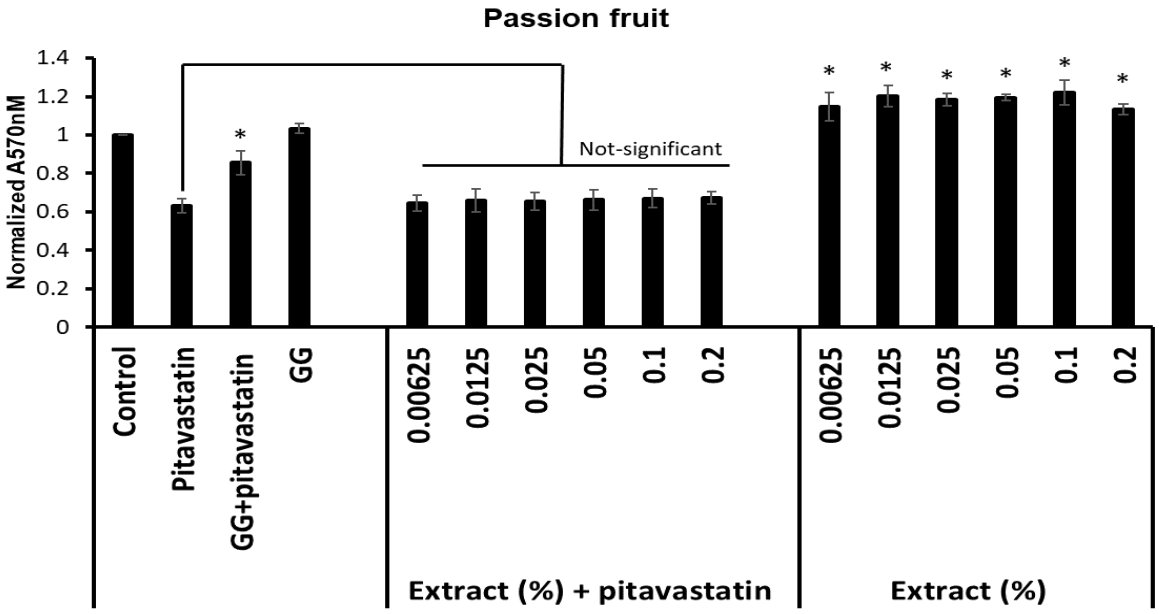


Figure (4-27): The effect of passion fruit extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

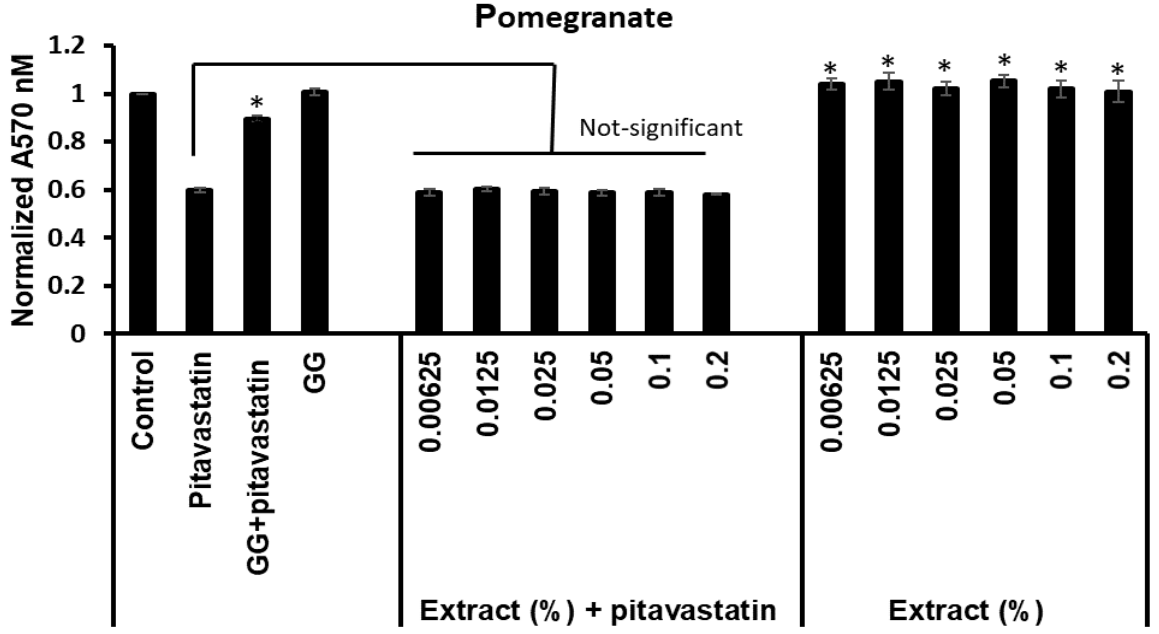


Figure (4-28): The effect of pomegranate extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

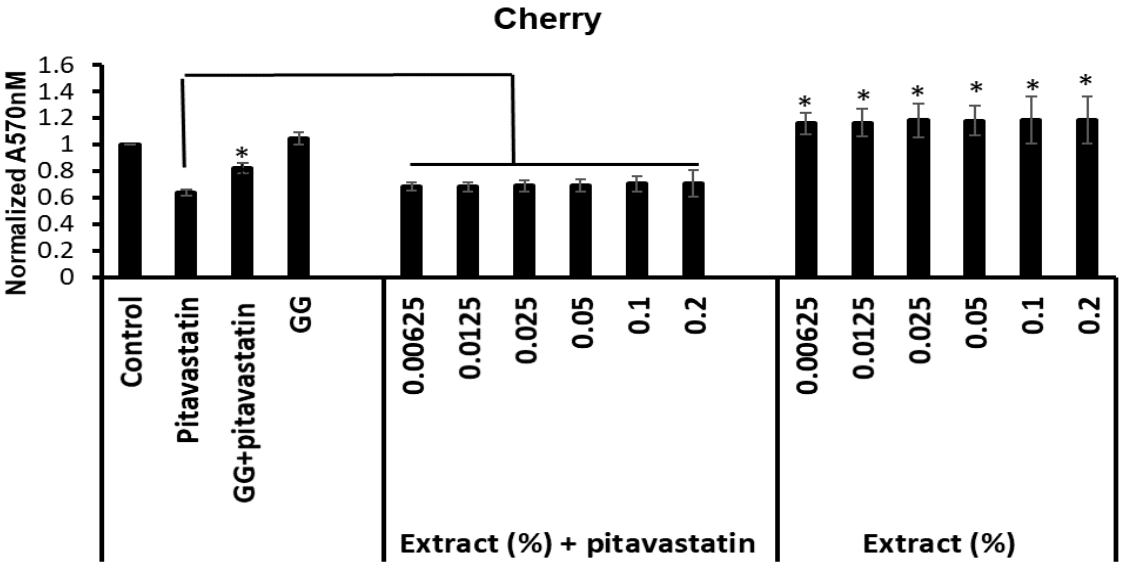


Figure (4-29): The effect of cherry extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

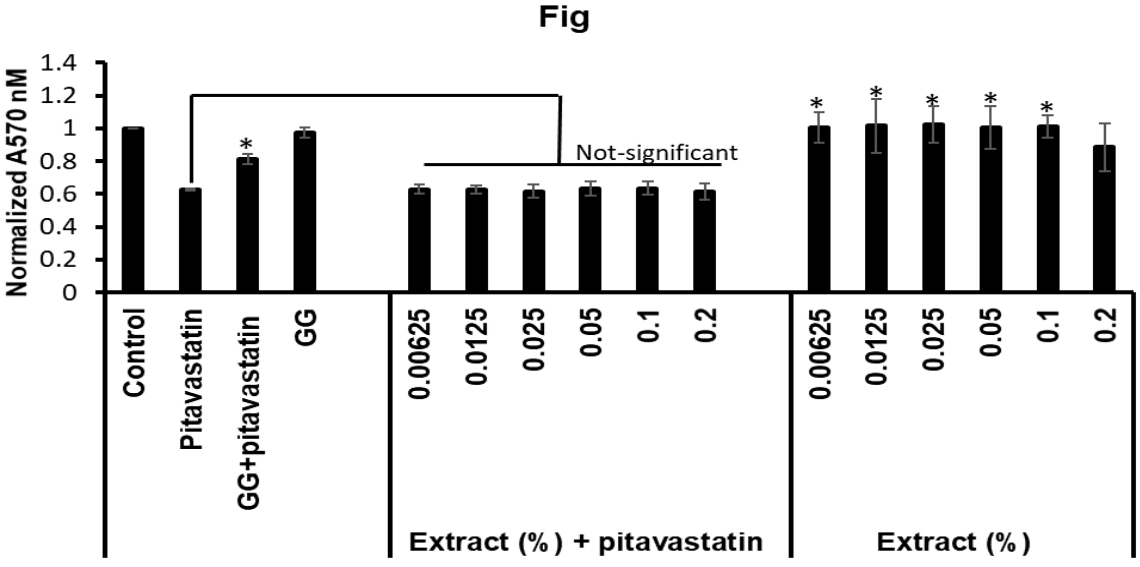


Figure (4-30): The effect of fig extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P<$ 0.05 and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

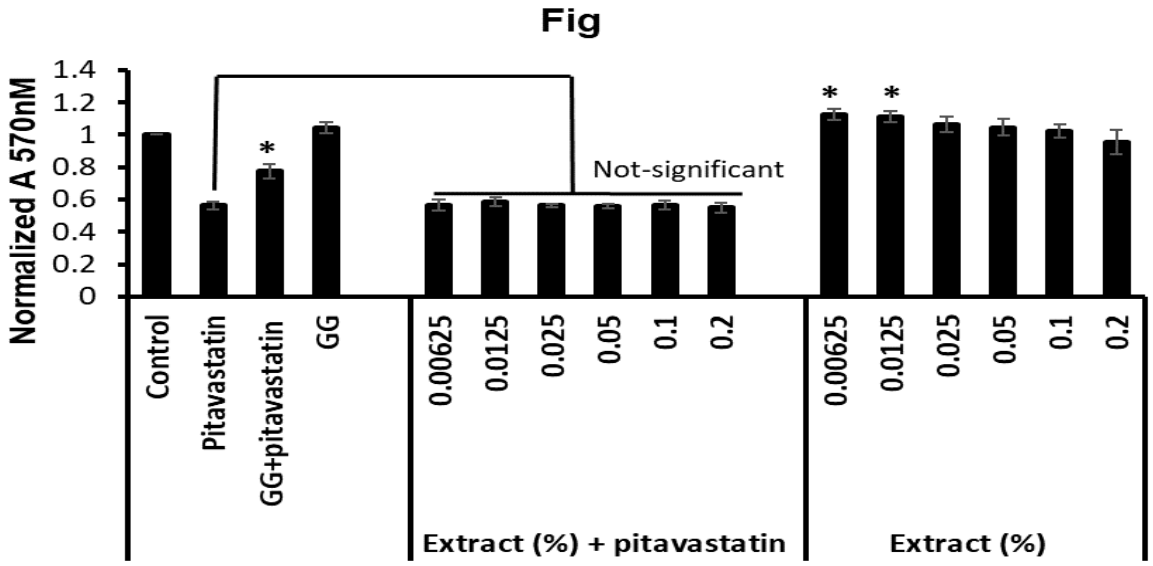


Figure (4-31): The effect of fig extract on pitavastatin activity against fuov1 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; One Way Anova).

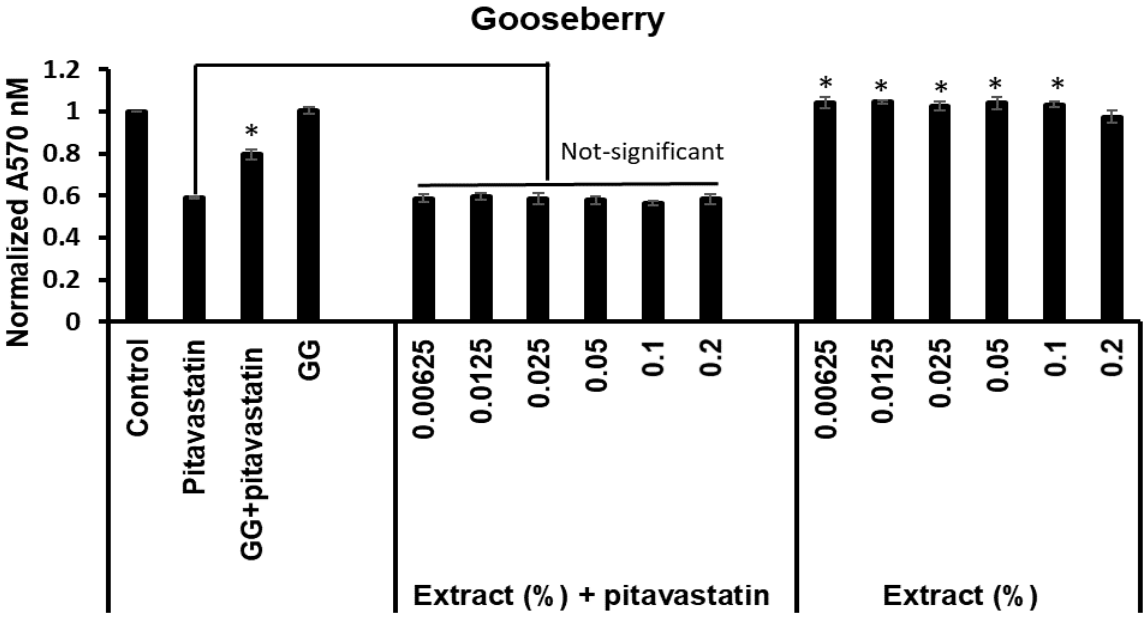


Figure (4-32): The effect of gooseberry extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; One Way Anova).

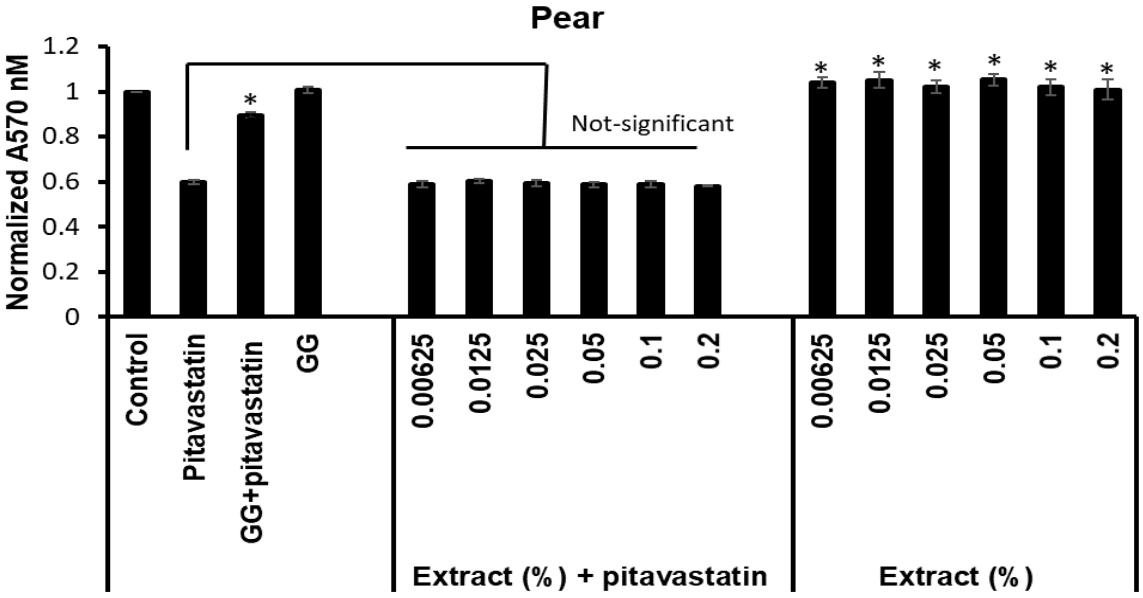


Figure (4-33): The effect of pear extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P<$ 0.05 and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

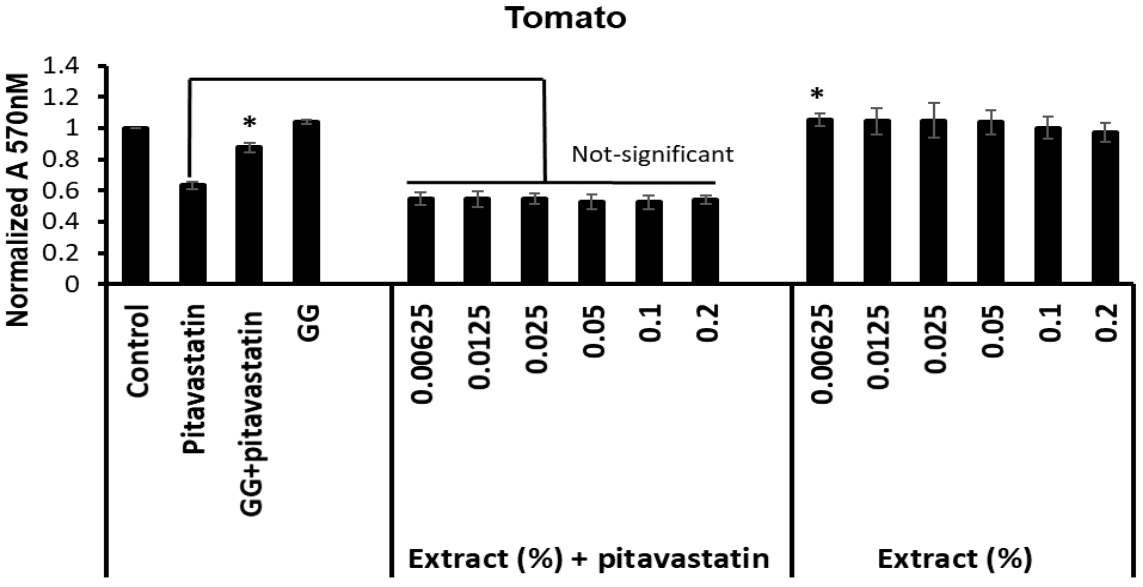


Figure (4-34): The effect of tomato extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P<$ 0.05 and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

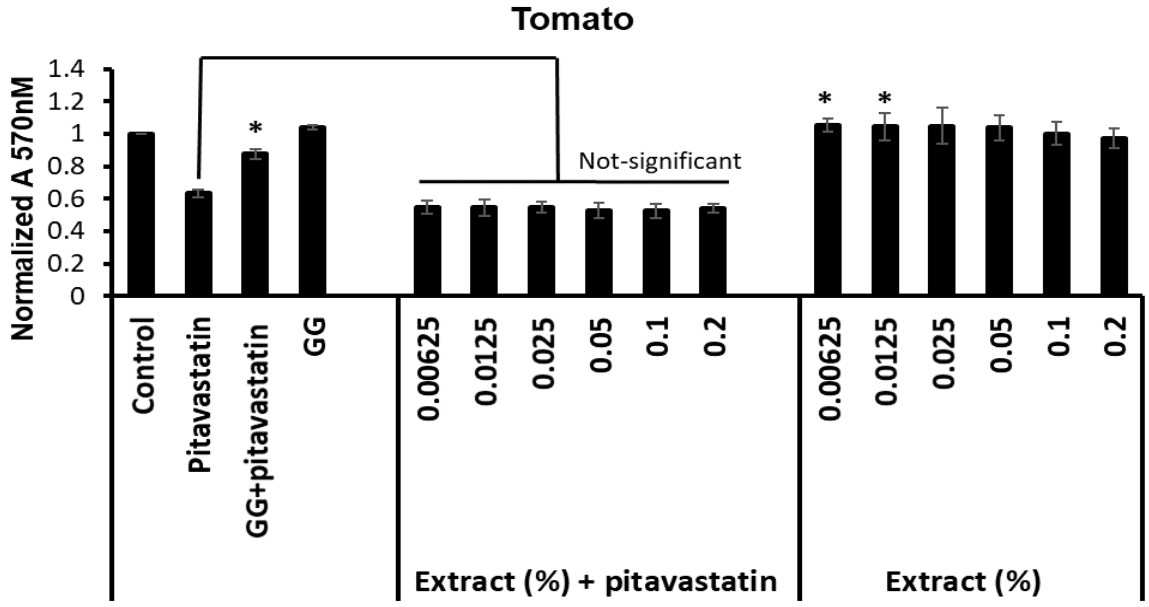


Figure (4-35): The effect of tomato extract on pitavastatin activity against fuov1 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

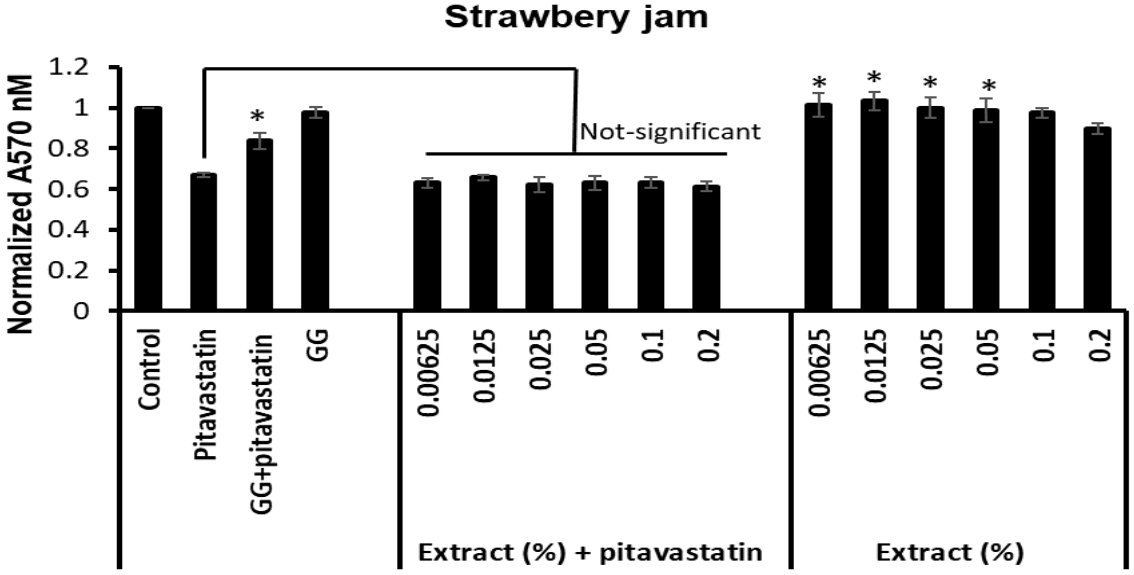


Figure (4-36**):** The effect of strawberry jam extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

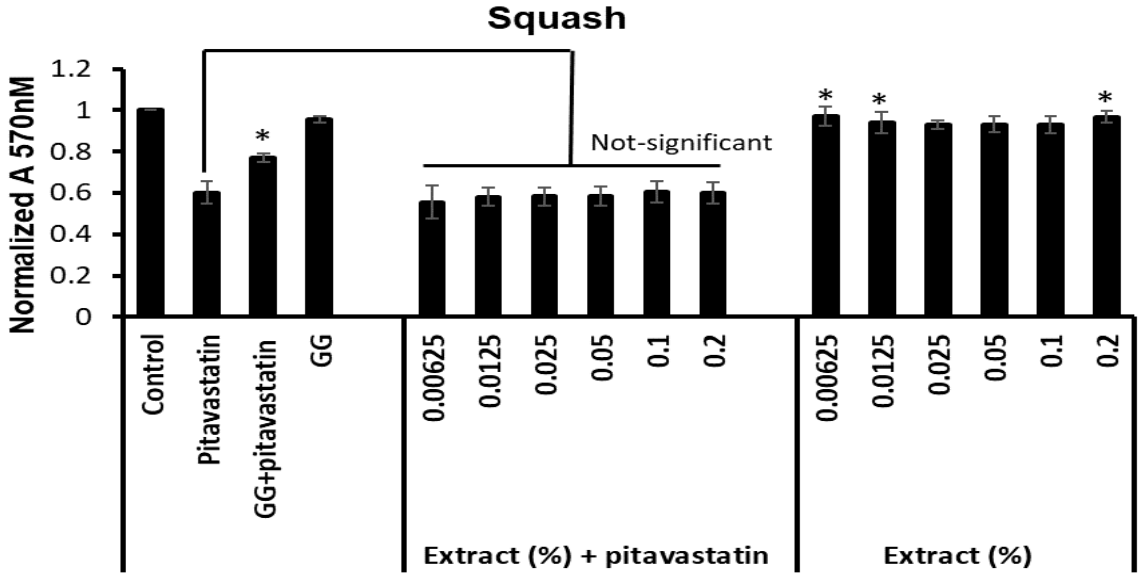


Figure (4-37): The effect of squash extract on pitavastatin activity against ovcar-4 ovarian cancer cell line. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of food extract. Extracts are expressed as a final concentration (v/v). After 72 hours, the number of surviving cells assessed by staining with SRB. The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells treated with solvent alone. GG denotes cells exposed to geranylgeraniol (5 μM) and were significantly different from this where indicated (*, $P< 0.05$ and **P ≤ 0.01, ***P ≤ 0.001; One Way Anova).

4.3 Trypan blue experiment

The ability of various food extracts to suppress the cytotoxic activity of pitavastatin was further evaluated by trypan blue staining of cells exposed to pitavastatin and the extracts to confirm the previous results (figure 4-35). The results were generally consistent with the results obtained in cell growth assays. The sunflower extract again significantly interfered with the activity of pitavastatin and increased the number of viable cells, although the extract had no measurable effect on its own. At the other hand, the extract from milk, which had limited effect in cell growth assays, proved unable to suppress the pitavastatin cytotoxic effect. In contrast, there are no significant effects of the milk extract when assessed in the trypan blue assay.

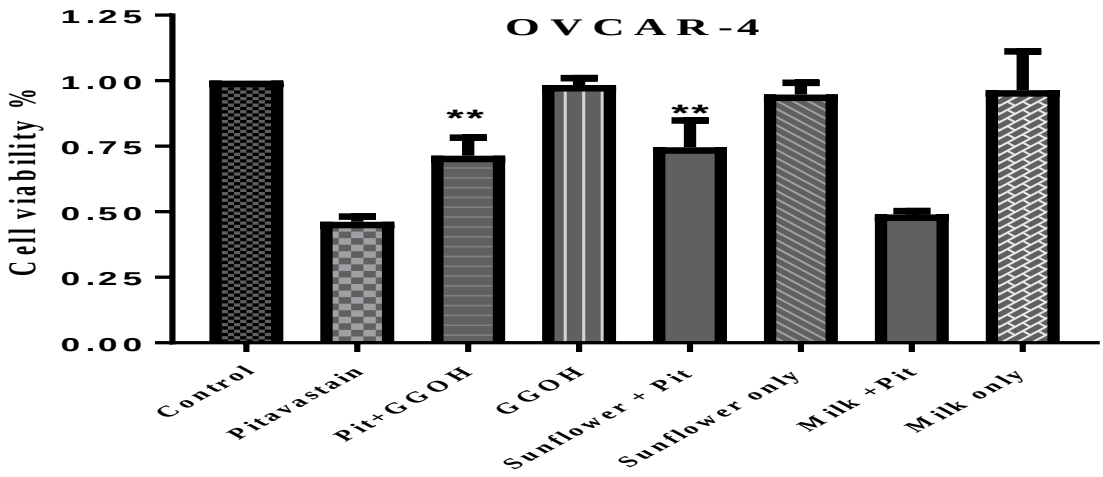


Figure (4-38): The effect of pitavastatin with/without geranylgeraniol and sunflower extract and milk extract with/without pitavastatin and the dead cells were measured by trypan blue staining after 72 of exposure ovcar4 to pitavastatin (10 μM) with/ without GGOH(5 μM) and sunflower or milk extract. The results (mean ± SD; n = 3) were compared to the effect expected for an additive interaction then calculated using mean and SD, Results were significantly different where shown (*, $P < 0.05$; **, $P < 0.01$; ***paired *t*-test).

4.2.4. GC/MS experiments

To confirm the presence of geranygeraniol in the food samples, some of the extracts were analysed by GC/MS. Geranylgeraniol was used to as a standard. Analysis of GC / MS data was performed by integrating each resolved chromatogram peak and normalising the area for the chromatogram total area. Peaks were tested for their mass distribution, and the geranylgeraniol library was used to classify such peaks. Sunflower oil and boiled black bean extract (figures (4-40, 4-41)) clearly contained geranylgeraniol. In contrast, free geranylgeraniol was not detected in extracts from boiled egg and lettuce.

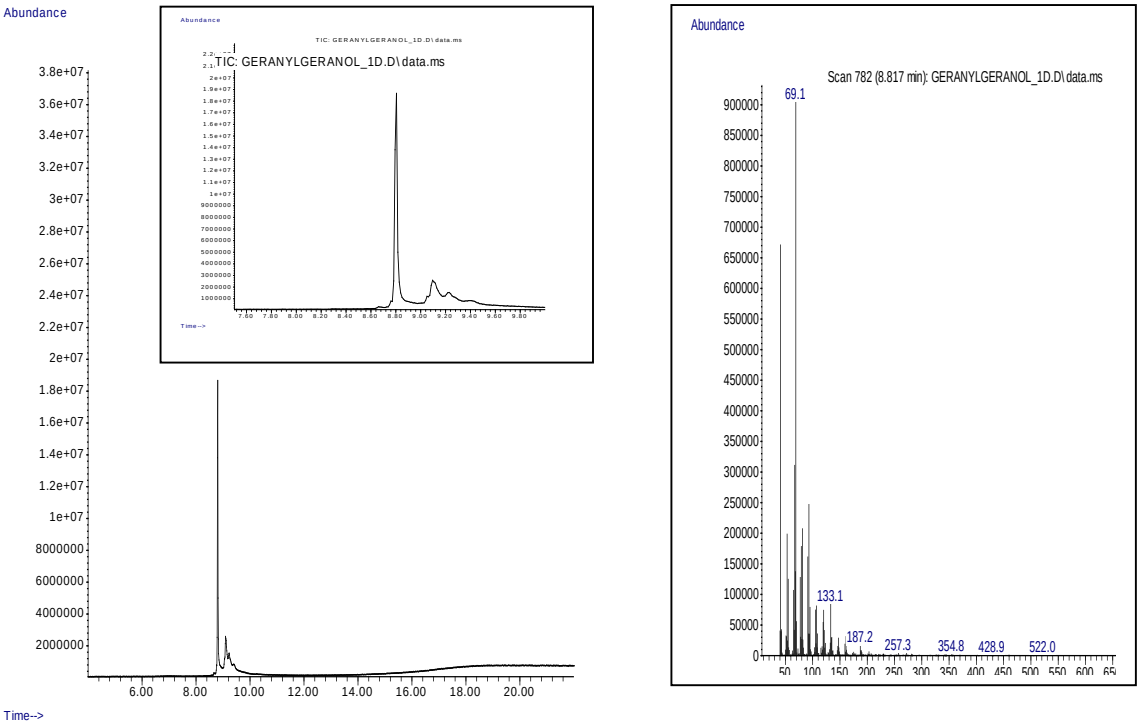


Figure (4-39): Shows the Standard geranylgeraniol of GC-MS chromatogram (A), and the electron ionization mass spectrum of geranylgeraniol at Rt 8.82 min (B)

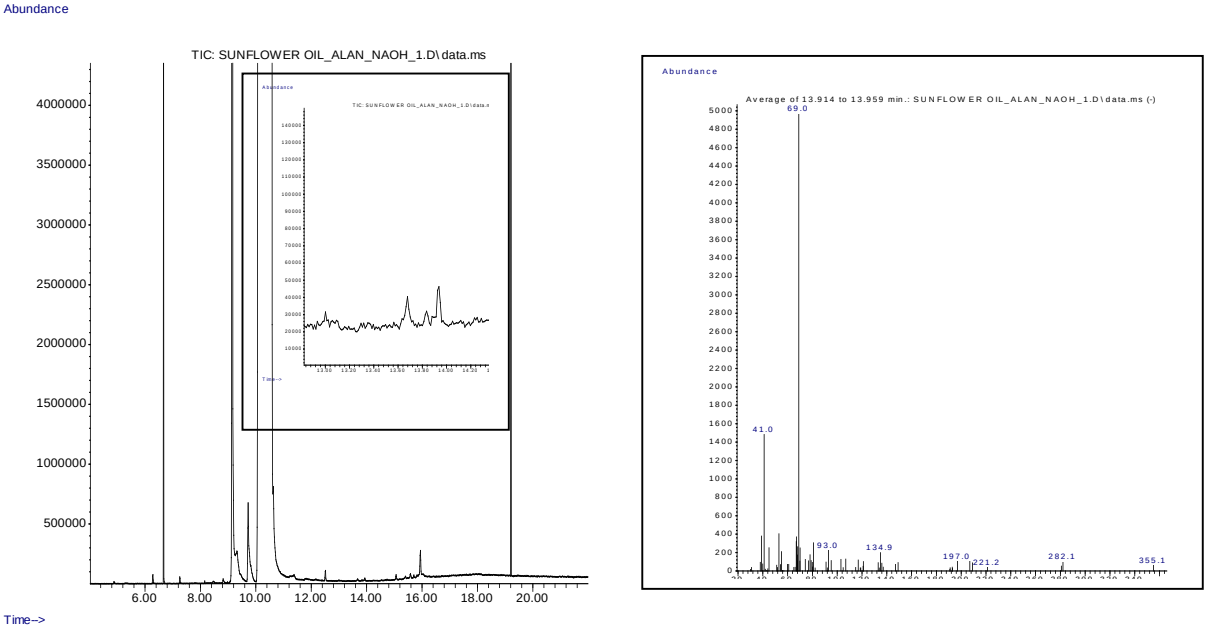


Figure (4-40): Shows the GC-MS chromatogram of sunflower oil, 20 mg/ml in hexane, 1 uL injected (A), and the electron ionization mass spectrum of a geranylgeraniol derivative at Rt 13.92 min (B).

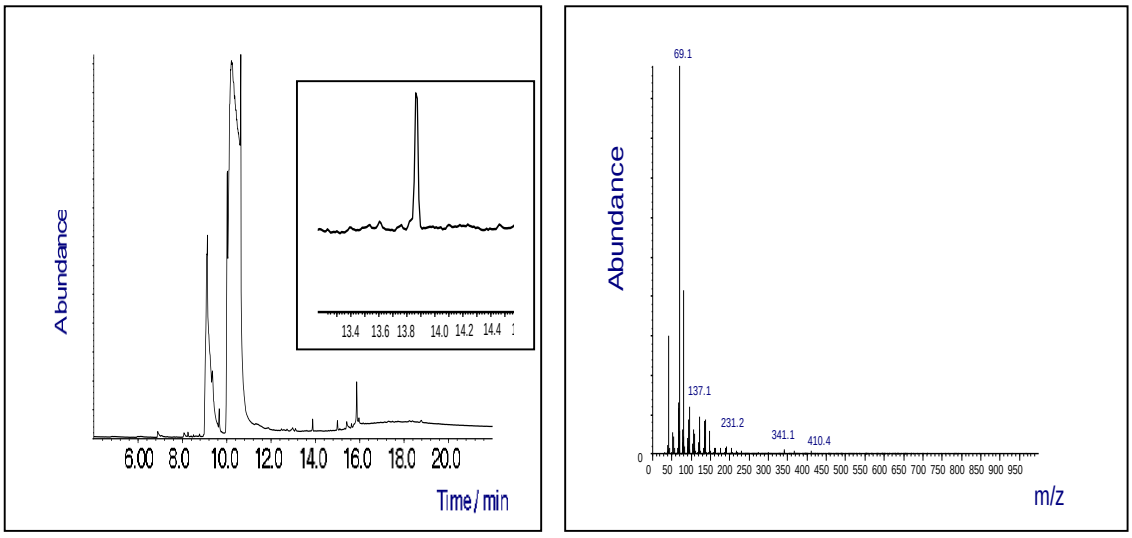


Figure (4-41): Shows the GC-MS chromatogram of boiled black bean extract, 20 mg/ml in hexane, 1 uL injected (A), and the electron ionization mass spectrum of a geranylgeraniol derivative at Rt 13.88 min (B).

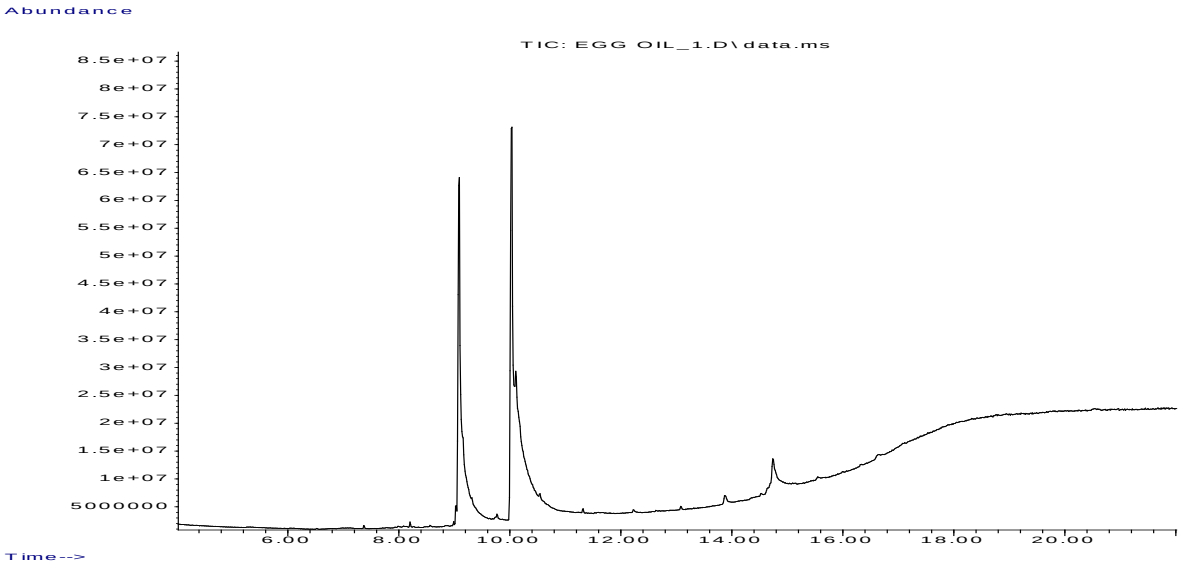


Figure (4-42): Shows the GC-MS chromatogram of egg extract oil, 20 mg/ml in hexane, 1 uL injected (no geranylgeraniol derivatives found).

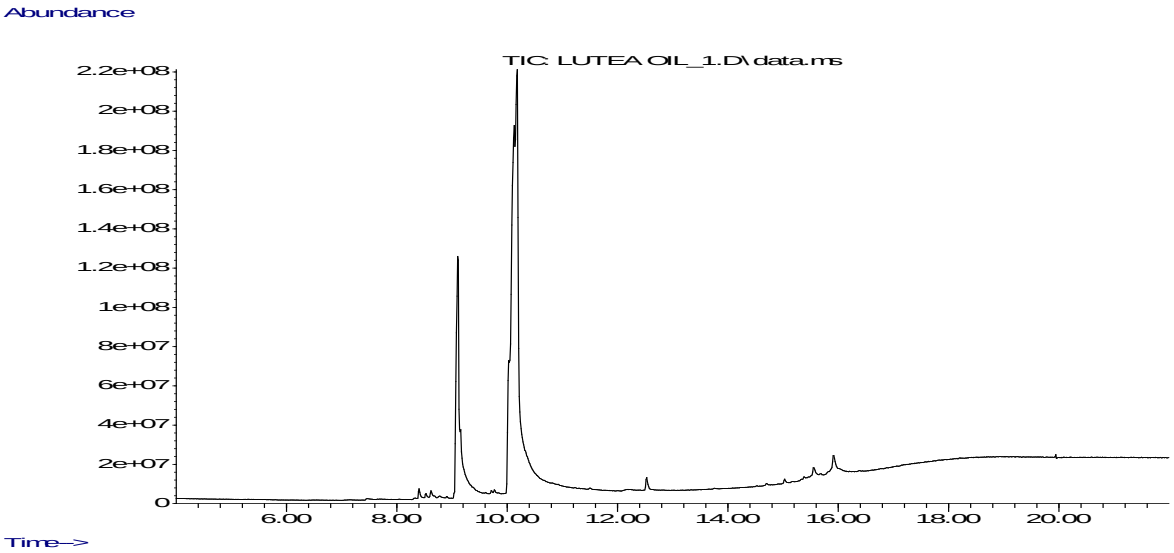


Figure (4-43): Shows the GC-MS chromatogram of lettuce extract, 20 mg/ml in hexane, 1 uL injected (no geranylgeraniol derivatives found).

4.2.5. The effect of mevalonate pathway intermediate metabolites on pitavastatin anticancer activity

Although it has previously been shown that geranylgeraniol can suppress the cytotoxic activity of pitavastatin, it was possible that other mevalonate pathway metabolites might also be able to do this. Consequently, several different metabolites of the mevalonate pathway were evaluated in combination with pitavastatin in cell growth assays using OVCAR-4 cells. Mevalonate & Geranylgeraniol showed nearly complete suppression of the cytotoxic activity of pitavastatin. In contrast, supplementation of cell culture medium with either coenzyme Q10 (5μM), Dolichol (5μM) or Cholesterol (5μM) did not have significant effects on the cytotoxic activity of pitavastatin (fig. (4-44)). To confirm these results, the experiments were also repeated using FUOV1 cells (fig (4-45)) and similar results were obtained.

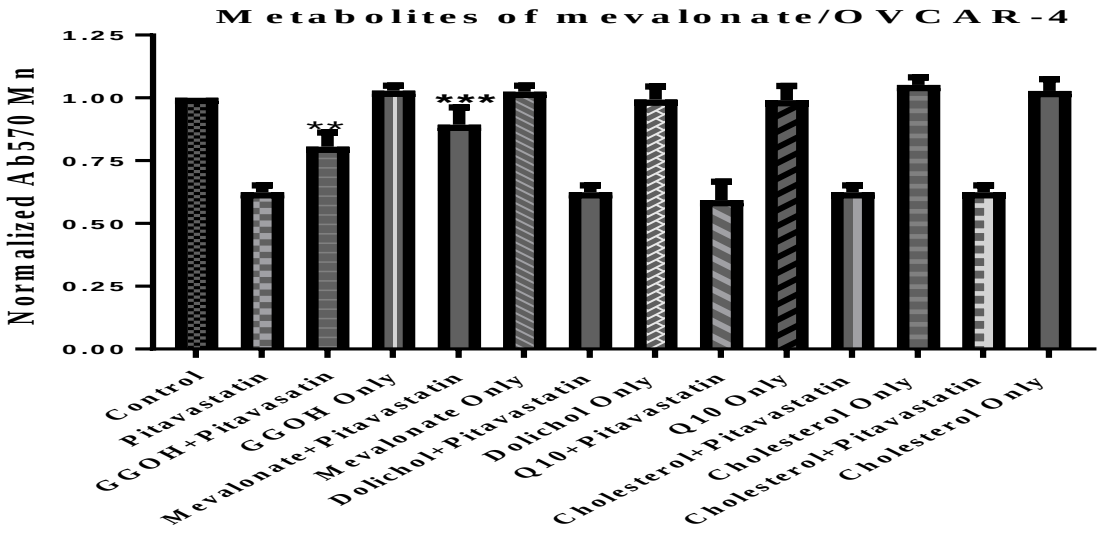


Figure (4-44): The effect of metabolites of the mevalonate pathway on pitavastatin's activity against Ovcar-4 ovarian cancer cells. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of metabolites (coenzyme Q10 (5μM), Dolichol (5μM) or Cholesterol (5μM)). After 72 hours, the number of surviving cells assessed by staining with SRB. GG denotes geranylgeraniol (5 μM) The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells untreated and were significantly different where indicated by an unpaired *t*-test (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; unpaired *t*-test).

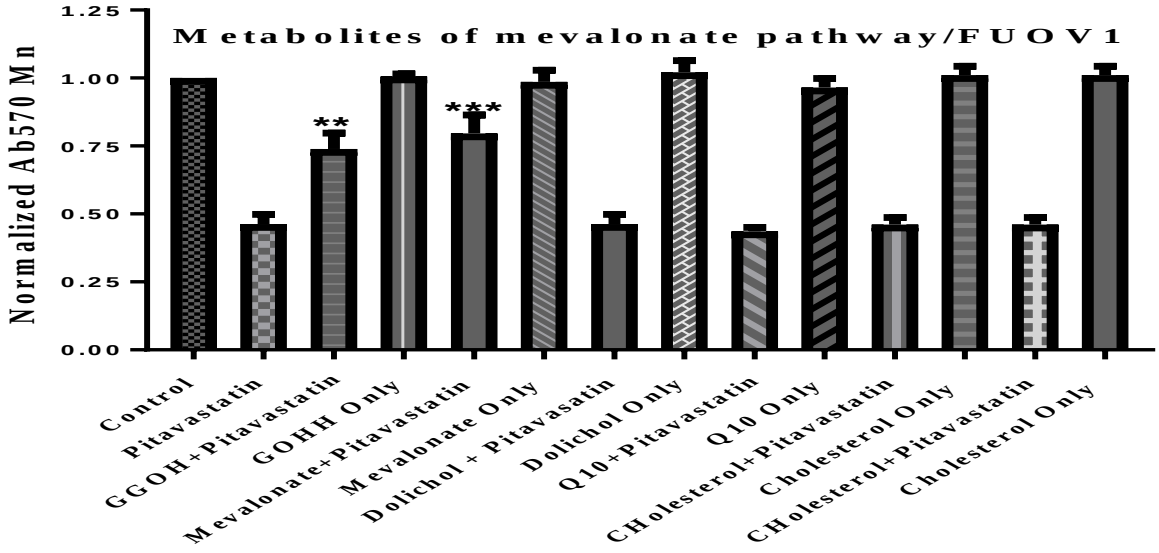


Figure (4-45): The effect of metabolites of the mevalonate pathway on pitavastatin's activity against fuov1 ovarian cancer cells. Cells were exposed to pitavastatin (10 μM) and/or the indicated concentration of metabolites(coenzyme Q10(5μM), Dolichol(5μM) or Cholesterol(5μM)). After 72 hours, the number of surviving cells assessed by staining with SRB. GG denotes geranylgeraniol (5 μM) The results (mean ± S.D., n =3) are expressed as a fraction of the absorbance measured in cells untreated and were significantly different where indicated by an unpaired *t*-test (*, $P< 0.05$ and **$P \leq 0.01$, ***$P \leq 0.001$; unpaired *t*-test).

4.4 Discussion

This study set out to evaluate if human foods could potentially impair the efficacy of pitavastatin as a drug to treat cancer. Unfortunately, statins have failed so far to show activity in prospective clinical trials as a cancer treatment. One plausible factor that contributes to this failure is that patients consume foods which contain geranylgeraniol and this bypasses the inhibitory effect of statins on the mevalonate pathway. Exposing cancer cells to pitavastatin in the presence of extracts collected from a variety of commonly consumed foods demonstrated that certain foods appear to contain a substance which inhibited the cytotoxic activity of pitavastatin against cultured ovarian cancer cells.

Some foods appear better able to inhibit the activity of pitavastatin than others do. The extract from sunflower showed almost a complete reversal of the inhibitory effects of pitavastatin in both cell lines evaluated (OVCAR-4 & FUOV-1, (figures 4-3 and 4-4)), while some extracts, such as corn oil, groundnut oil, coconut oil, grape seed oil, lettuce, Dolmio (pasta sauce tomato), oats, boiled black bean, pecan nuts and boiled egg, only partially reversed the effects (see figures 4-5 to 4-23). These results were supported by GC-MS analysis which revealed the presence of geranylgeraniol in sunflower and boiled black bean extracts (fig. (4-40,4-41)). In contrast geranylgeraniol was not detected in the extract from milk, and milk extracts were also inactive in the bioassay. The boiled egg and lettuce extracts (figures (4-42, 4-43)) partly suppressed the cytotoxic activity of pitavastatin but geranylgeraniol was not detected by GCMS. There are several potential explanations for this minor discrepancy. Possibly only small amounts of geranylgeraniol were present in the extract, and not detected by GCMS. Alternatively, different sources and types of lettuce may have been tested (unfortunately different extracts were tested). Alternatively, there may be compounds other than geranylgeraniol present in the extract which inhibit the activity of pitavastatin such as

mevalonate which is also able to block the cytotoxic effects of statins. In general, the oils seem to be a relatively rich source of geranylgeraniol and this is supported by a limited number of published analyses. Green foods may also contain geranylgeraniol than others. The results presented here provide identify some foods which patients could consume while receiving pitavastatin as cancer treatment as part of a clinical trial. However, there may be further complications because patients may prefer specific type food according to the traditional foods in the country or due to family habits. This could play an important role in the amount of geranylgeraniol that patients ingest. This may also contribute to some of the differences in retrospective clinical trials which have either claimed statins have an anti-cancer effect or have no effect. Differences in food consumption between different patients may lead to differences in the therapeutic effect of statins as anti-cancer agents. An added complication is that geranylgeraniol may be liberated from other compounds in intestine like wax esters or Geranylgeranyl diphosphate (GGPP) for example by intestinal phosphatase [389] or esterases, or even acid hydrolysis. However, the bioavailability of the liberated geranylgeraniol, and the parent compounds, is still unknown and further studies are needed to estimate the plasma level of geranylgeraniol which would result from their consumption.

Some of the extracts, for example those from corn, coconut, walnut and boiled eggs inhibited the growth of the ovarian cancer cell cultures when they were tested on their own (in the absence of pitavastatin). This was particularly evident when these extracts were tested at higher concentrations (figures (4-5,4-10, 4-11and 4-22)). This made it impossible to evaluate whether these extracts contained a substance able to suppress the activity of pitavastatin, at least when tested at the higher concentrations. We have not yet identified the compounds present in the different foods, which are responsible for the inhibitory activity present in these extracts. It may simply reflect the effect of large amounts of oil acting in a non-pharmacological manner, for example by disrupting cell membrane structure. In addition, the oils may have biophysical

mechanism of inhibition by creating droplets that effectively sequester the pitavastatin due to its lipophilic structure.

Blockage of the mevalonate pathway by statins results in reduction of the level of cholesterol and simultaneously other products of the pathway for instance geranylgeranyl pyrophosphate (GGPP), farnesyl pyrophosphate (FPP), dolichols, Co-enzyme Q10 and Cholesterol (Mullen et al., 2016). It was, therefore, important to evaluate if compounds other than geranylgeraniol could suppress the activity of pitavastatin. Only mevalonate and geranylgeraniol, had a significant effect (see figures (4-44 and 4-45). Mevalonate significantly, but not fully, reversed the effect of pitavastatin. The inability to completely supress the effect of pitavastatin may reflect technical problems in the assay. Mevalonate pathway metabolites can down-regulate HMGCR protein levels through feedback regulation of the SREBP transcription factor that regulates expression of *HMGCR* gene. It may be technically challenging to provide exactly the right concentration of, for example, mevalonate, to restore full pathway activity without activating the negative feedback pathways which would diminish the production of other pathway intermediates. Another potential explanation for the failure to completely suppress the effects of pitavastatin is that high concentrations of, for example, mevalonate is toxic through another mechanism and so it is impossible to compensate for mevalonate pathway inhibition by pitavastatin without causing toxicity by another mechanism. In addition, the prenylation of proteins by geranylgeranyl transferases requires geranylgeraniol pyrosphosphate. These experiments tested geranylgeraniol rather than its pyrophosphate. Using the alcohol rather than the pyrophosphate increased the likelihood of the isoprenoid being membrane permeable and becoming available inside the cells. The solvent extracts may have also contained geranylgeraniol rather than its pyrophosphate due to the alkaline hydrolysis step which would be expected to convert any geranylgeraniol present as a pyrophosphate to the corresponding alcohol. In addition, geranylgeraniol rather than the pyrophosphate may be liberated from

compounds in food. Geranylgeraniol is known to be present as wax esters in various vegetable oils. The wax esters often contain geranylgeraniol as esters of palmitic acids, and unsaturated C18 acids in particular [390] or even fatty acids containing up to 24 carbon atoms. The literature contains conflicting data whether wax esters can be hydrolysed to release free geranylgeraniol, but hydrolysis would release geranylgeraniol as its unmodified alcohol[391]. Regardless of the source of the geranylgeraniol (from wax esters or added directly into the assay as a control), it is not clear how cells can generate geranylgeraniol pyrophosphate from the corresponding alcohol. Normally, the pyrophosphate form of the isoprenoids is derived from phosphorylation of mevalonate by mevalonate kinase. The phosphorylation of geranylgeraniol by this enzyme may be relatively inefficient and so all the geranylgeraniol may not be converted to the pyrophosphate. This could again contribute to the failure of the isoprenoids to completely suppress the effect of pitavastatin.

The other metabolites that were tested included co-enzyme Q10, dolichol and cholesterol and had no significant effect on the cytotoxic activity of pitavastatin. This is particularly important because of the issue of myopathy caused by statins, particularly at high doses. Although all statins are relatively safe drugs, they are associated with a significantly increased risk of myopathy, ranging in severity from asymptomatic increases in creatine kinase (CK) and muscle weakness, aches, and fatigue to fatal rhabdomyolysis. Even though the prevalence of rhabdomyolysis is thankfully very small (0.1 percent of all statin users), it is notable the number of patients who do not tolerate statins due to myopathic symptoms (1–10 percent)[392]. Importantly, co-enzyme-Q10 is sometimes recommended to patients to minimize the likelihood of statins causing myopathy. However, the evidence that co-enzymeQ10 is effective in suppressing statin-induced myopathy is not particularly convincing. Several studies were conducted to determine the association of co-enzyme Q10 concentration in serum and muscle coenzyme levels and with myopathy symptoms following statin administration. Few studies

have shown that the statins have reduced circulating levels of co-enzyme Q10, while other studies have not confirmed a decrease in muscle co-enzyme Q10 levels during statin therapy[393]. In some studies, co-enzyme Q10 supplementation can help to increase the plasma levels of co-enzyme Q10 in those taking statins[394]. In contrast, other studies show that the supplementation of coenzyme Q10 may boost myopathy symptoms[381], [395]. Consequently, a causal correlation between reduced co-enzyme Q10 and myopathy has not been definitively demonstrated[396]–[398]. In addition, randomised, double-blind clinical trials have failed to demonstrate that co-enzyme Q10 supplementation decreases myopathy associated with statins[399]. Despite these discrepancies, co-enzyme Q10 continues to be recommended to some patients to relieve statin-induced myopathy. Thus, it was particularly important to evaluate the effects of co-enzyme Q10 on the activity of pitavastatin. The observation that co-enzyme Q10 failed to alter the activity of pitavastatin in the bioassay suggests that if patients taking pitavastatin to treat cancer are also recommended to take co-enzyme Q10 to reduce the risk of myopathy, it will not interfere with the anti-cancer activity of pitavastatin.

In summary, the goal of these studies was to explore the potential for metabolites of mevalonate pathway within food to interfere with the anticancer activity of pitavastatin in clinical trials. The data presented here suggested that patients using statins to treat cancer should avoid most vegetable oils as well as some “green” food. A number of foods were identified that patients could consume in addition to Ensure and this may increase the chance of clinical trial of pitavastatin being successful. In clinical trials, it will be important to assess whether an appropriate dose of pitavastatin can be found that is effective against cancer cells without causing unacceptable toxicity.

Chapter 5

Exploration of the mechanism of action of pitavastatin as an anti-cancer agent

5. Introduction

Cancer is a heterogeneous multifactorial disease that continues to be one of the leading causes of death worldwide, accounting for 21% of total death and is the second-largest disease affecting the people in developed countries[400], [401]. Despite substantial advancement in cancer research and the production of medications, the long-term survival of most cancer patients remains limited even though, in recent years, an significant body of evidence has accumulated supporting the notion that a wide range of both congenital and somatically acquired ribosomal abnormalities, as well as regulation of ribosomal function by oncogenic factors, lead to a phenotype for cancer [401]. However, phenotypic cancer symptoms tend to represent specifically changes in protein synthesis and function, which are also the targets of most drugs [402], genomics and transcriptomics have been the focus of study for years.

The inadequacy of current screening services to tackle the deadliest cancers highlights the need for innovative methods to strengthen cancer screening, diagnosis, and care. Biomarkers are one such tool. For example, biomarkers with susceptibility may indicate screening modalities, if any, should be used to evaluate the risk level. Predictive biomarkers can be used for highly selective therapies to prospectively identify appropriate patients for treatment, and biomarkers for the prognosis can be used to track treatment response. The use of biomarkers in both clinical development and marketplace will be essential to success[403]. Biomarkers are classified as indicators of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention which can be calculated objectively[404]. They may take several forms — genes, proteins, carbohydrates, ions, cells or physiological measurements that are radioactively identified[405]. However, the detection and development of biomarkers for cancer may pose a greater research challenge due to the biological nature of cancer, the varied settings where biomarkers can be useful in cancer screening, diagnosis and care include the identification of patients who are predisposed to cancer; the conclusive detection of disease and

the classification of disease subtypes and prognoses ; choice of the most suitable treatment for the particular patient, including dose titration and minimization of adverse events; and control of therapy, the needs of biomarkers used in early cancer diagnosis vary from those used in disease staging and treatment decisions[403]. No present screening approach for the early detection of ovarian cancer has influenced ovarian cancer patients' survival. Developing a successful ovarian cancer screening approach is challenging because it is not a single disease and can be divided into at least five distinct histological subtypes with distinct recognizable risk variables, each with distinct bio-related and clinical characteristics [406].

Statins are potent cholesterol decreasing drugs that have been shown to suppress in vitro proliferation of tumour cells and growth of tumours in animal models. In addition, longitudinal analyses of human cohorts showed decreased cancer-specific mortality in patients taking statins[407]. However, previously included E-cadherin membrane expression as both a marker and a mechanism for the resistance to atorvastatin-mediated growth suppression of cancer cells; however, a statin sensitivity transcriptome-based biomarker signature has not yet been identified[407]. Therefore, we investigated the sensitivity of biomarkers for statin therapy using the bioinformatics-experimental approach.

Several studies have recently identified numerous mevalonate pathway (MP) enzymes as crucial to the survival of various transformed cells by supplying sterol and isoprenoids and other products [408], [409], therefore, the role of MP in many aspects of carcinogenesis has contributed to its being considered a therapeutic target. MP deregulation may lead to malignant transformation in OC. Recent findings from our laboratory have indicated that pitavastatin is superior to other oncological statins as it is the only statin that is both lipophilic, making it more active than hydrophilic statins, and has a long half-life (t1/2 ~11 hours) [308], [309], [410], [411]. The latter property of pitavastatin is essential because it has been shown that continuous

inhibition of HMGCR is sufficient to induce cell death, and the gaps in plasma drug concentration between prolonged dose intervals with short half-life statins are likely to compromise statin activity [412]. HMGCR activity can be deregulated in tumours and become resistant to negative sterol feedback regulation, which can help to provide an abundance of isoprenoids to promote transformed cell growth. However, despite a good understanding of the mevalonate pathway itself, there is no clear mechanistic understanding of how inhibiting it causes cancer cell death. This lack of mechanistic understanding also makes it difficult to predict which individual cancer patients are mostly likely to benefit from treatment with a statin. The research described in this chapter set out to address these deficiencies. The Broad Institute has conducted an analysis of the sensitivity of a panel of 500 cancer cell lines to a range of therapeutic drugs. The expression profile of these cell lines is has also been reported. This allows identification of genes whose expression correlations with drug sensitivity and which may correspondingly be involved in setting the cellular sensitivity to the drug. The expression of these genes may then both provide biomarkers to identify which patients are most likely to respond to the drug as well as provide insight into the mechanism by which the drugs are cytotoxic.

Isoprenoids are used to alter many small GTPases of superfamily proteins post-translationally and to help their membrane localisation[413], and provide an obvious potential pathway affected by pitavastain Superfamily members are important regulators for most cell processes, including differentiation, proliferation, dynamics and transport of vesicles and organelles, nuclear dynamics, and cytoskeleton regulation[414]. The Rho family's small GTPaes are master regulators for actin cytoskeleton rearrangements and cell morphology[415]. GTPases also control microtuble structure, dynamics, plus-end capture and actin cytoskeleton[416].

5.1. Microtubules and MAPs

Microtubules are vital elements of the cytoskeleton and play a critical role in many cellular procedures, including cell division, cell motility, intracellular trafficking, and cell cytoskeleton maintenance[417]. Microtubules are intrinsically dynamic polymers composed of αβ-tubulin heterodimers and their dynamic properties are crucial for the assembly of the mitotic spindle and for the attachment and separation of chromosomes during mitosis [418].

Tubulin protein family members share a strongly homologous structure consisting of a globular body created from the N-terminal and intermediate domains and an extremely flexible and disordered carboxy-terminal (C-terminal) tail area[419]. The C-terminal tail of the tubulin proteins stretches outside the microtubule wall, where it is the location for a broad spectrum of post-translation modifications and protein interactions that control the dynamics of microtubules and other signalling effectors[419], [420]. The C-terminal tails are the most divergent areas among different isoforms of β-tubulin, making this area a prominent candidate in identifying the particular function of the tubulin proteins[421]. The structure of tubulin isoforms is a key element of the tubulin code, together with post-translation modifications.

Several microtubules associated proteins (MAPs) have been identified. Maps can alter the dynamic parameters of microtubules and organize microtubules into complicated structures [422]. The MAP family also involves microtubule motors that slide along the MTs. The motors use ATP hydrolysis to move cargo along microtubules, carrying vesicles, chromosomes, DNA, RNA and even other microtubules[423], [424]. Therefore, MT networks and related MAPs play important roles in different biological processes, including cell division, intracellular trafficking and cell morphogenesis[424], [425]. MAPs are a family of proteins that bind to and stabilize microtubules, MAP protein structures include members of the proteins MAP1, MAP2, MAP4, and Tau; they are called "structural" MAPs because they are non-enzymatic proteins

that bind along the microtubular surface [426], [427]. There are several comprehensive reports on the interactions of MAPs with microtubules, which concentrate on the actin binding and function of the MAPs — MAP1/2/4 and Tau structures as microtubule – actin cross linking activities[426].

One member of family of MAPs is Ensconsin / MAP7 and it is connected with the cytoskeleton interphase microtubules, but its over-expression in cultivated cells does not appear to influence the dynamics of microtubules during interphase; instead mitotic defects often happen during cell division[428].

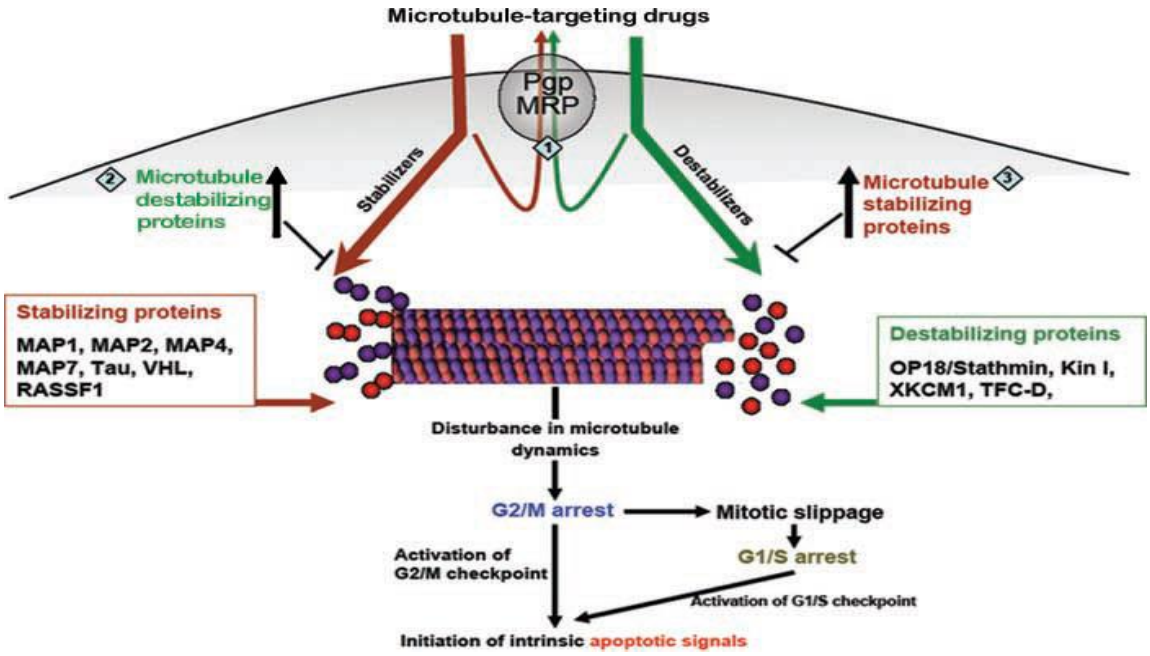


Figure (5-1): MAPs and cancer cell resistance / sensitivity to the action of microtubule-targeting medicines**,** Intracellular proteins control the action of agents that stabilise or destabilise microtubules which affect the dynamics of the microtubules and how the microtubule-targeting drugs induce apoptosis[429].

Microtubule-associated proteins (MAPs) were first identified as proteins that bound to microtubules and stabilise them. Currently, a still growing number of MAPs shows a more complex image of such proteins as microtubule cytoskeleton organisers with a broad range of functions, MAPs require microtubules to participate in a variety of cellular processes such as the assembling of mitotic and meiotic spindles, the development of neurons and the formation

of ciliary axonemas[430]. MAP4 is widely expressed, whereas MAP1 and MAP2 are predominantly expressed in neurons and MAP7 is limited to epithelial cells [431]. All members of the MAPs family have a similar organisation, with two preserved domains predicted to be helical, linked by an unstructured linker. The neuronal MAPs tau and MAP2 hinder motility of kinesin-1[432]–[434]. Monroy and their colleagues found the reconstitutions *in vitro* with purified proteins showed that MAP7 recruited kinesin-1 to MTs and reduced motor speed but only had a mild effect on kinesin-1 running length[432]. MAP7 was extremely immobile in these trials and was not co-transported with the engine, indicating that MAP7 affects the initial recruitment of kinesin to MTs but has little effect on kinesin-1 movement [432]. Some researchers measured function of three MAPs which are required for kinesin-1, initially using the distribution of mitochondria as a readout, because it strongly depends on kinesin1 KIF5B; in the absence of KIF5B, mitochondria were no longer distributed in the cytoplasm but clustered around the nucleus [435]. The MAPs family members are firmly established as positive kinesin-1 regulators[423], [436], [437]. MAP7 proteins are represented in flies by one homologue (ensconsin), and in mammals by four isoforms encoded by different genes (MAP7, MAP7D1, MAP7D2, and MAP7D3)[438]–[440]. All members of the MAP7 family have a similar organisation, with two retained domains expected to be helical, connected by an unstructured linker. Study in flies, however, have shown that ensconsin's C-terminal fragment lacks the MT-binding domain [423], [436], [437]. Kinesin-1 is well known to be auto-inhibited by its C-terminal cargo-binding domains[441], and it has been proposed that ensconsin may play a role in alleviating kinesin auto-inhibition [423]. Hooikaas and his colleagues tried to produce a functional triple knockout of MAP7, MAP7D1, and MAP7D3, but these cells were not viable [442]. Unlikely, the lack of kinesin-1-mediated transport, as KIF5B knockout cells display no clear growth or proliferation deficiencies, and the other two kinesin-1 isoforms, KIF5A and KIF5C, do not appear to be expressed in HeLa cells [443], while they found that the protein of MAP7 has been shown to be phosphorylated and therefore inactivated during mitosis as a result

of its downstream phosphorylation of Cdk1/cycline B. This contributes to a decrease in interphase stability of the microtubules, which can contribute to the growth of centrosomally nucleated microtubules [444]. MAP7 proteins may contribute to cell division, as ensconsin is known to be involved in spindle formation in flies [445]. At the meantime, eliminating three MAP7 counterparts using MAP7D1 and MAP7D3 siRNA-mediated knockdowns in a stable MAP7 knockout line, resulting in an effective loss of all three MAP7 family members, phenocopiedthe impact of KIF5B knockout, leading to a tight perinuclear clustering of mitochondria. They concluded that members of the MAP7 family function redundantly in the localization of mitochondria and that this effect is unlikely to be due to changes in the structure of the MT network [442]. Additionally, the MT-affinity regions are located in the MAP7 and MAP7D3 C-terminal connector [439], [446]. MAP7 is necessary for proper kinesin1-dependent nuclear distribution in mammalian myotubes [438]. Conversely, a protective mechanism to inhibit runway proliferation of oncogene-transformed cells similar to senescence and apoptosis could be an improper expression of MAPs and disruption of microtubule dynamics, as in early cutaneous melanoma [447].

MAP7 also allows the motor protein kinesin-1 (KIF5) to interact with microtubules. Both MAP7 and kinesin-1 are needed in to support nucleus placement and spacing in multinuclear muscles cells, processes necessary for muscle function [448]. There is a direct interaction between Kinesin-1 and the Ensconsin C-terminal domain (the kinesin-binding domain, or KBD) [449]. The binding of the ensconsin N-terminal MT-binding domain (MBD) and the kinesin-1 engine domain is therefore necessary to rescue nucleus-positioning flaws after kinesin-1 or ensconsin is knocked down [438].

The majority of cellular tubulin is cytoplasmic but a significant fraction is associated with the plasma membrane and other membranes This association is thought not to be an artefact of

membrane isolation procedures, because association with plasma membrane changes some of the properties of tubulin. The association of tubulin with membranes is relatively strong because neutral detergent is required to release the dimer from the membranes[450]. It was a mystery how tubulin, which is a very polar protein, can become so strongly associated with the membrane considering hydrophobic interactions are more likely to be required[450]. Morphological cells structures suggest that microtubules can be bound to plasma or organelle membranes by connectors proteins that have not yet been identified. A bifunctional membrane anchor may result in a hydrophobic interaction with the membrane on the one side and a second, potentially but not necessarily more polar interaction with tubulin on the other[451]. A possible protein connecting microtubules to the plasma membrane is 2,3 -cyclic nucleotide-3 -phosphodiesterase (CNP)[452]. CNP is both prenylated and palmitoylated[453], which provides a hydrophobic moeity for membrane intercalation. CNP protein binds to microtubules and this affect their in vitro polymerization activity [453][451]. CNP can also directly associate with F-actin [454].

Table (5-1): The function of highly expression genes which used in these experiments

Genes	Mechanism of gene action
Rab11-FIP4	Rab11-FIP4's over-expression considerably increased the mobility and invasiveness of in vitro HCC cells[455], A significantly favorable correlation between the expression of Rab11-FIP4 and HIF-1α was noted in HCC tissues and a more precious predictor of bad prognosis for HCC patients were shown the combination of Rab11-FIP4 and HIF-1α. To conclude, Rab11-FIP4 is a HIF-1α target gene and has a pro-metastatic function in HCC, indicating that Rab11-FIP4 may be a promising candidate for HCC therapy [455]. Some studies have shown that Rab11-family interacting proteins (Rab11-FIPs) are tumour-related and can act as tumour promoters in certain cancers[456].

GRhL-2	Grainyhead-like 2 (Grhl2), a transcription factor belonging to the grainyhead-like(Grhl) family, plays a major role in establishing epithelial polarity and in acquiring and maintaining epithelial-specific functions[457]. Grainyhead-like 2 (GRHL2) knockout eliminates the development of oral cancer by reciprocal control of the signalling pathways for MAP kinase and TGF-β[458]. In addition, Grainyhead transcription factor 2 over-expression is associated with poor prognosis in human pancreatic carcinoma[458].
BSPRY	BSPRY (B-box and SPRY-domain containing protein) Originally recognized as a binding partner in the epithelial Ca^{2+} channel family of transient receptor potential vanilloid 5 (TRPV5). BSPRY's overexpression in kidney epithelial cells is an adverse regulator of Ca+ transport [459].
PRR15	This is a family of proteins found in eukaryotes that consists of proline-rich 15 (PRR15) and proline-rich 15-like proteins. PRR15 is expressed almost exclusively in post-mitotic cells both during foetal development and in adult tissues, such as the intestinal epithelium and the testis. Its expression in mouse and human gastrointestinal tumours is linked, directly or indirectly, to the disruption of the Wnt signalling pathway[460]. The patient developed eventually peritoneal metastases and died following surgery for 15 months. Molecular analyses have identified a KRAS mutation and a novel PRR15L-RSPO2 fusion in both SuSA and adenocarcinoma components, which preserves the entire coding area of RSPO2[461].
HMGCS1	This enzyme condenses acetyl-CoA with acetoacetyl-CoA to create HMG-CoA, the substratum for HMG-CoA reductase (HMGCS1), catalyzes the

	same response in the biosynthetic sterol pathway, and HMGCS2 diverges from HMGCS1 [462].
RBM35A	ESRP1(epithelial splicing regulatory protein 1) and ESRP2 manage alternative splicing occurrences linked to cell epithelial phenotypes[463], and ESRP1 is over-expressed in ovarian cancer and promotes the flipping of ovarian cancer cells from mesenchymal to epithelial[464].
CCDC64B	BICD2 is a multifunctional adapter for motor proteins with minus end-directed dynein-dynactin and plus end-directed kinesin-1 kinesin family 5A for microtubules. Adapter proteins mediate the particular binding between the motor protein and its cargo. In this way, adapters control the motor protein's function and location. Motor proteins have significant tasks not only in microtubular transport, but also in controlling the dynamics of microtubular polymerization and microtubular length [465].
FXYD3	FXYD domain ion transport regulator 3 (FXYD3), FXYD3 is a Na / K-ATPase regulator recently associated with the development and progression of different cancers; thus, FXYD3 has been proposed as a possible therapeutic target for these cancers[466].
MAP7	MAPs are a family of proteins that bind microtubules and stabilize them [467]. While MAP4 is omnipresent, MAP1 and MAP2 isoforms are mainly expressed in neurons and MAP7 is limited to epithelial cells[422], [423]. Aberrant expression and significance of MAPs to the resistant phenotype of a broad spectrum of microtubular targeting agents. MAP7 connects branch sites to microtubules to support branch maturation in neuronal cells [446].

Table (5-2): The function of poorly expressedgenes used in these experiments

Genes	Mechanism of gene action
Frizzled-2(FZD-2)	FZDs play key roles in cell polarity regulation, embryonic development, cell proliferation, neural synapse formation, and many other developmental and adult organism growth procedures. The proteins of the WNT secreted glycoproteins and FZD ligands consist of nineteen members that can attach to the cell surface complex, a FZD family member and a co-receptor Low density lipoprotein-related protein 5/6 (LRP5/6), each FZD member can combine individually with the several distinct WNT proteins to trigger either canonical WNT/β-catenin or non-canonical WNT / PCP signalling pathways (planar cell polarity) and WNT / Ca^{2+} [468], [469]
FGF	Growth factors for fibroblasts (FGFs) are a large family of growth and differentiation variables (24 members). FGFs mediate their impacts by life on the cell surface with FGF receptors (FGFRs). FGFs and the receptors ' signalling axis play significant roles in controlling cell proliferation, migration, angiogenesis, wound healing, and differentiation. It has been shown that the FGF – FGFR axis modulates tumour stroma and cancer development. On the other side, in certain situations, FGF signals may have tumour-suppressive features. FGF signalling mechanisms have been shown to be involved in cancer development by causing mitogenesis, cell migration and tumour angiogenesis. Consequently, aberrant signals of FGF can encourage the growth of cancer[470], [471]
AXL	AXL plays a major role in controlling cell invasion and migration in tumour cells. In tumour cells with low metastatic potential, over-expression of AXL encourages migration and invasion. Furthermore, blocking AXL in metastatic tumour cells is adequate to suppress in vitro migration of

	tumour cells. In vivo, breast and glioma tumour models have lately defined a function for AXL in invasion and metastasis[472]–[474].
VIM	This gene encodes an intermediate protein of the type III filament. The cytoskeleton consists of intermediate filaments, microtubules and actin microfilaments. The encoded protein is accountable for keeping the cytoplasm's cell form and integrity and for stabilizing cytoskeletal interactions. This protein is engaged in the transportation of neuritogenesis and cholesterol and acts as an organizer of several other critical proteins engaged in cell attachment, migration and signalling. [475]–[477]
CYR61	CYR61 involves various biological processes, including cell proliferation, inflammation, cell adherence, migration, embryogenesis, and wound repair[478]. Intriguingly, CYR61-Mediated Multidrug Resistance in Gastric Adenocarcinoma AGS Cells[479]. Furthermore, Cyr61/CCN1 regulates the dCK and CTGF of pancreatic ductal adenocarcinoma and induces gemcitabine resistant phenotype[480].
PTRF	PTRF protein translocates from caveolae to the cytosol after stimulation of insulin[43]. Caveolae comprise truncated forms of PTRP protein and may be the site of proteolysis dependent on phosphorylation. Caveolin is an integral membrane protein, both exposed to the cytosolic surface of the plasma membrane with N-and C-termini[481]. Caveolin's N-terminal domain can interact with and concentrate a variety of signal transduction proteins on the surface of the caveolae, such as G-proteins, Src-like kinases, C and H-Ras proteins[482].
MAP7D1	MAP7D1 (MAP7 Domain Containing 1) is a Protein Coding gene, The microtubules connected with the Map7 and Map7D1 (Map7/7D1) proteins form an interdependent regulatory loop with the critical Wnt signalling

	signal transducer Disheveled, which connects directly to the cortical location of Disheveled and facilitates the cortical targeting of MT plus-ends in reaction to Wnt5a in Hela cells [483].
MARVELD3	MarvelD3 is a tight junction transmembrane element. Tight junctions also control processes of signalling that guide the proliferation of cells[484]. Occludin, tricellulin and MarvelD3 share a common MARVEL among the transmembrane proteins (MAL and associated vesicle trafc and membrane connection proteins) Domain that differentiates them from the other proteins of the claudin family junctional tetraspan transmembrane [484]. The junctional MARVEL domain proteins can modulate junctional permeability characteristics, they are believed to work mostly as regulators of tight junctions or elements of signalling processes that signal tight junctions to the cell interior[485].

5.2. Results: -

5.2.1. Identification of genes potentially associated with cellular sensitivity to statins.

The Broad Institute's cancer therapeutics response portal of online data on the website: - (http://portals.broadinstitute.org/ctrp/?compoundId=27894&compoundName=simvastatin) was interrogated to identify the high or low p-value gene expression in ovarian cancer cell lines was associated with sensitivity to simvastatin, fluvastatin and lovastatin in 500 cell lines from a range of cancer types. Genes were selected for further investigation that were associated to more than one statin. The genes selected are described in tables (5-1, 5-2). No specific level of significance (P value) was used to select the genes for investigation; rather the 15 genes which showed the most significant association with statin sensitivity were selected. Further studies were then performed to evaluate whether these expressions of these genes predicted sensitivity to pitavastatin in ovarian cancer cell lines.

5.2.2. Pitavastatin growth inhibition activity in six cells line of ovarian cancer

To compare gene expression profiles of ovarian cancer cells to their sensitivity to pitavastatin, the potency of pitavastatin was first evaluated in cell growth assays (figure (5-2)). The results showed that four of the six cell lines had broadly similar sensitivities to pitavastatin, but that Ovcar-8 and Ovsaho were notably more sensitive (table 5-3).

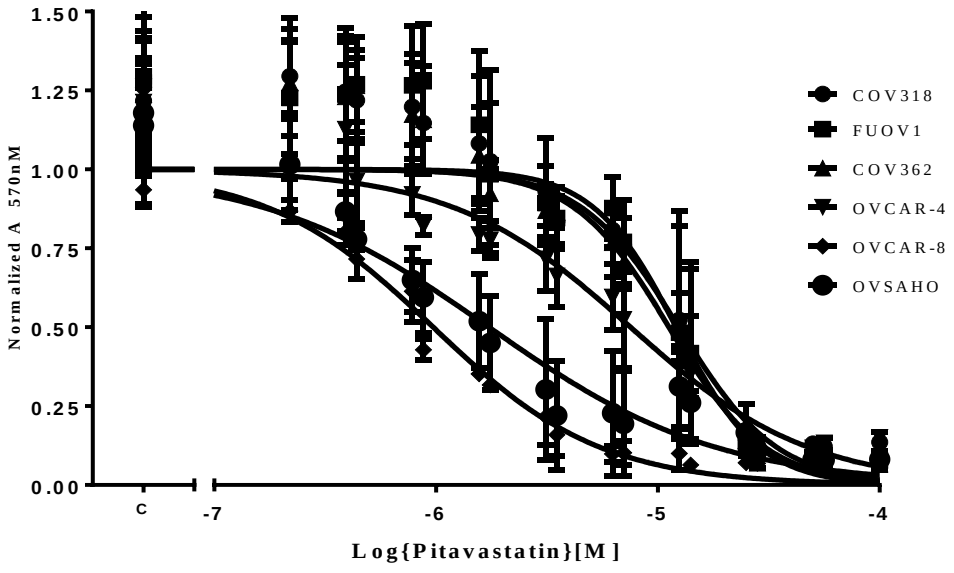


Figure (5-2): Dose response curve of cell proliferation assays. The cells were exposed to the indicated concentration of pitavastatin (starting with 100μM) for 72 hours and the relative number of surviving cells determined by staining with SRB. The results are expressed as a fraction of the relative cell number measured in samples treated with solvent (mean ± SD; n = 3). "C" on the x-axis indicates a samples treated with drug solvent alone.

Table (5-3): IC_{50}s of pitavastatin growth inhibition activity in six cells line of ovarian cancer (mean ± S.D, n= 3)

No	Cell line	growth inhibition activity (IC50, μM)
1	**Cov318**	7.7 ± 1.4
2	**Cov362**	8.6 ± 0.7
3	**Fuov-1**	6.2 ± 0.9
4	**Ovcar-4**	5.6 ± 1.2
5	**Ovcar-8**	0.82 ± 2
6	**Ovsaho**	0.78± 1.9

5.2.3. Results of gene expression

The expression of the genes identified as correlating with sensitivity to statins was then measured in the panel of 6 ovarian cancer cell lines by both RT-qPCR and western blotting. Notably, some genes were highly expressed in single cell line when measured by western blotting, for example Rab-11fip4, BSPRY and FXYD3 in OVCAR-4 while some genes show highly expression in all cell lines used in this experiment, e.g. FGF5, AXL, VIM, GRHL-2 and PPR15Lin both western blotting and qPCR experiments. In addition, the results showed high expression of CDCC64B gene in COV362 cell lines in both experiments (western blotting and qPCR). In general, the result from the qPCR analysis were consistent with the results from western blotting. There were a few exceptions to this however. Notably, AXL showed high levels of protein in blotting experiments using OVSAHO while very lows of expression when measured by qPCR in the same cells. In contrast, the same gene showed low protein levels in blotting experiments using FUOV-1 cells but relatively high expression when measured by qPCR.

Most of these observations did not reveal a correlation between expression of the protein and the cells' sensitivity to pitavastatin. However, Map7 was highly expressed in the two cell lines more sensitive to pitavastatin in both western blotting (Fig 5-3) and RT-qPCR analysis suggesting it may be a candidate marker for cellular sensitivity to pitavastatin or may be a key target in the cytotoxic activity of pitavastatin.

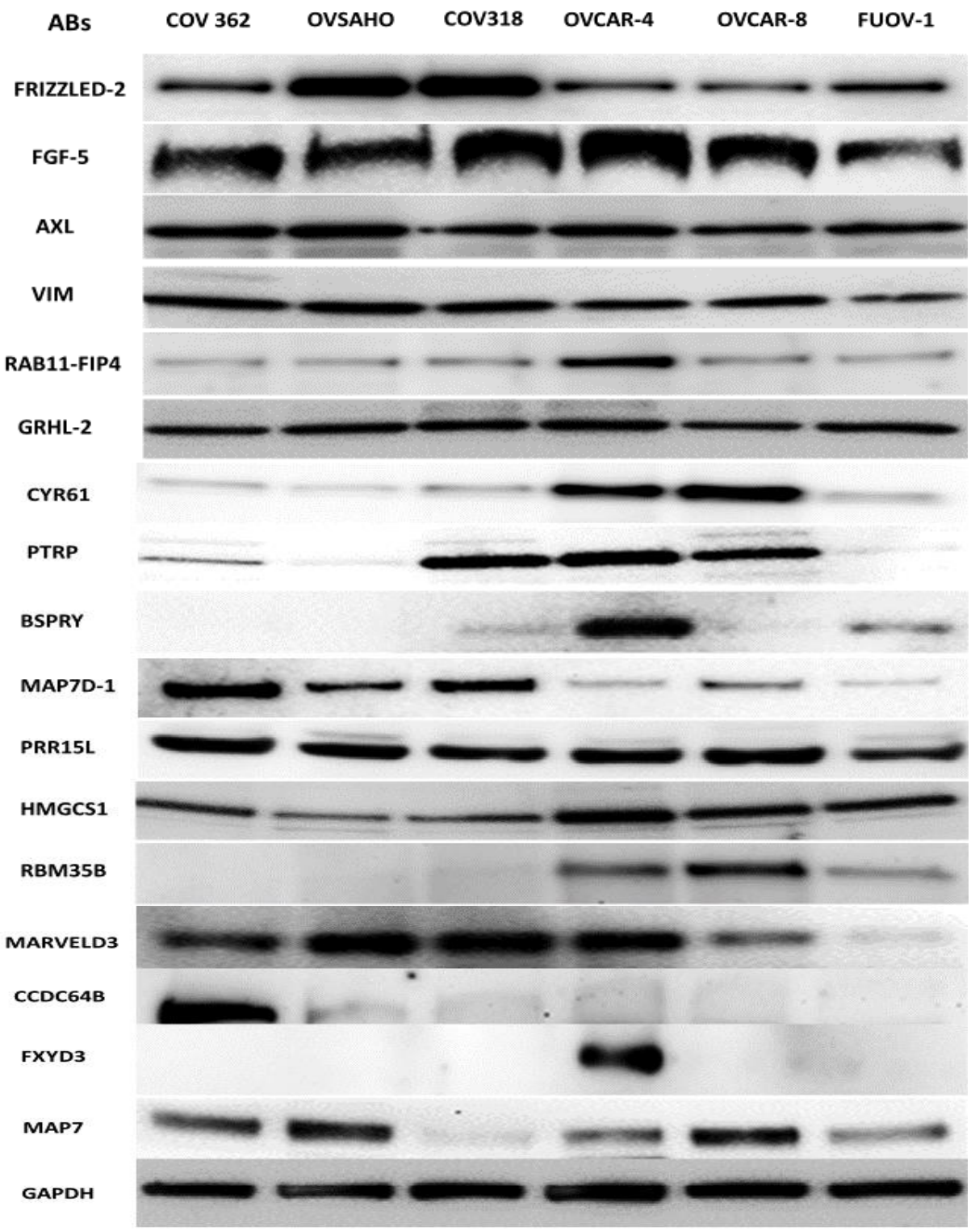


Figure (5-3): Evaluation of the expression of 18 genes in ovarian cancer cells by western blotting**.** Protein lysates were obtained from six cell lines of ovarian cancer (COV362, Ovsaho, COV318, Ovcar-4, Ovcar-8 and Fuov-1) without any drugs treatment. The results repeated three times.

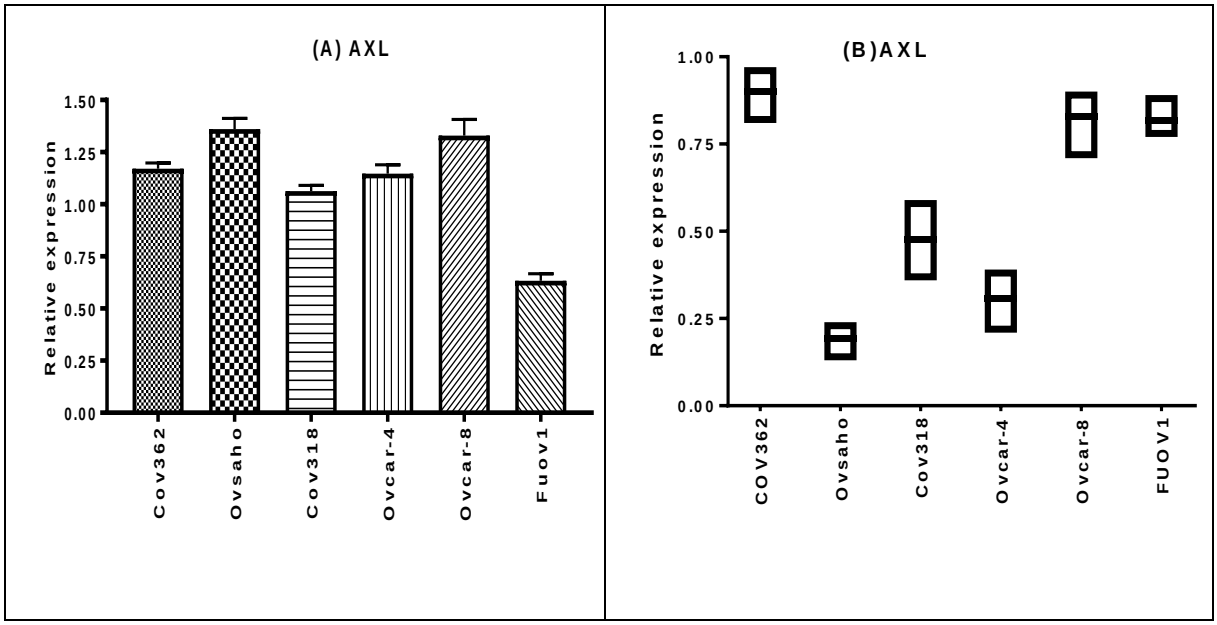


Figure (5-4): A. Quantification of the level of AXL protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of AXL in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

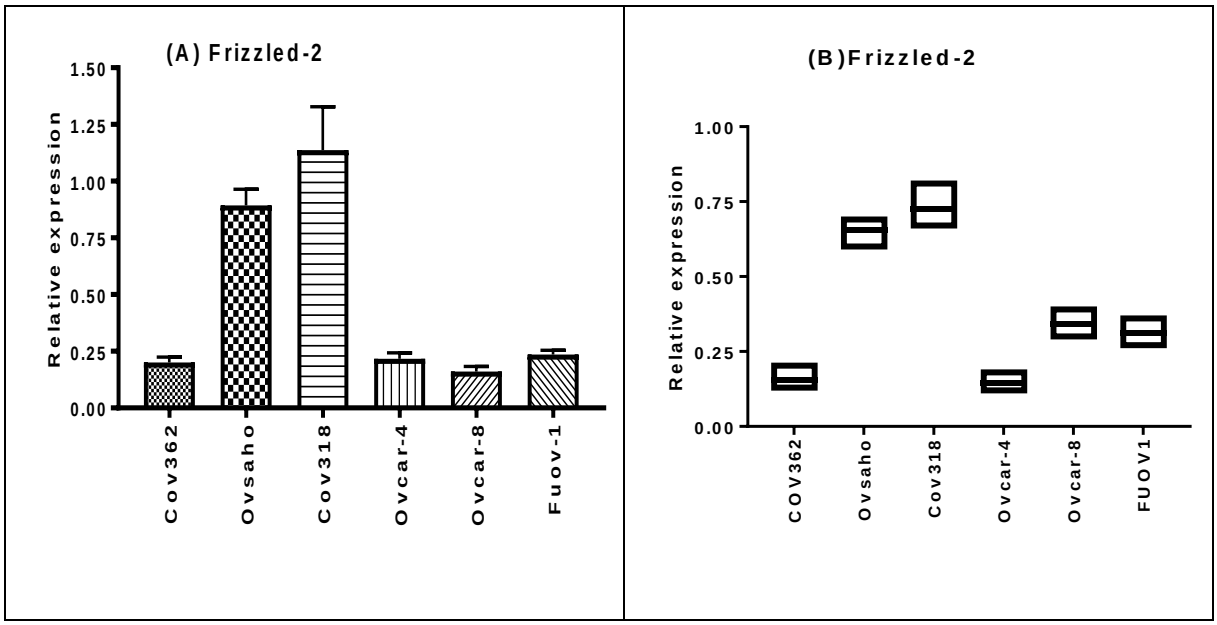


Figure (5-5): A. Quantification of the level of Frizzled-2 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of Frizzled-2 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

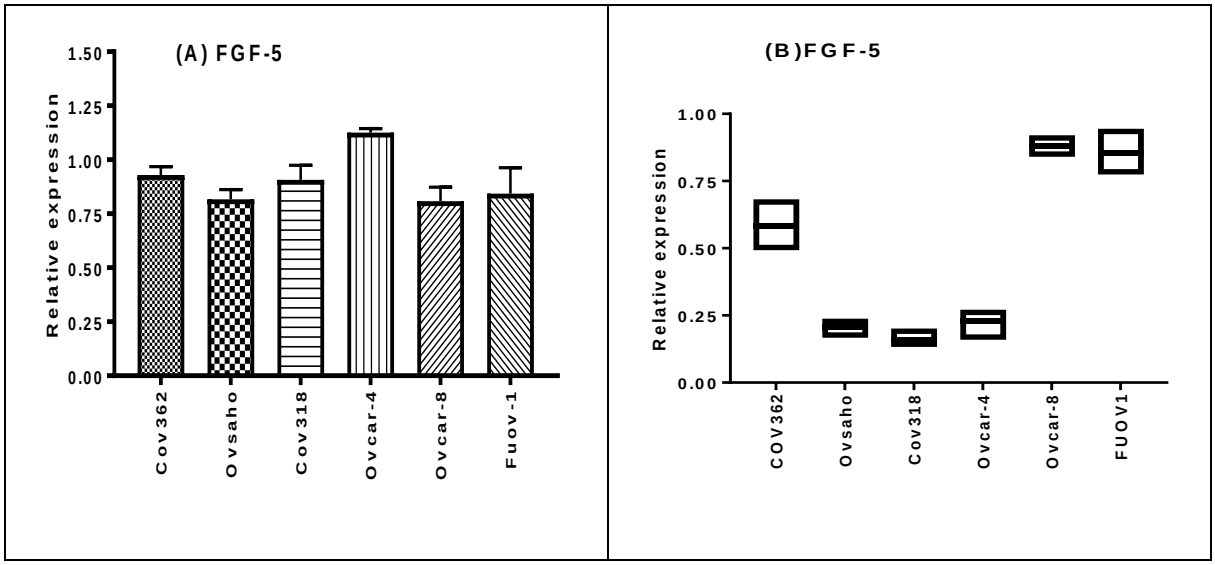


Figure (5-6): A. Quantification of the level of FGF-5 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of FGF-5 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

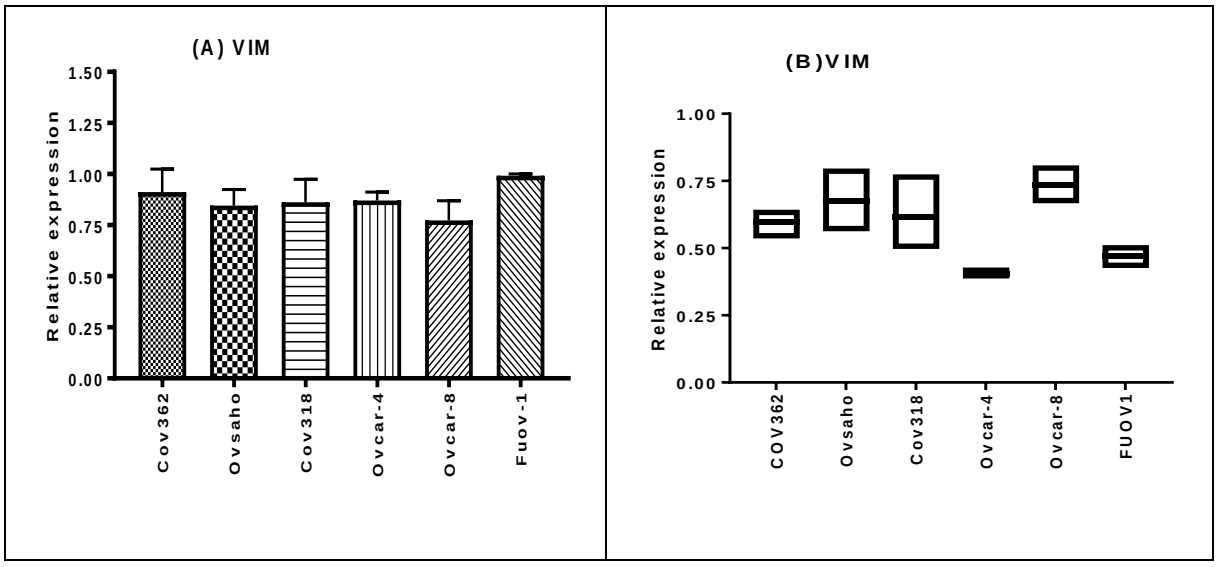


Figure (5-7): A. Quantification of the level of VIM protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of VIM in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

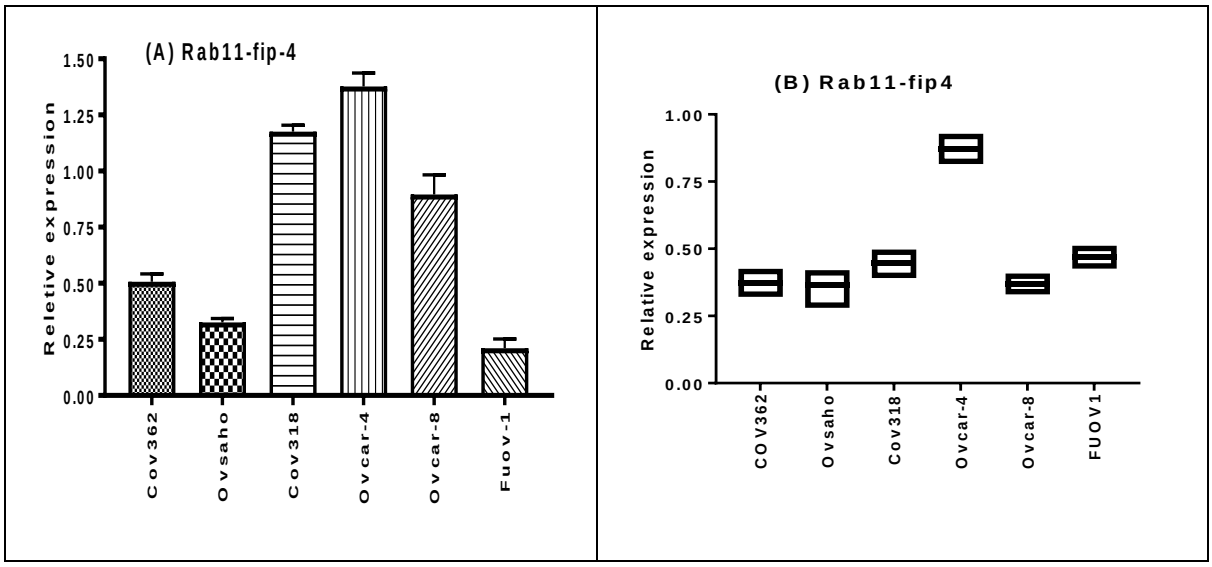


Figure (5-8): A. Quantification of the level of Rab11-fip-4 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of Rab11-fip-4 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

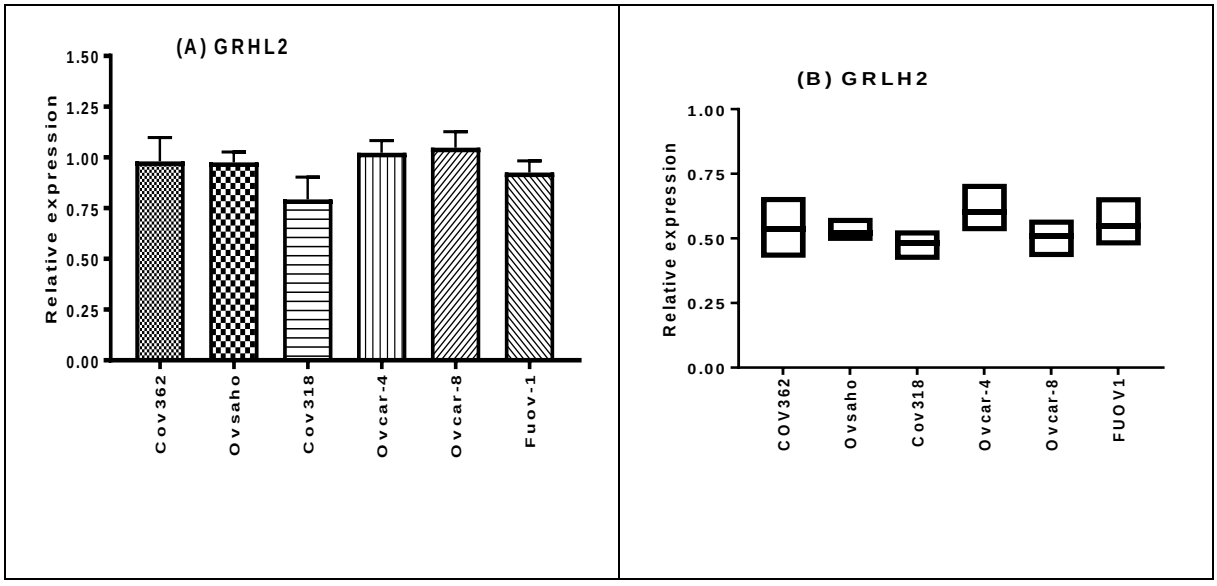


Figure (5-9): A. Quantification of the level of GRHL2 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of GRHL2 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

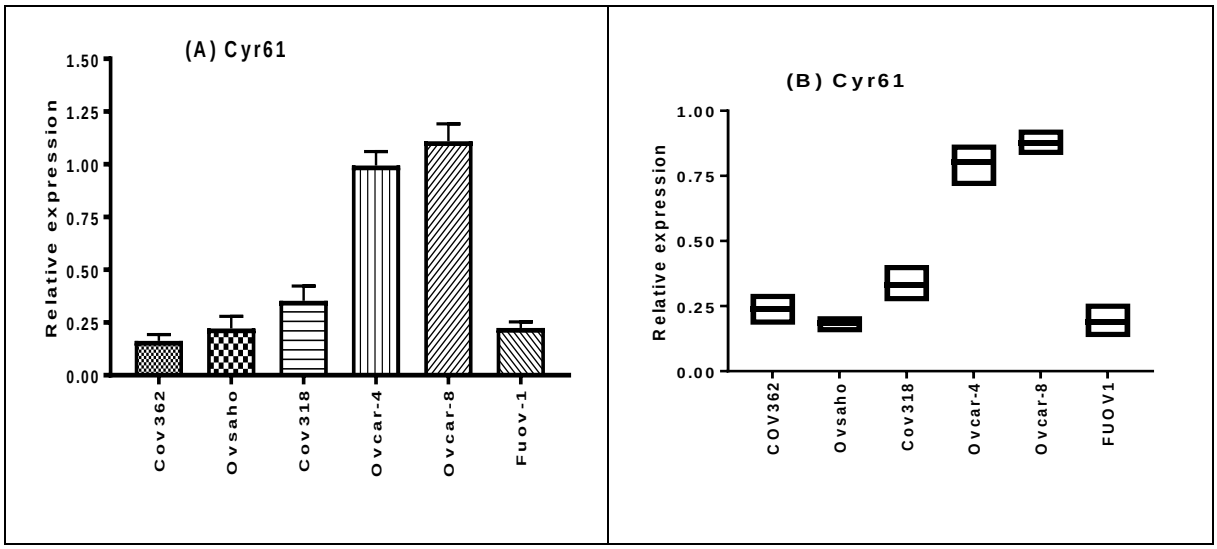


Figure (5-10): A. Quantification of the level of Cyr61 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of Cyr61 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

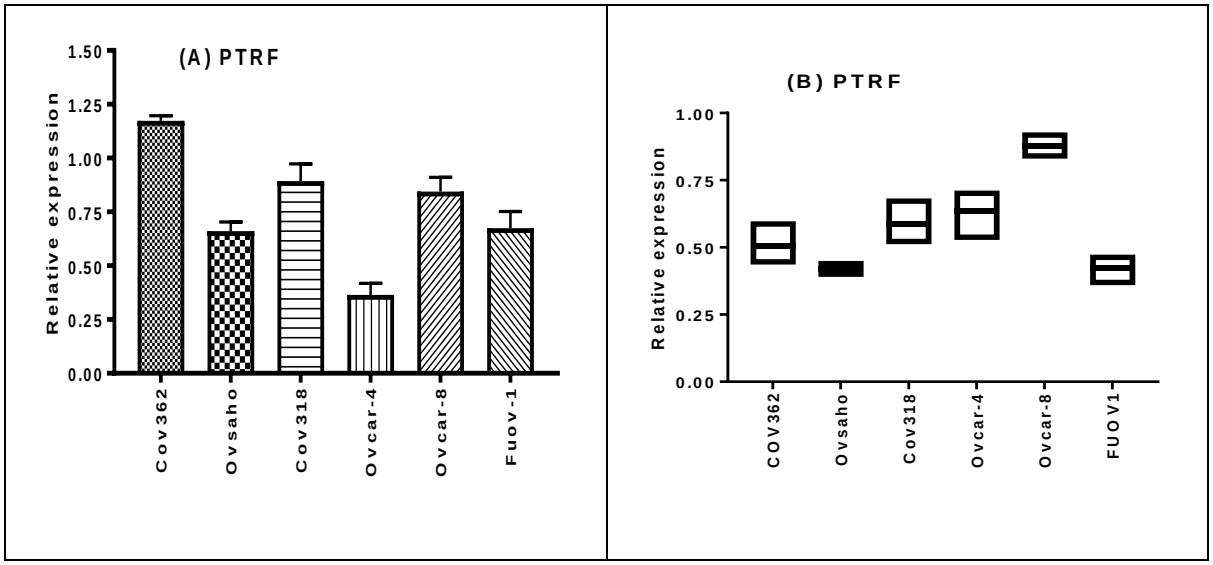


Figure (5-11): A. Quantification of the level of PTRF protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of PTRF in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

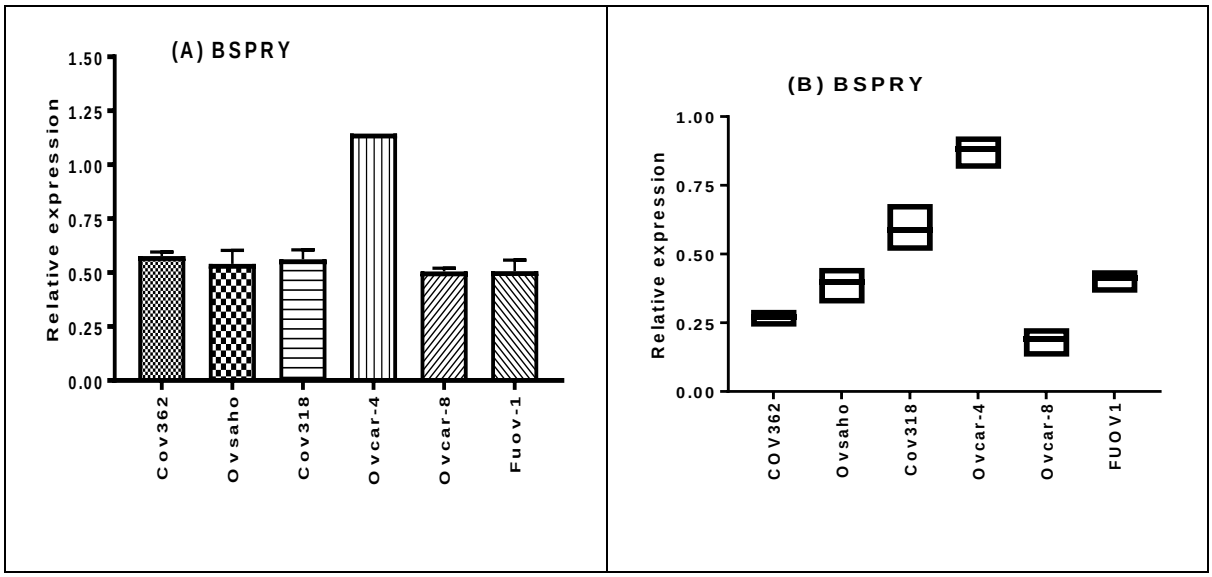


Figure (5-12): A. Quantification of the level of BSPRY protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of BSPRY in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

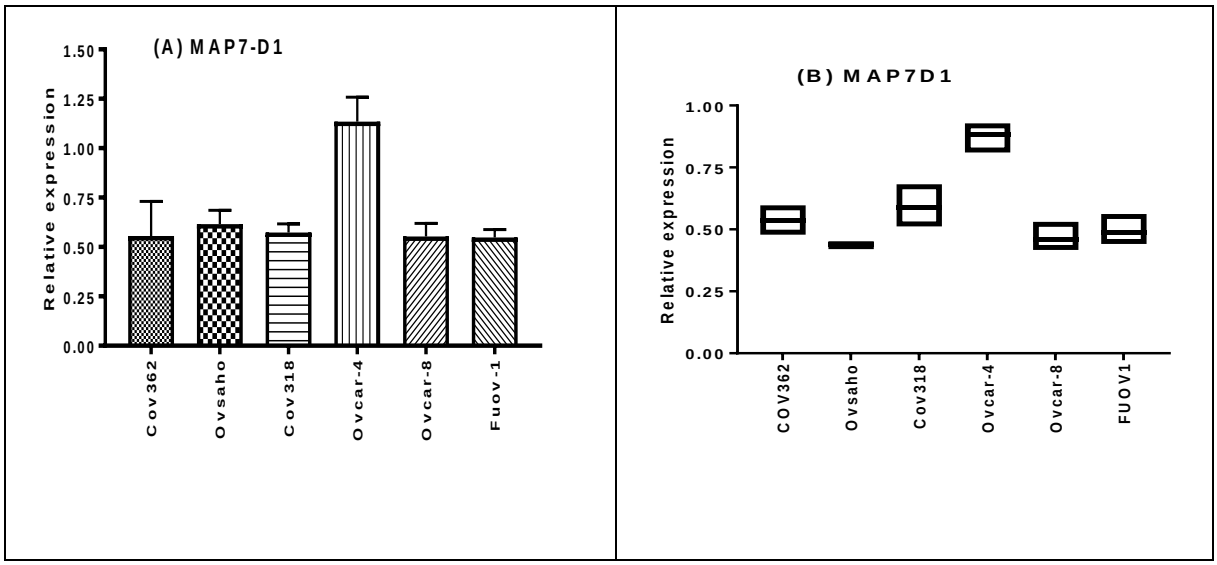


Figure (5-13): A. Quantification of the level of MAP7-D1 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of MAP7-D1 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

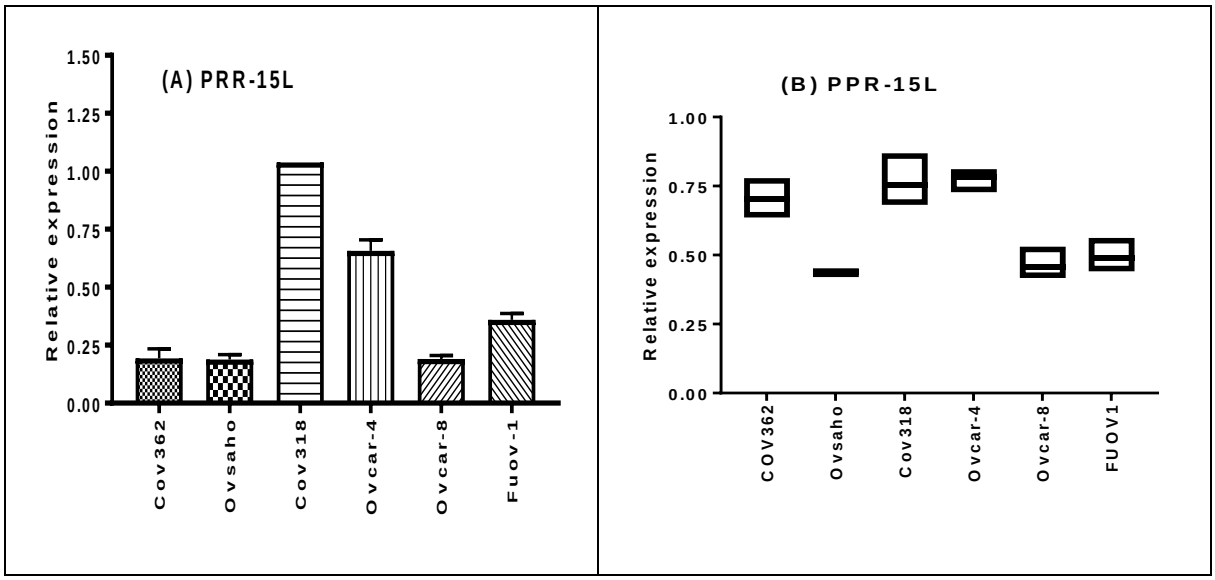


Figure (5-14): A. Quantification of the level of PRR-15L protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of PRR-15L in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

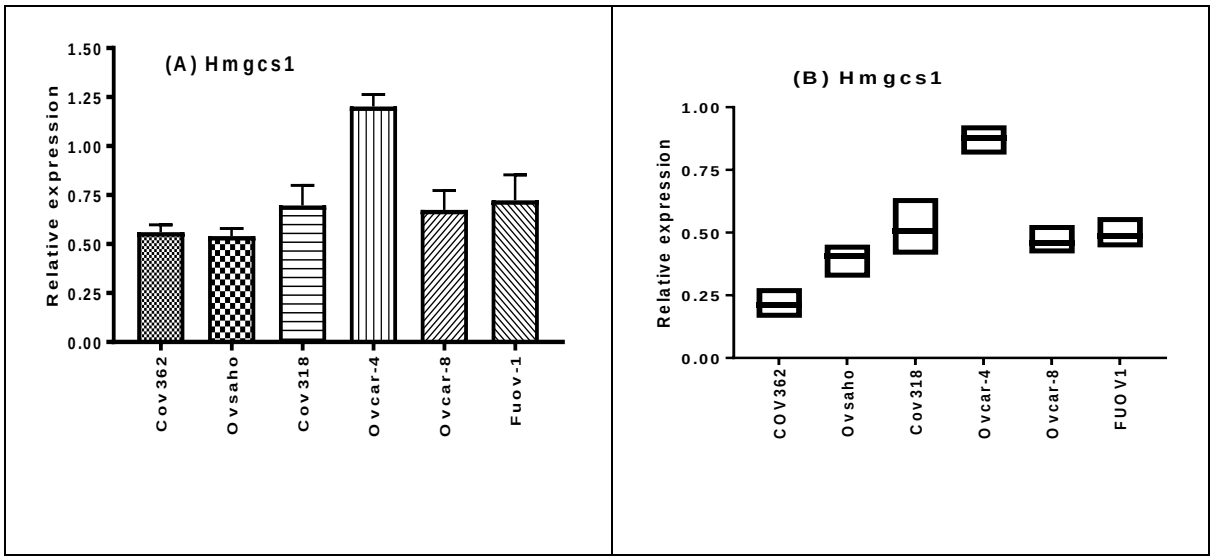


Figure (5-15): A. Quantification of the level of Hmgcs1 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of Hmgcs1 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

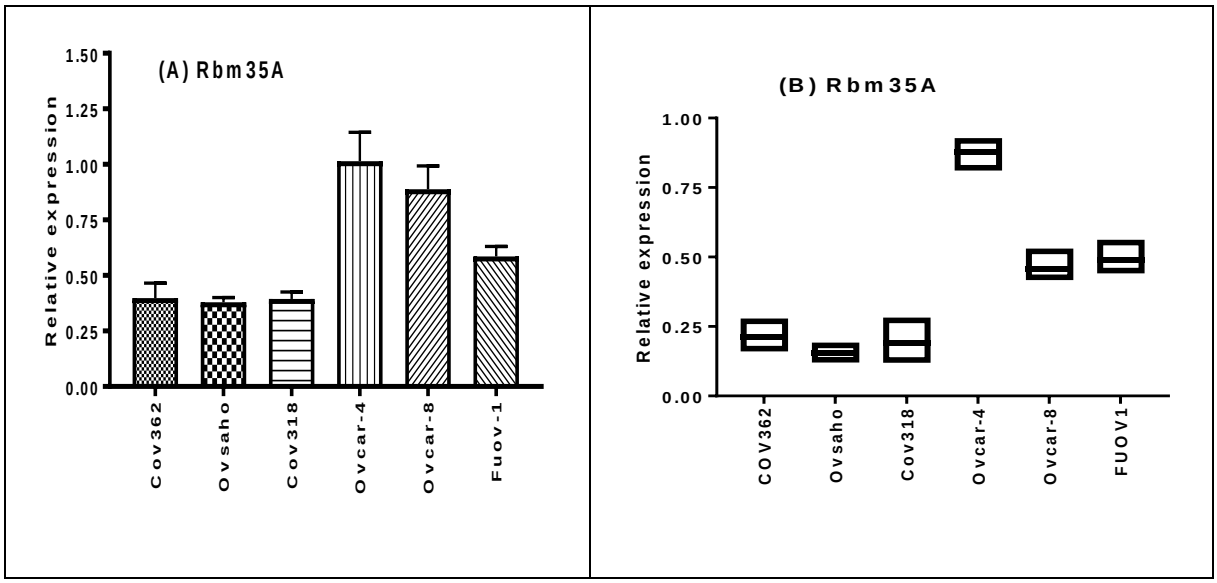


Figure (5-16): A. Quantification of the level of Rbm35A protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of Rbm35A in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

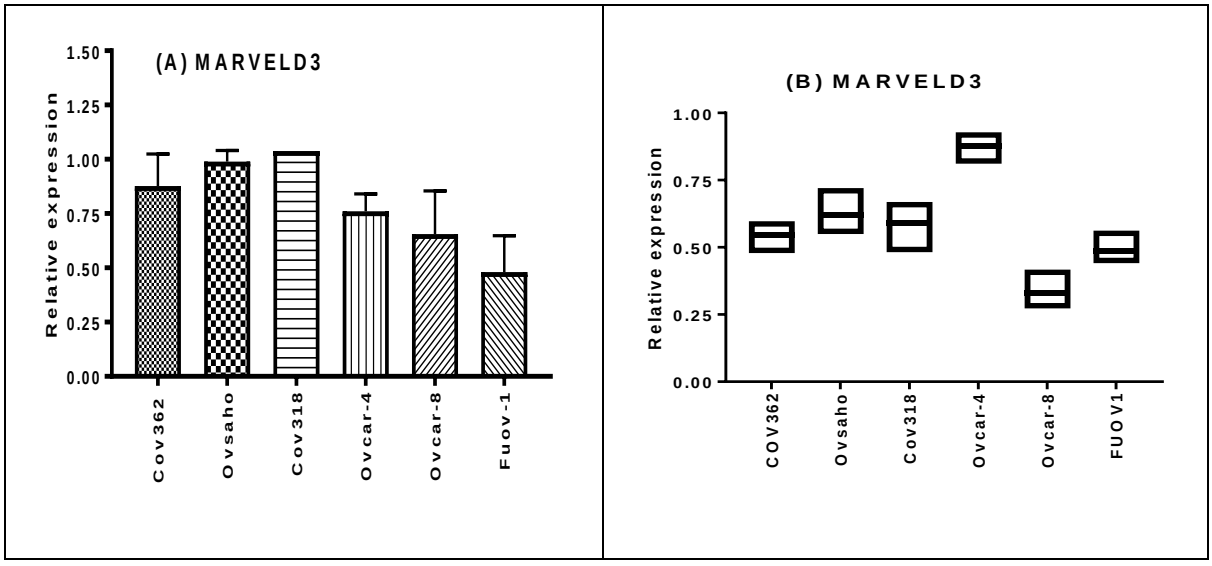


Figure (5-17): A. Quantification of the level of MARVELD3 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of MARVELD3 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

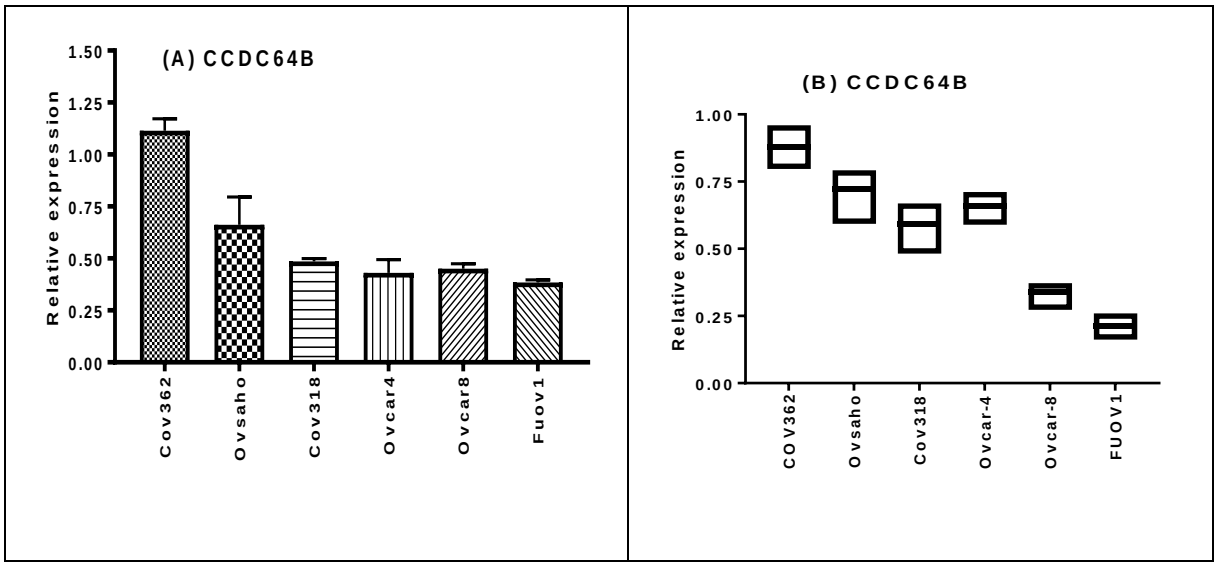


Figure (5-18): A. Quantification of the level of CCDC64B protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of CCDC64B in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

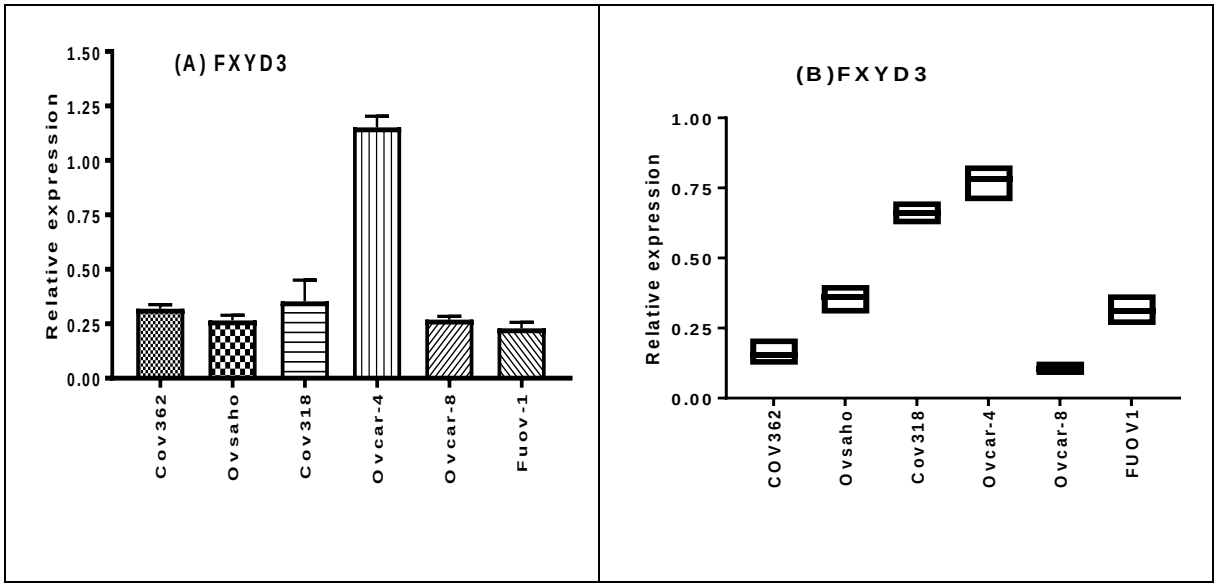


Figure (5-19): A. Quantification of the level of FXYD3 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of FXYD3 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

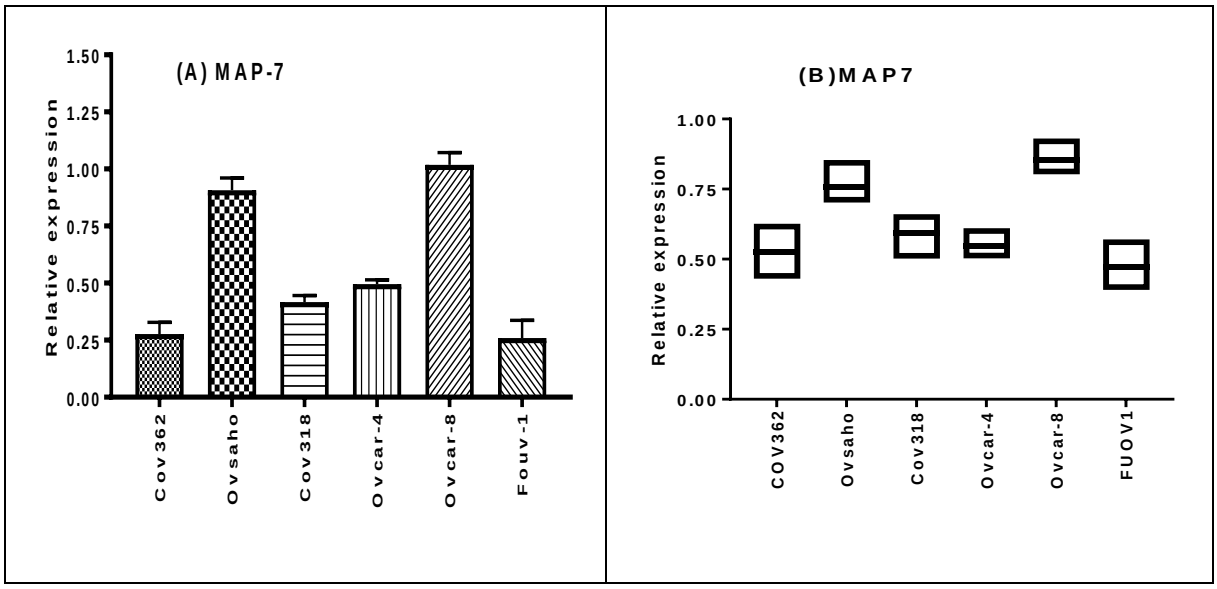


Figure (5-20): A. Quantification of the level of MAP7 protein measured in 6 ovarian cancer cell line measured by western blotting. The results (mean ± S.D., n=3) which normalized to GAPDH as a control. (B) Relative of mRNA expression of MAP7 in 6 ovarian cancer cell line was measured by using qPCR. The results (mean ± S.D., n =3) were normalized to actin.

5.2.3. Knock-downs of the pitavastatin-sensitive genes in Ovcar-8 and Ovsaho cell line

The previous data suggested that the expression of MAP7 correlated with the sensitivity of ovarian cancer cells to pitavastatin. To explore this further, the expression of MAP7 was repressed by RNAi and the effect on pitavastatin sensitivity determined. Considering that one of the major binding partners of MAP7 is tumour Kinesin-1 (KIF5B), its expression was also repressed by RNAi.

In order to ensure efficient transfection without noticeable toxicity, the expression of the candidate genes after RNAi and the transfection conditions for each siRNA were optimized before evaluating the effect of knock-down. OVCAR-8 and OVSAHO cells were transfected with 4 separate siRNA corresponding to each gene and the number of surviving cells compared to the number of cells surviving after transfection a non-targeting siRNA.

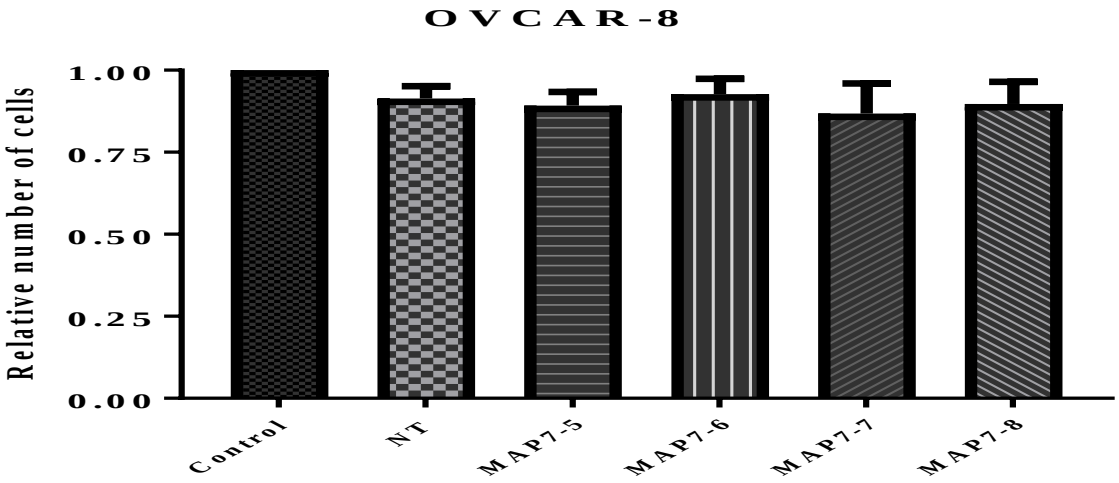


Figure (5-21): OVCAR-8 cells were transfected with each of the indicated siRNA to MAP7 (100nM) and after a further 72 hours the cells stained with SRB. The results (n=3, mean ± S.D.) the expressed measured in cells transfected with a non-targeting siRNA (NT-1). The results were not significantly different from the cell growth measured in cells transfected with a NT-1 where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

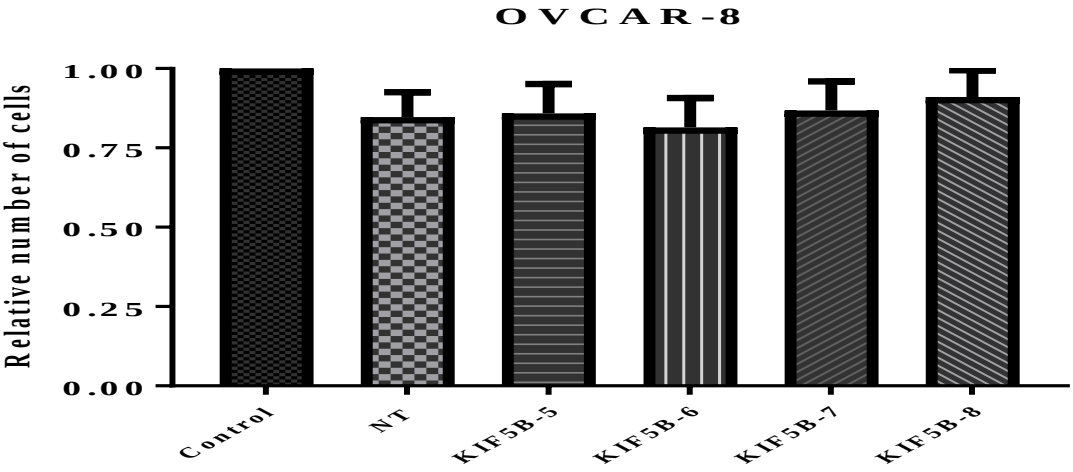


Figure (5-22): OVCAR-8 cells were transfected with each of the indicated siRNA to KIF5B (100nM) and after a further 72 hours the cells stained with SRB. The results (n=3, mean±S.D.) the expressed measured in cells transfected with a non-targeting siRNA (NT-1). The results were not significantly different from the cell growth measured in cells transfected with a NT-1 where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

Having established non-toxic transfection conditions, the impact of knockdown on protein levels was determined using 3 separate siRNAs. The results revealed a variable extent of knock-down of the various targeted genes, but at least 2 siRNAs were found for each gene that caused significant knock-downs (defined as greater than 40% reduction in the protein).

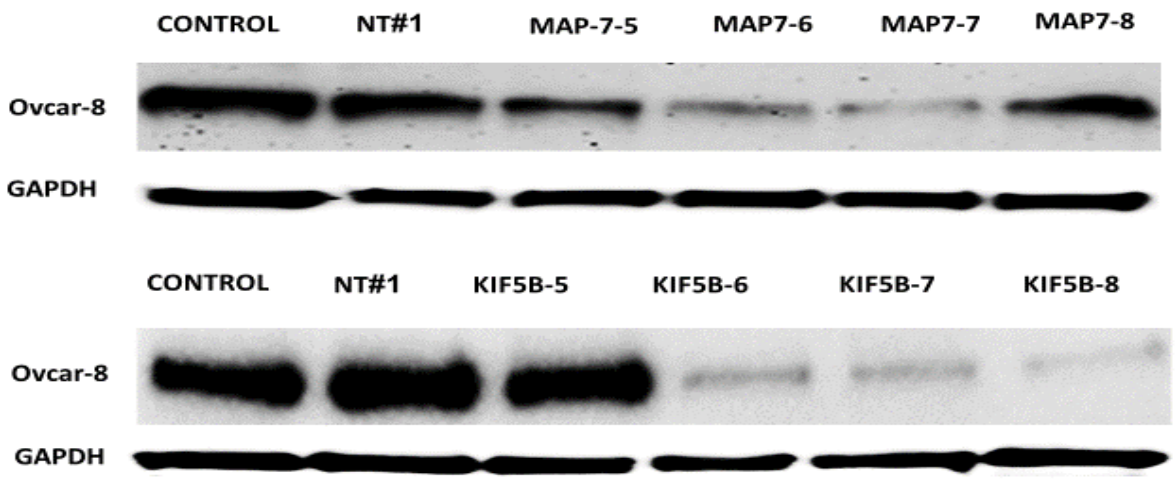


Figure (5-23): Knock-down of OVCAR-8 with MAP-7 and KIF5B was measured by western blotting. The expression of MAP7 and KIF5B protein was determined by western blotting of protein lysates obtained from OVCAR-8 cell lines after 48 hours of transfection with the indicated MAP7 and KIF5B siRNAs.

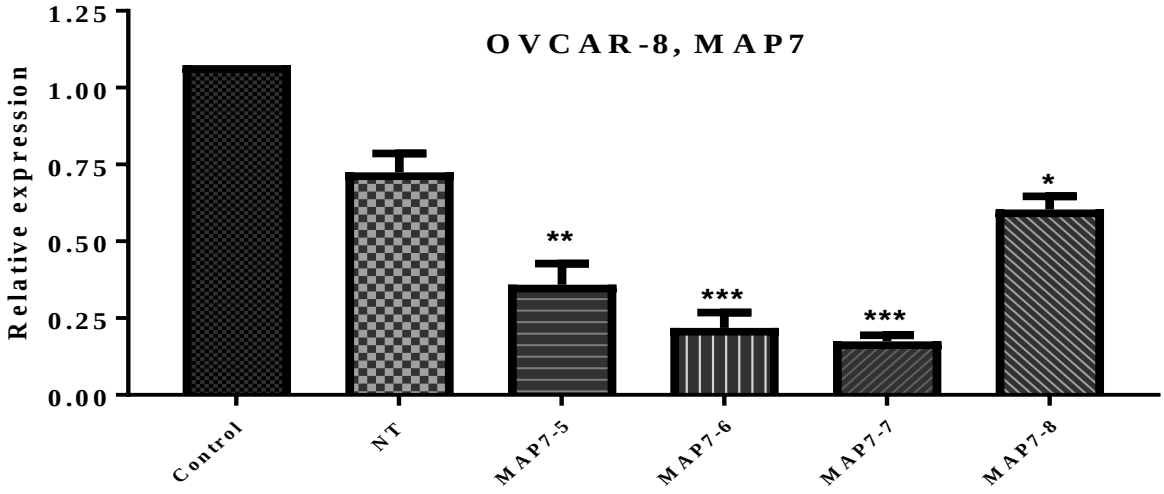


Figure (5-24): Quantification of knock-down of MAP-7 in OVCAR-8 cells was measured by western blotting. The results of the knockdown of MAP-7 were quantified by image analysis and are expressed (n=3, mean±S.D.) as a fraction of that measured in cells transfected with a non-targeting siRNA (NT-1) then normalized with GAPDH. The results were significantly different from the expression measured in cells transfected with a NT-1 where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

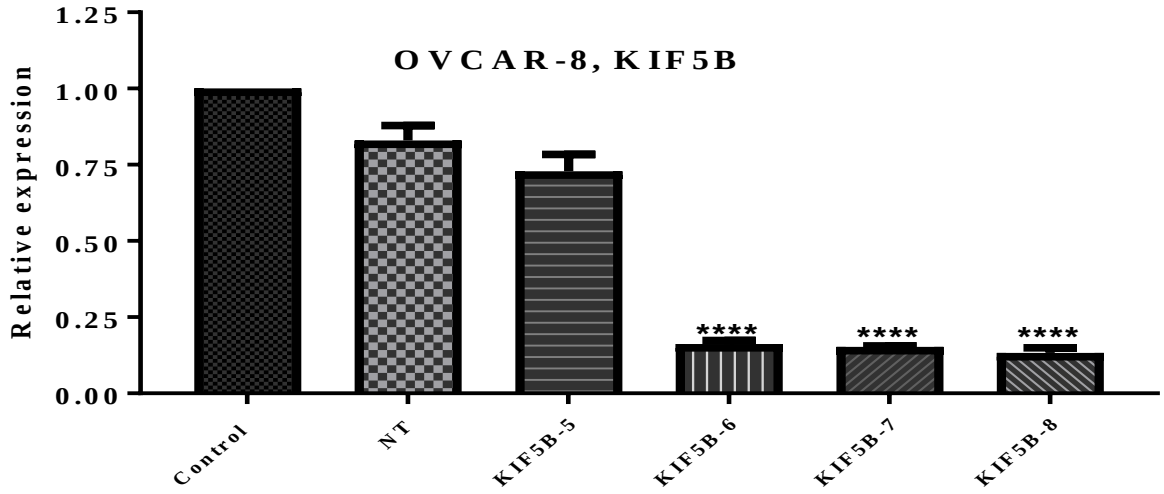


Figure (5-25): Quantification of knock-down of KIF5-B in OVCAR-8 cells was measured by western blotting. The results of the knockdown of KIF5B were quantified by image analysis and are expressed (n=3, mean±S.D.) as a fraction of that measured in cells transfected with a non-targeting siRNA (NT-1) then normalized with GAPDH. The results were significantly different from the expression measured in cells transfected with a NT-1 where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

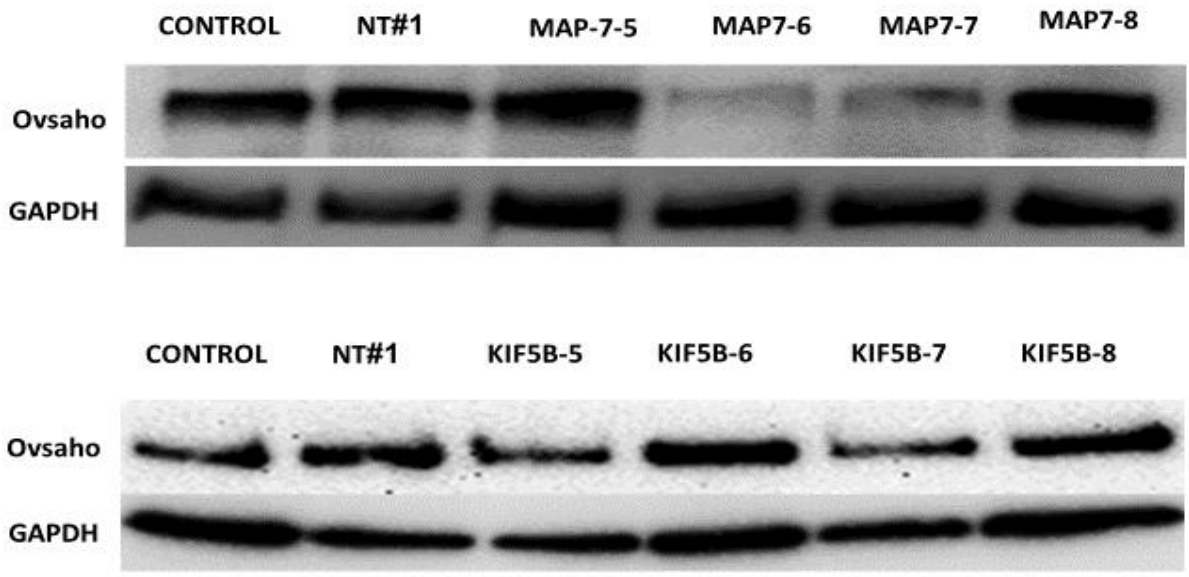


Figure (5-26): Knock-down of OVSAHO cells with MAP-7 and KIF5B was determined by western blotting of protein lysates obtained from OVSAHO cell lines after 48 hours of transfection with the indicated MAP7 and KIF5B siRNAs.

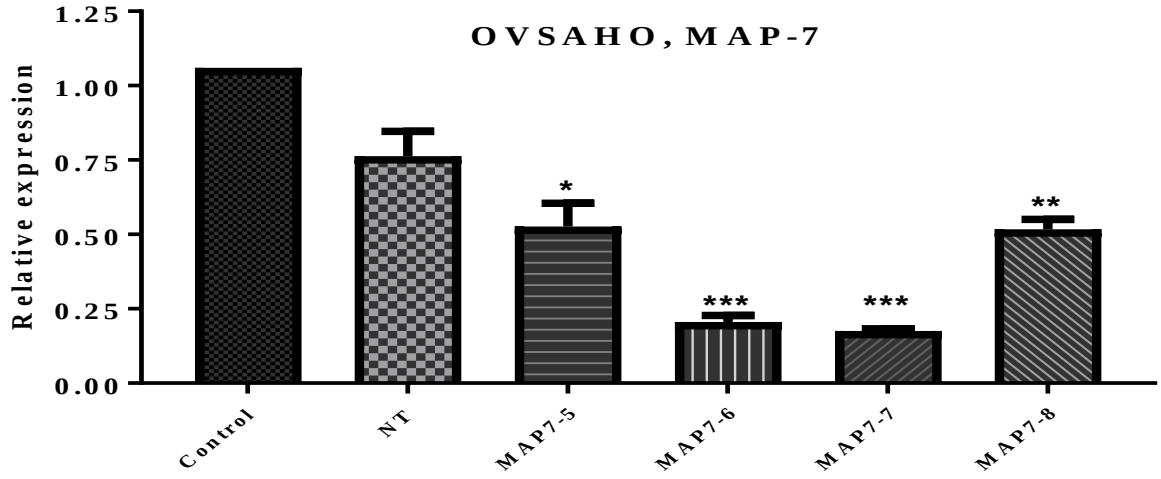


Figure (5-27): Quantification of knock-down of MAP-7 in OVSAHO cells was measured by western blotting. The results were quantified by image analysis and are expressed (n=3, mean±S.D.) as a fraction of that measured in cells transfected with a non-targeting siRNA (NT-1) then normalized with GAPDH. The results were significantly different from the expression measured in cells transfected with a NT-1 where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

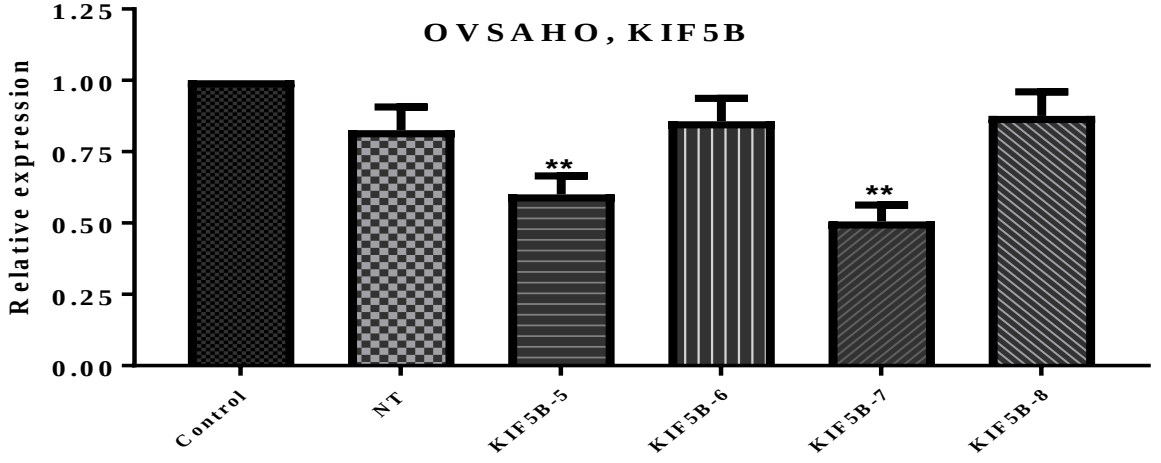


Figure (5-28): Quantification of knock-down of KIF5-B in OVSAHO cells was measured by western blotting**.** The results were quantified by image analysis and are expressed (n=3, mean±S.D.) as a fraction of that measured in cells transfected with a non-targeting siRNA (NT-1) then normalized with GAPDH. The results were significantly different from the expression measured in cells transfected with a NT-1 where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

5.2.4. Effect of genes knock-down on the sensitivity to pitavastatin in cell growth assays

To determine the effect of knockdown of the candidate drug-sensitivity genes, siRNA which caused at least 50 % knockdown were used to transfect OVCAR8 and OVSAHO cells and the

effect on the potency of pitavastatin measured in cell growth assay and compared to cells transfected with non-targeting siRNA or an siRNA to the mRNA encoding CD45, which is not expressed in epithelial cells.

In OVCAR-8 cells, knockdown of MAP7 or KIF5B using three separate siRNA had no significant effect on the sensitivity to pitavastatin (Figs 5-29, 5-30). In contrast, in OVSAHO cells, two siRNA directed to MAP7 decreased the sensitivity to pitavastatin modestly as did one of two siRNA directed to KIF5B.

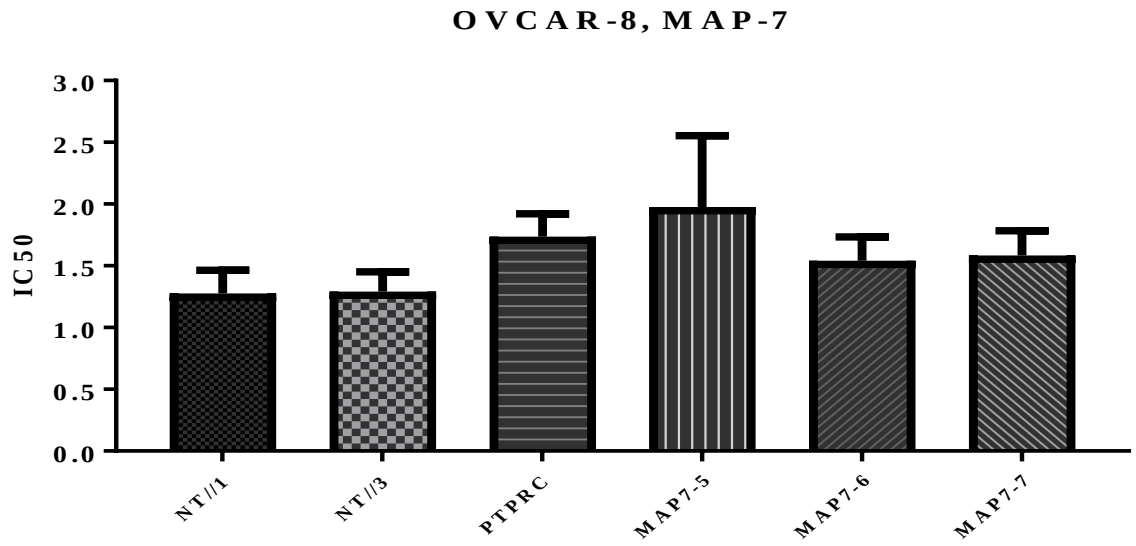


Figure (5-29): OVCAR-8 cells were transfected with either 100 nM NT-1 or 100 nM of the siRNA targeting the indicated genes. After 24 hours a range of concentrations of pitavastatin were added and after a further 72 hours the cells stained with SRB The IC_{50} of pitavastatin (n=3, mean± S.D.) The results were non-significant different from the expression measured in transfected with a NT-1, NT-3 (Non targeting) and PTPRC (targets an irrelevant mRNA) where indicated (One Way Anova; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

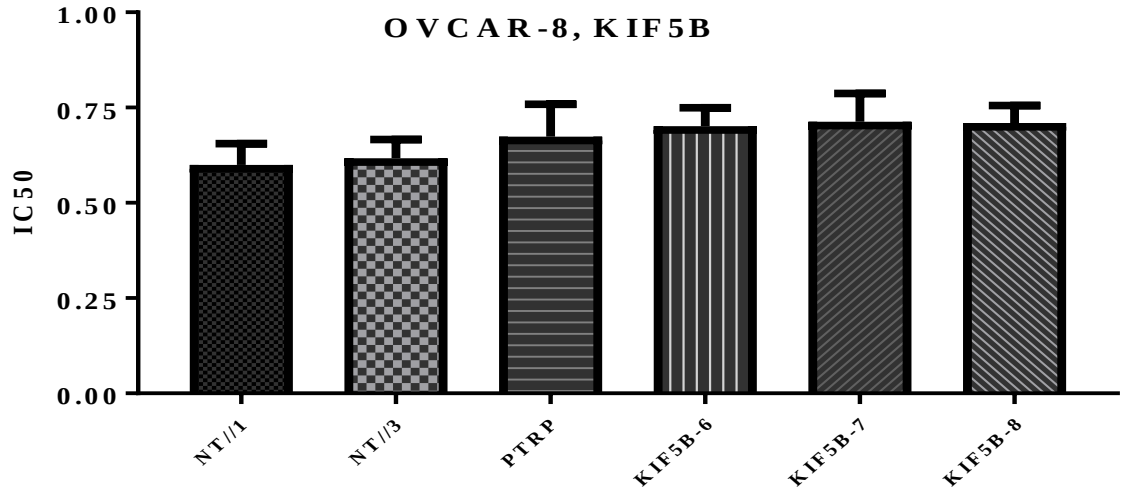


Figure (5-30): OVCAR-8 cells were transfected with either 100 nM NT-1 or 100 nM of the siRNA targeting the indicated genes. After 24 hours a range of concentrations of pitavastatin were added and after a further 72 hours the cells stained with SRB The IC_{50} of pitavastatin (n=3, mean± S.D.) The results were non-significant different from the expression measured in transfected with a NT-1, NT-3 (Non targeting) and PTPRC (On targeting) where indicated (One Way Anova *, *P*< 0.05; **, *P*< 0.01; *** *P*< 0.001).

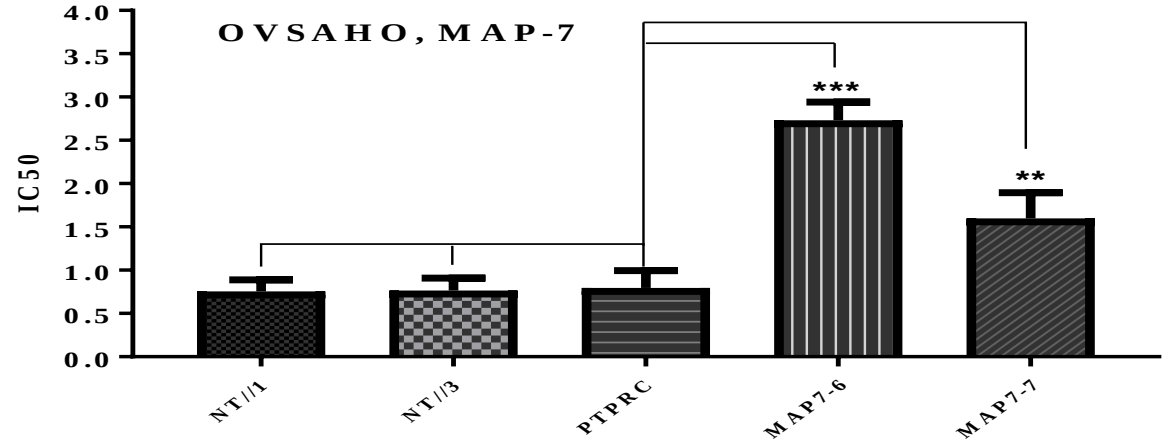


Figure (5-31): OVSAHO cells were transfected with either 100 nM NT-1 or 100 nM of the siRNA targeting the indicated genes. After 24 hours a range of concentrations of pitavastatin were added and after a further 72 hours the cells stained with SRB The IC_{50} of pitavastatin (n=3, mean± S.D.) The results were non-significant different from the expression measured in transfected with a NT-1, NT-3 (Non targeting) and PTPRC (On targeting) where indicated (One Way Anova; *, *P*< 0.05; **, *P*< 0.01; *** *P*< 0.001).

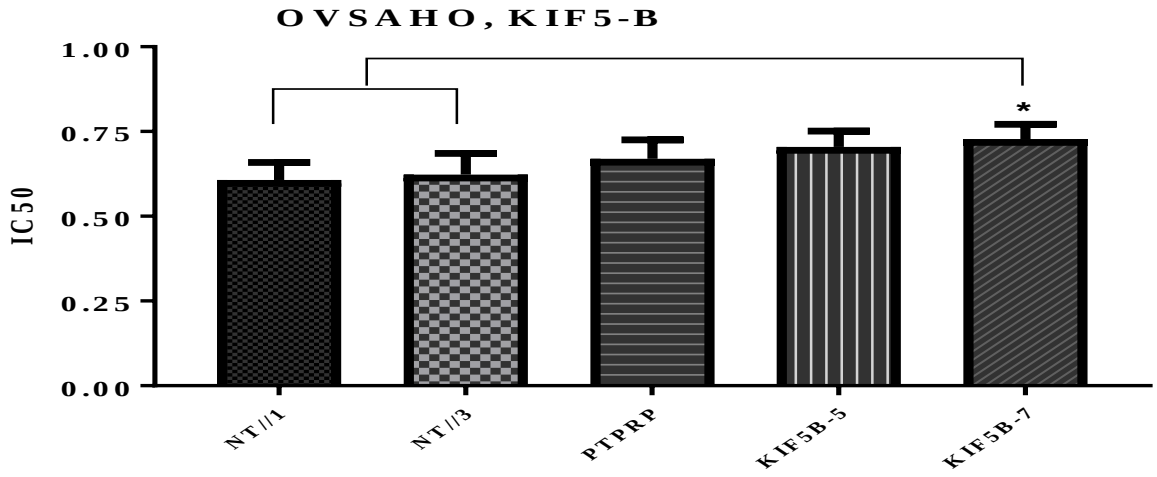


Figure (5-32): OVSAHO cells were transfected with either 100 nM NT-1 or 100 nM of the siRNA targeting the indicated genes. After 24 hours a range of concentrations of pitavastatin were added and after a further 72 hours the cells stained with SRB The IC_{50} of pitavastatin (n=3, mean± S.D.) The results were non-significant different from the expression measured in transfected with a NT-1, NT-3 (Non targeting) and PTPRC (an irrelevant protein) where indicated (One Way Anova; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

5.2.5. Expression of -tubulin in ovarian cancer cell treated with pitavastatin

Considering that MAP7 and KIF5B are both involved in regulating microtubule function, the effect of pitavastatin on microtubules was explored. Expression of tubulin was assessed by western blotting in OVCAR-8 and OVSAHO cells after exposure to pitavastatin for different time of (24h, 48 and 72h). The concentration of pitavastatin used in each cell line was one or two times the IC_{50} measured in cell growth assays. Pitavastatin reduced the level of tubulin compared to untreated cells at both concentrations. Interestingly, the tubulin levels showed a massive decrease after 24 and 48 hours while there was a slight recovery of tubulin expression after 72 hours of treatment at both concentration (Figure 5-33).

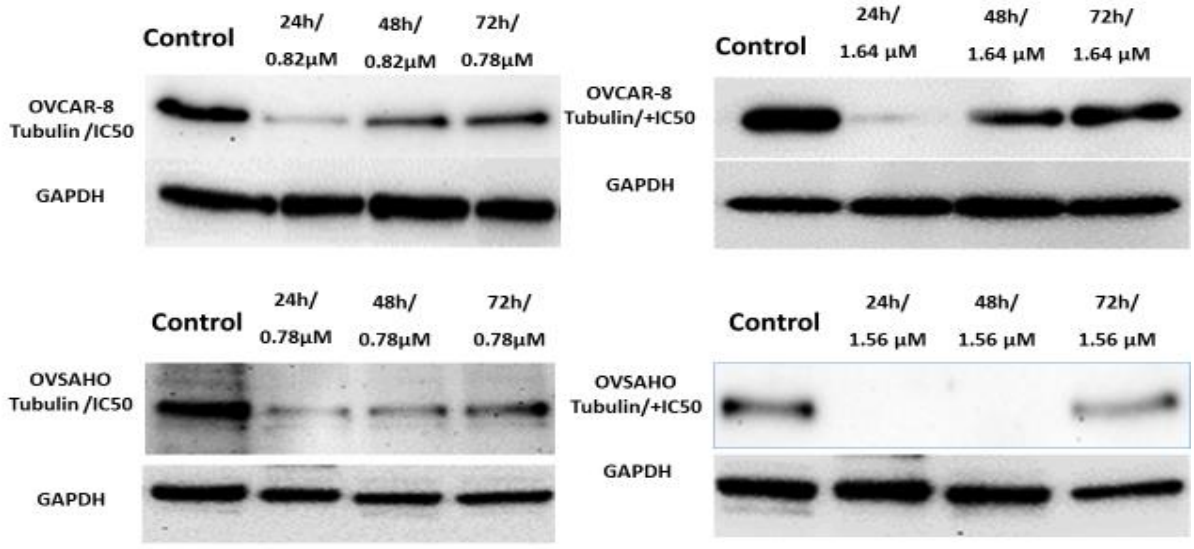
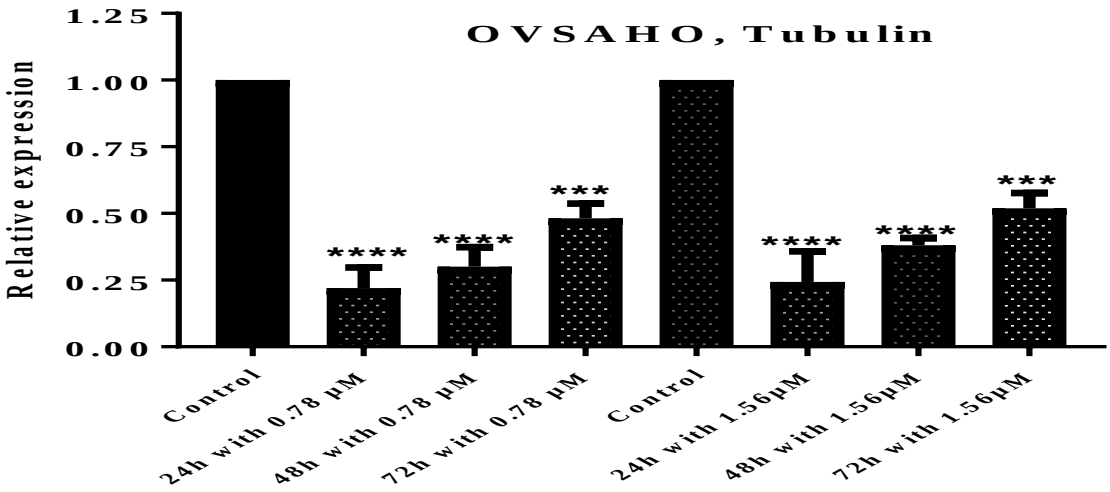
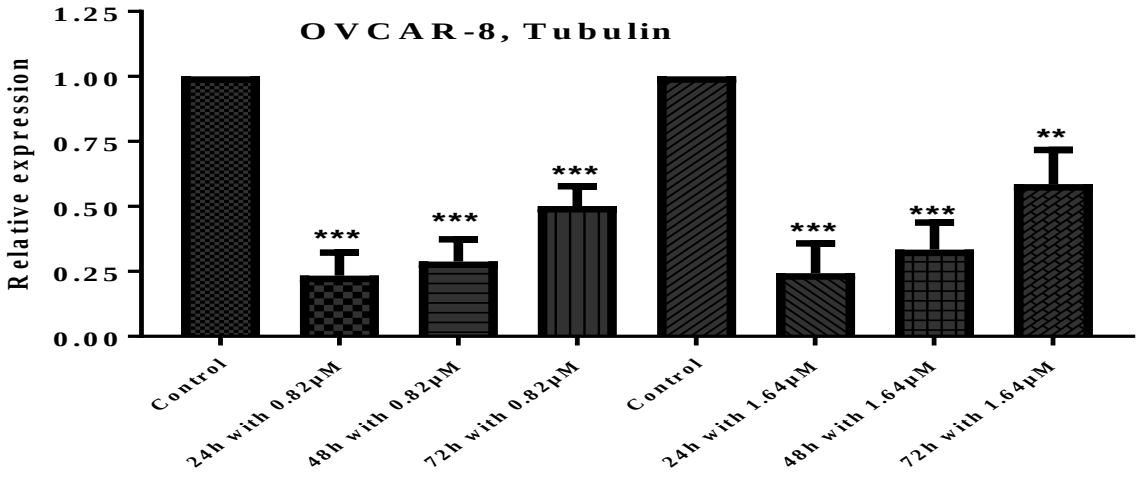


Figure (5-33): The expression of tubulin protein was determined by western blotting of protein lysates obtained from OVCAR-8 and OVSAHO cell lines after 24, 48 and 72 hours of treatment with IC_{50} (0.78µM) and + IC_{50} (1.56µM) of pitavastatin for OVSAHO and IC_{50} (0.82µM) and + IC_{50} (1.64µM) of pitavastatin for OVCAR-8.



Figures (5-34): Tubulin expression in OVSAHO cells were measured by western blotting after exposure to the indicated concentration of pitavastatin for 24, 48 or 72 hours. The results and were quantified by image analysis (n=3, mean±S.D.) and are expressed as a fraction of the tubulin measured in cells treated with drug solvent and then normalized to GAPDH. The results were significantly different from the expression measured in untreated cells where indicated (One Way Anova; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).



Figures (5-35): Tubulin expression in OVCAR-8 cells were measured by western blotting after exposure to the indicated concentration of pitavastatin for 24, 48 or 72 hours. The results and were quantified by image analysis (n=3, mean±S.D.) and are expressed as a fraction of the tubulin measured in cells treated with drug solvent and then normalized to GAPDH. The results were significantly different from the expression measured in untreated cells where indicated (One Way Anova; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

5.2.6. Immunostaining of -tubulin expression in OVCAR-8 and OVSAHO ovarian cancer cell treated with pitavastatin

Considering that the significant changes the in levels of tubulin expression measured by western blotting in both cell lines (OVSAHO and OVCAR-8) these experiments were repeated using immunofluorescence in OVCAR-8 and OVSAHO to visualize microtubules. There was again a rapid effect of pitavastatin on the microtubules after 24, 48 hours of treatment and again this partly recovered after 72 hours' exposure to the drug (fig. (5-36) (5-38)).

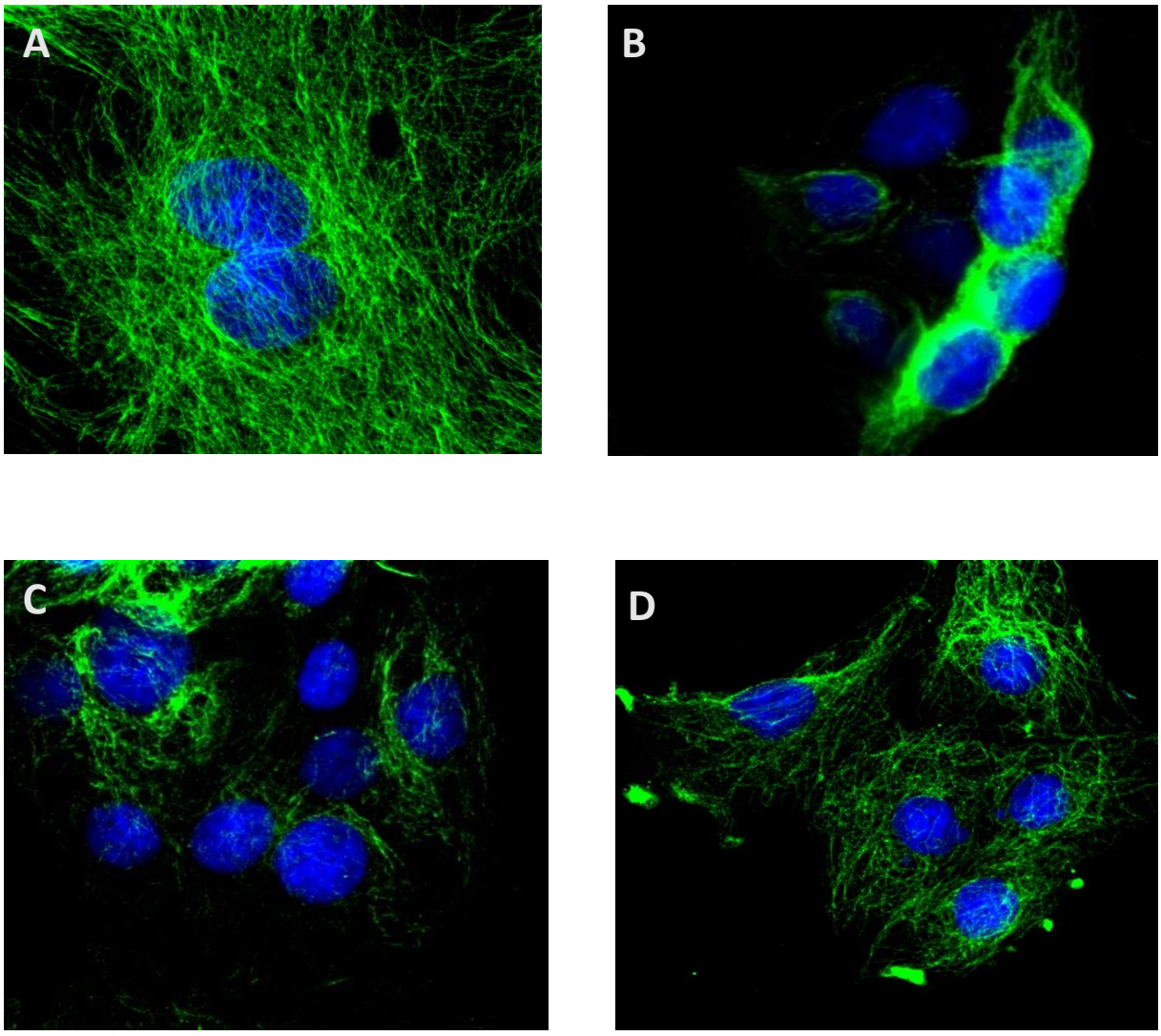


Figure (5-36): Measurement of tubulin by immunocytochemistry in Ovcar -8 cells. The cells were treated with drug solvent (A) or with the IC_{50} (0.82 µM) pitavastatin for 24 hours (B), 48 hours (C) or 72 hours (D) and stained for α-tubulin (Green) and with DAPI (nuclei, blue). The results are representative of three experiments. the images have taken by confocal microscope with 50 µm scale.

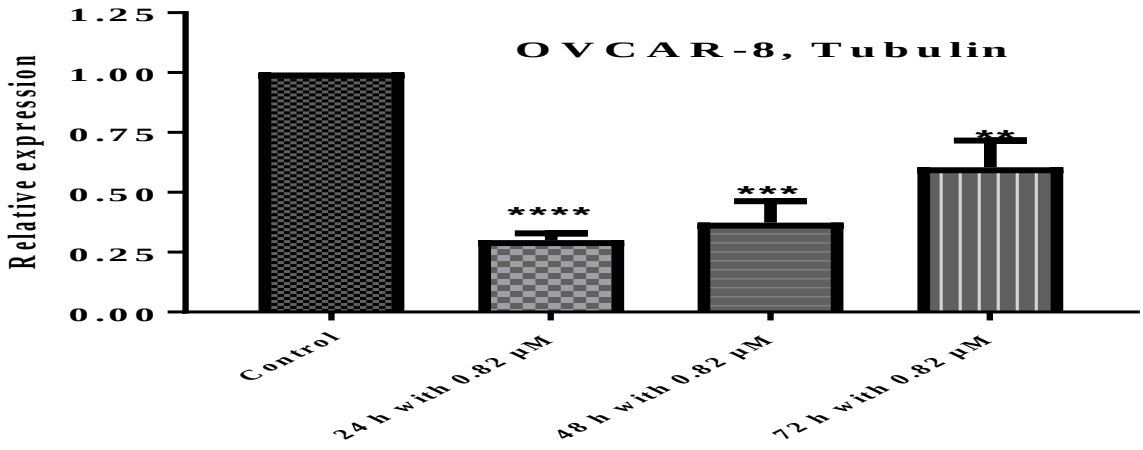
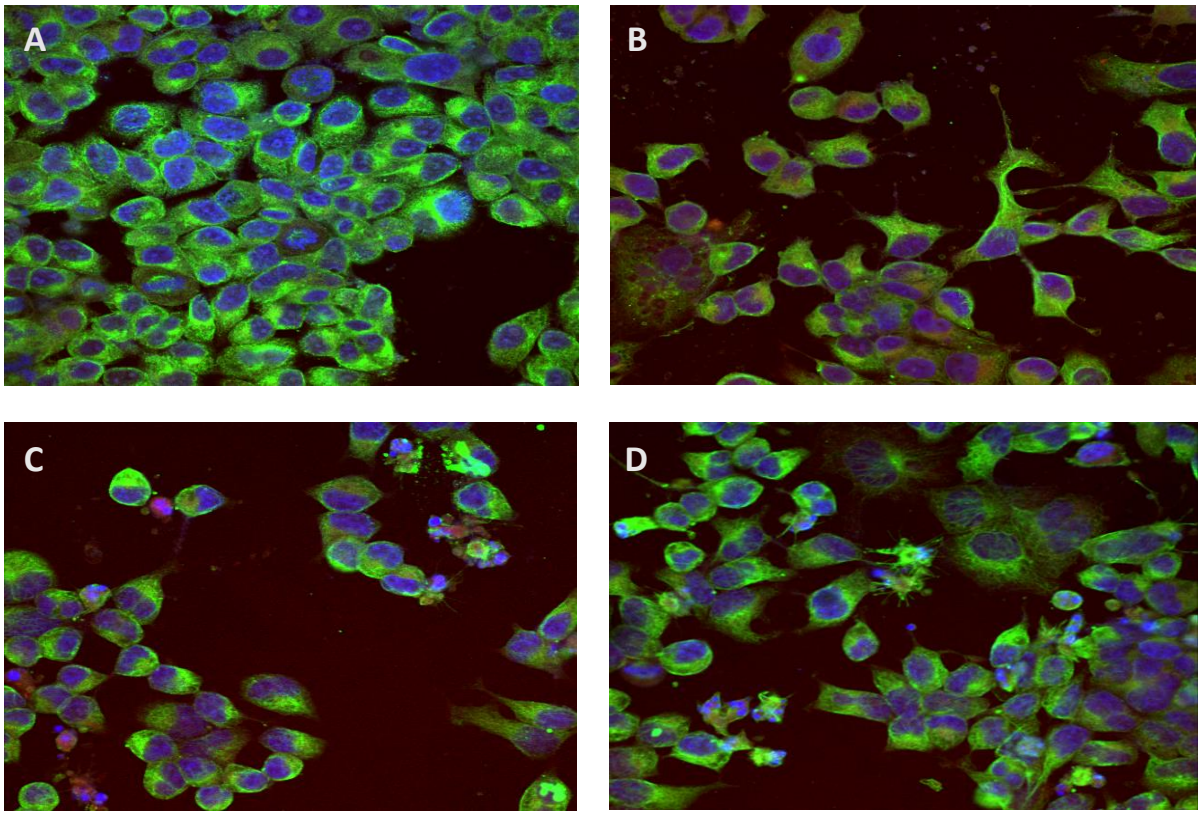


Figure (5-37): Quantification the effect of pitavastatin on microtubules tubulin expression in OVCAR-8 counted by Fiji (computer program). Cells were grown on a slide chamber for 24 hours then treated with pitavatatin at a concentration equal to its IC_{50} in cell growth assays for 24 ,48 or 72 hours. The results were significantly different from the expression measured in untreated cell as a control where indicated (One Way Anova; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).



Figures (5-38): Measurement of tubulin by immunocytochemistry in OVSAHO**.** The cells were treated with drug solvent (A) or with the IC_{50} (0.78 µM) pitavastatin for 24 hours (B), 48 hours (C) or 72 hours (D) and stained for α-tubulin (Green) and with DAPI (nuclei, blue). The results are representative of three experiments. the images have taken by confocal microscope with 50 µm scale.

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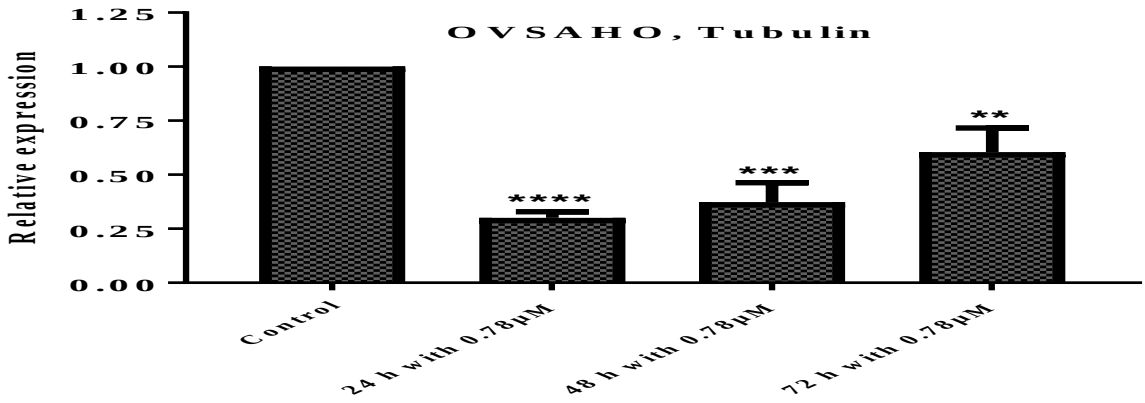


Figure (5-39): Quantification the effect of pitavastatin on microtubules tubulin expression in OVSAHO counted by Fiji (computer program). Cells were grown on a slide chamber for 24 hours then treated with pitavatatin at a concentration equal to its IC_{50} in cell growth assays for 24 ,48 or 72 hours. The results were significantly different from the expression measured in untreated cell as a control where indicated (One Way Anova; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

5.2.7. Expression of Actin in ovarian cancer cell after treatment with pitavastatin

Considering the effects of pitavastatin on the microtubule cytoskeleton, the effect on the actin cytoskeleton were also investigated. Expression of Actin was assessed by Western blotting of OVCAR-8 and OVSAHO cells. The western blotting revealed a decreased in actin levels after 24 hours' exposure to pitavastatin, but by 48 hours this had essentially recovered to the amount in the control cells (fig (5-40)).

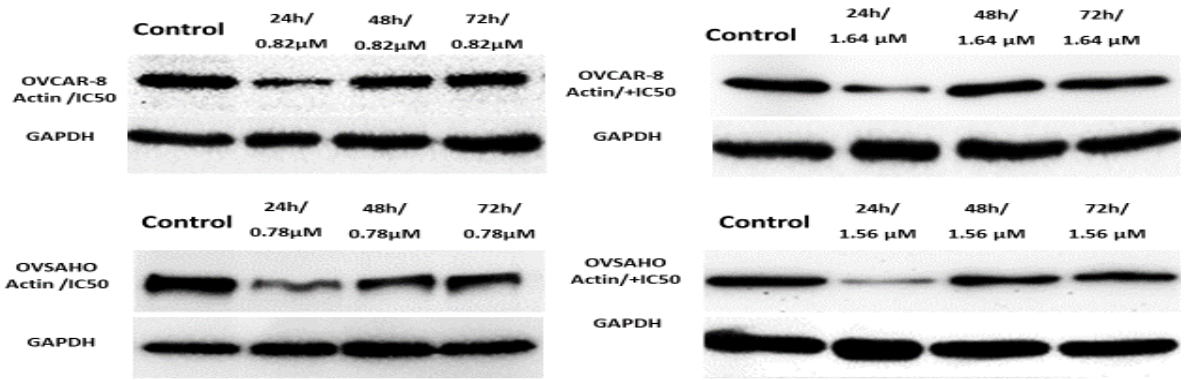


Figure (5-40): The expression of actin protein was determined by western blotting of protein lysates obtained from OVCAR-8 and OVSAHO cell lines after 24, 48 and 72 hours of exposure to pitavastatin. The drug concentrations were equal to the IC_{50} (0.78µM) and 2x IC_{50} (1.56µM) measured in cell growth assay using with OVSAHO cells or the IC_{50} (0.82µM) and 2x IC_{50} (1.64µM) of pitavastatin using OVCAR-8 cells.

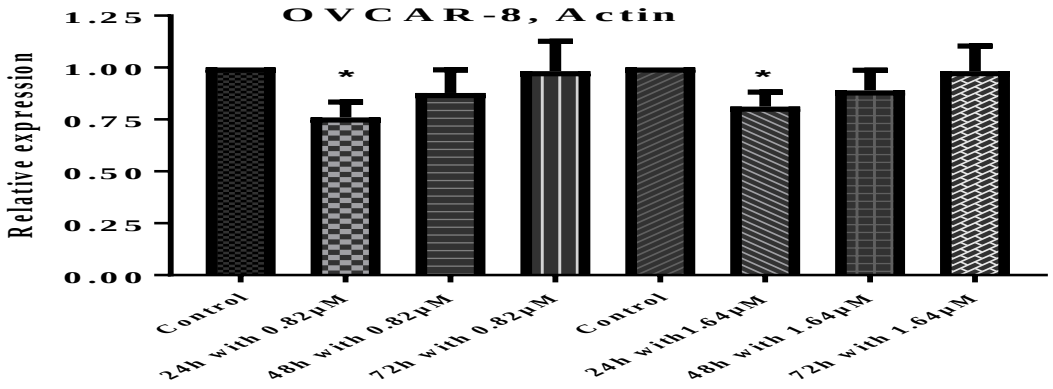


Figure (5-41): Actin expression in Ovcar-8 were measured by western blotting. after exposure to the indicated concentration of pitavastatin for 24, 48 or 72 hours. The results and were quantified by image analysis (n=3, mean±S.D.) and are expressed as a fraction of the actin measured in cells treated with drug solvent and then normalized to GAPDH. The results were significantly different from the expression measured in untreated cells where indicated (One Way Anova; *, *P*< 0.05; **, *P*< 0.01; *** *P*< 0.001).

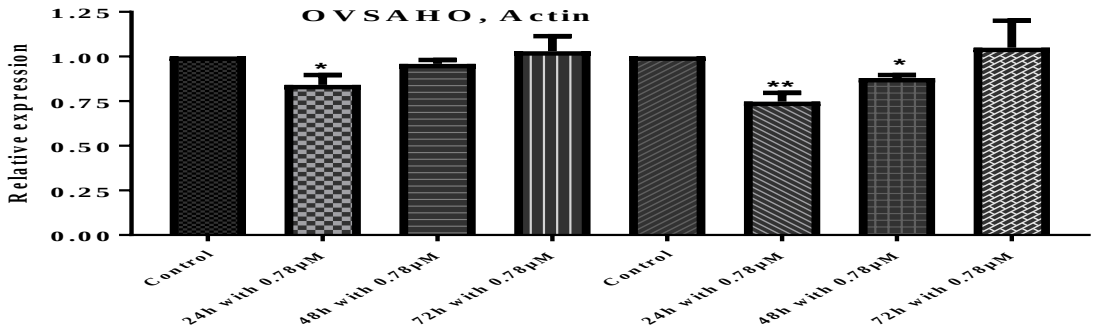


Figure (5-42): Actin expression in Ovsaho were measured by western blotting. after exposure to the indicated concentration of pitavastatin for 24, 48 or 72 hours. The results and were quantified by image analysis (n=3, mean±S.D.) and are expressed as a fraction of the actin measured in cells treated with drug solvent and then normalized to GAPDH. The results were significantly different from the expression measured in untreated cells where indicated (One Way Anova; *, *P*< 0.05; **, *P*< 0.01; *** *P*< 0.001).

5.2.8. CNP expression and immunohistochemistry

CNP is post-translationally modified by an isoprenylation process at its C terminus. CNP is a potential protein connecting the microtubules to the plasma membrane. This made it a potential candidate for the effects of pitavastatin on the microtubules. Ovcar-8 and Ovsaho were treated with pitavastatin with or without geranylgeraniol or and farnesol for 24 hours. The cells were fractionated to obtain membrane and cytosolic fractions and the CNP content of each

fraction determined by western blotting. Pitavastatin caused the loss of CNP from both the cytosolic and cell membrane fractions and this could be restored by supplementation with either farnesol or geranylgeraniol (figures (5-43), (5-44), (5-45) and (5-46)).

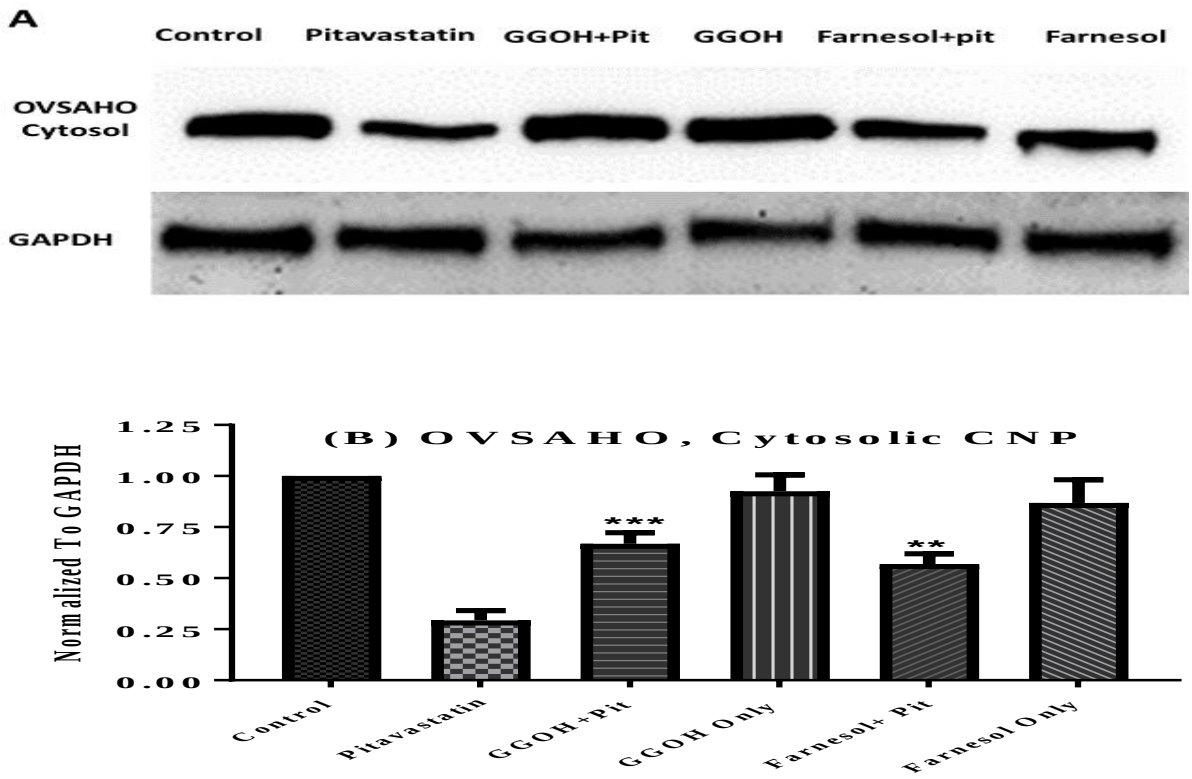


Figure (5-43): (A) The expression of CNP protein was determined by western blotting of protein lysates obtained from cytosol of OVSAHO cell lines 24 hours after pitavastatin treatment (IC_{50} 0.78µM) with/without isoprenoids (GGOH (5 µM) and Farnesol (5 µM)). (B)cytosolic CNP expression in Ovsaho were measured by western blot. The results (n=3, mean±S.D.) are expressed as a fraction of that measured in treated cells IC_{50} of pitavastatin alone and with/without GGOH and farnesol for 24 hours then normalized with GAPDH. The results were significantly different from the expression measured in Untreated cell as a control where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

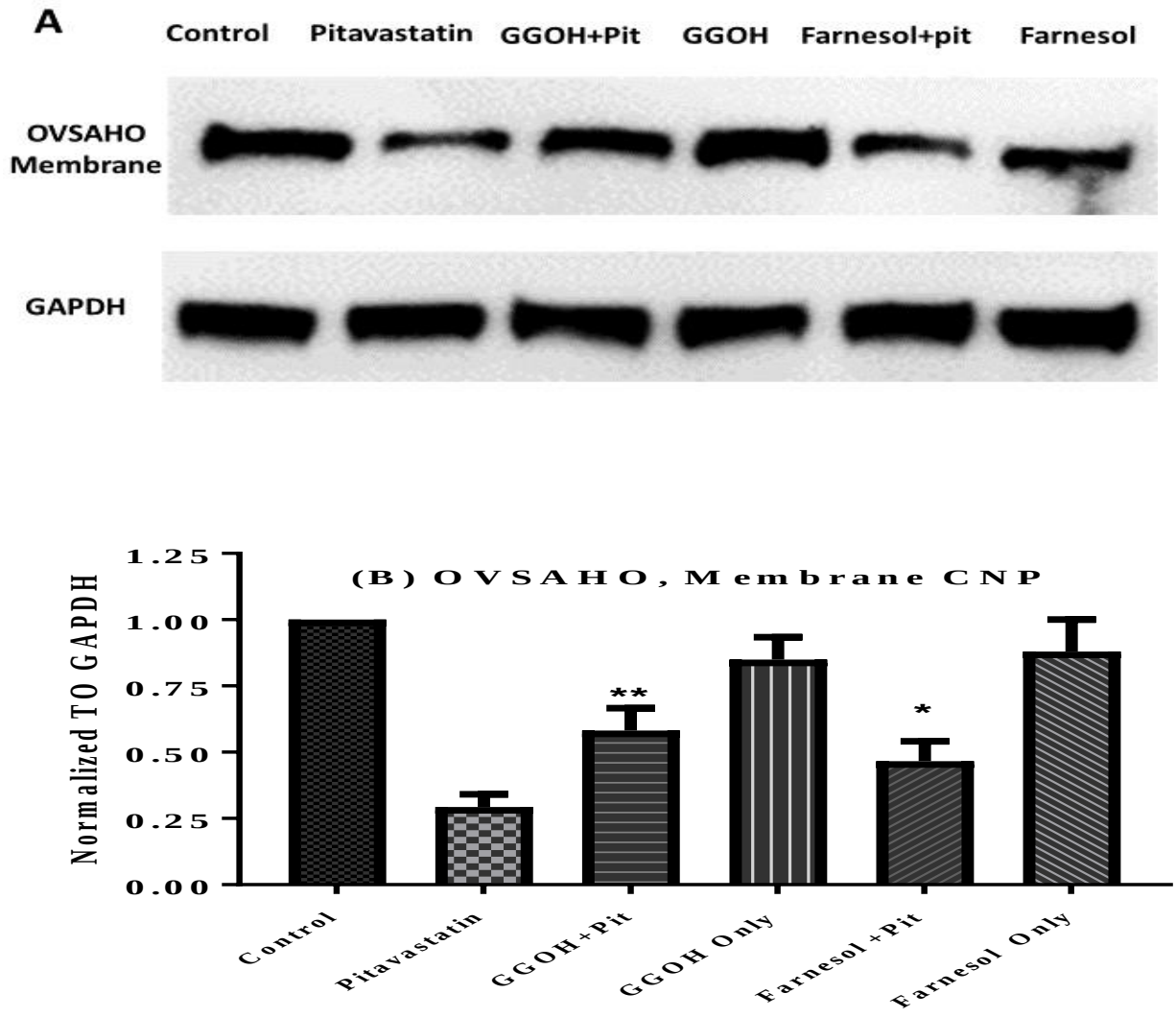


Figure (5-44): (A) The expression of CNP protein was determined by western blotting of protein lysates obtained from membrane of OVSAHO cell lines 24 hours after pitavastatin treatment (IC_{50} 0.78µM) with/without isoprenoids (GGOH (5 µM) and Farnesol(5 µM)). (B)Membrane CNP expression in Ovsaho were measured by western blot. The results (n=3, mean±S.D.) are expressed as a fraction of that measured in treated cells IC_{50} of pitavastatin alone and with/without GGOH and farnesol for 24 hours then normalized with GAPDH. The results were significantly different from the expression measured in Untreated cell as a control where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

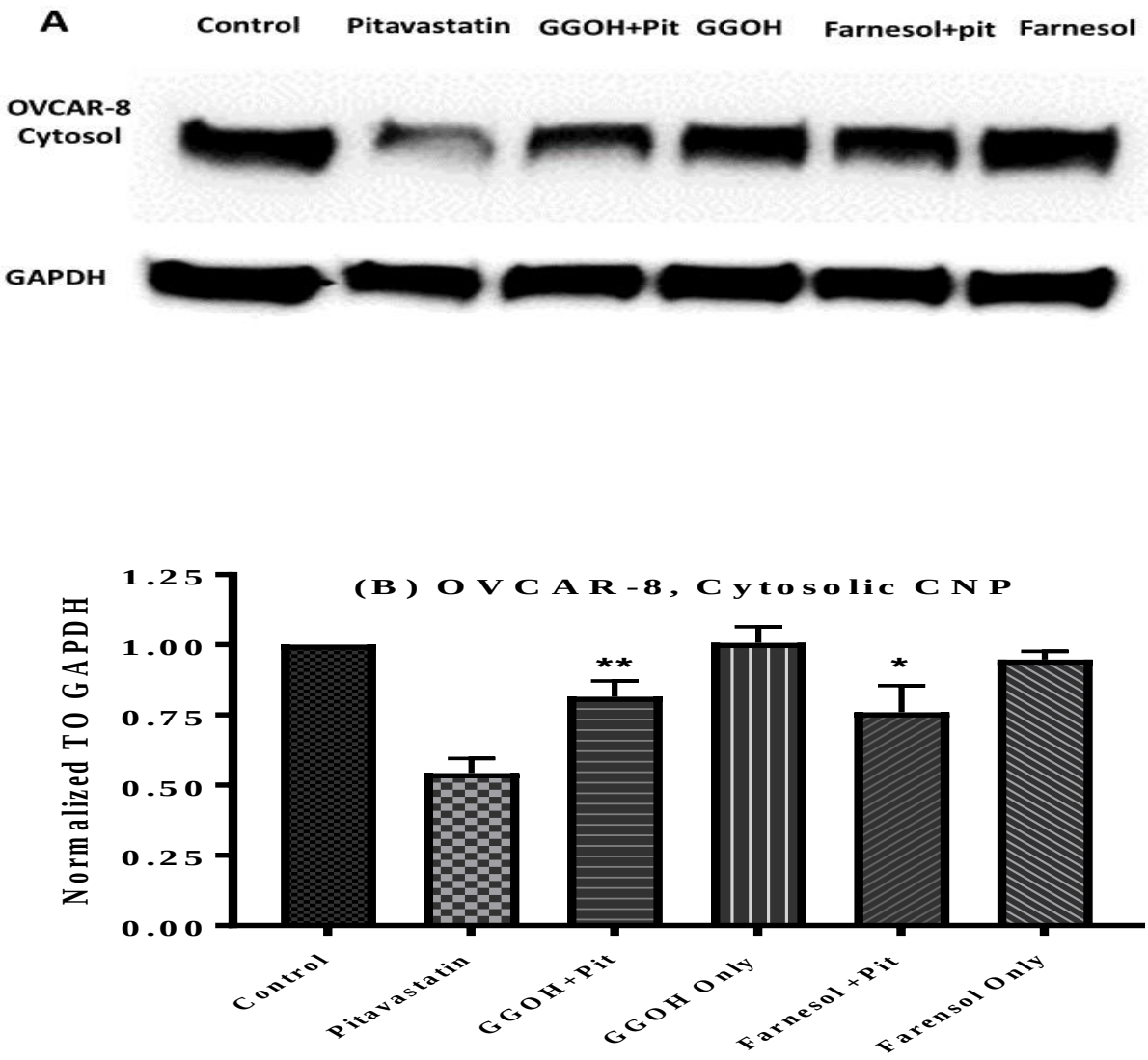


Figure (5-45): (A) The expression of CNP protein was determined by western blotting of protein lysates obtained from cytosol of OVCAR-8 cell lines 24 hours after pitavastatin treatment (IC_{50} 0.82µM) with/without isoprenoids (GGOH (5 µM) and Farnesol(5 µM)). (B)cytosolic CNP expression in Ovcar-8 were measured by western blot. The results (n=3, mean±S.D.) are expressed as a fraction of that measured in treated cells IC_{50} of pitavastatin alone and with/without GGOH and farnesol for 24 hours then normalized with GAPDH. The results were significantly different from the expression measured in Untreated cell as a control where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

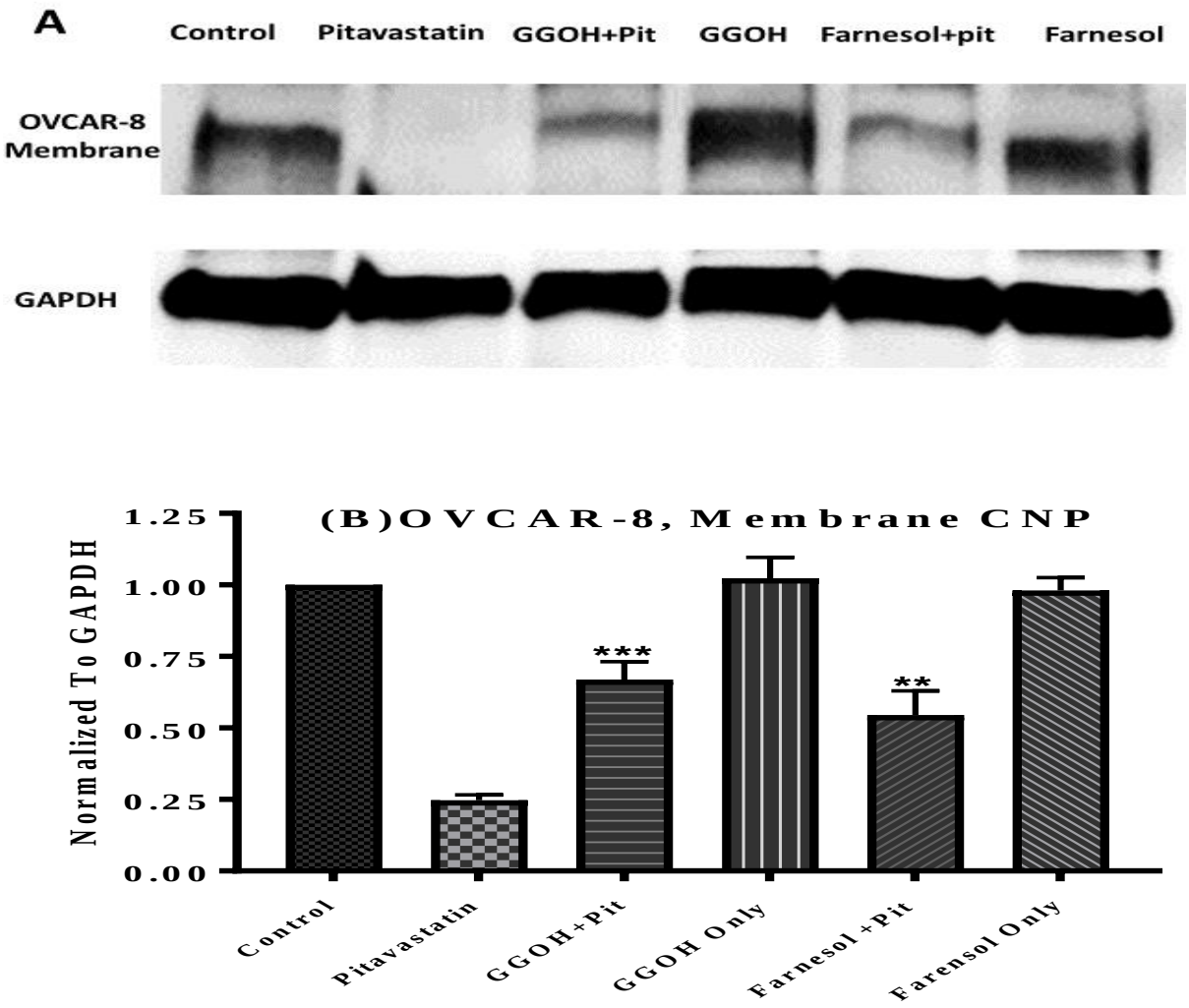
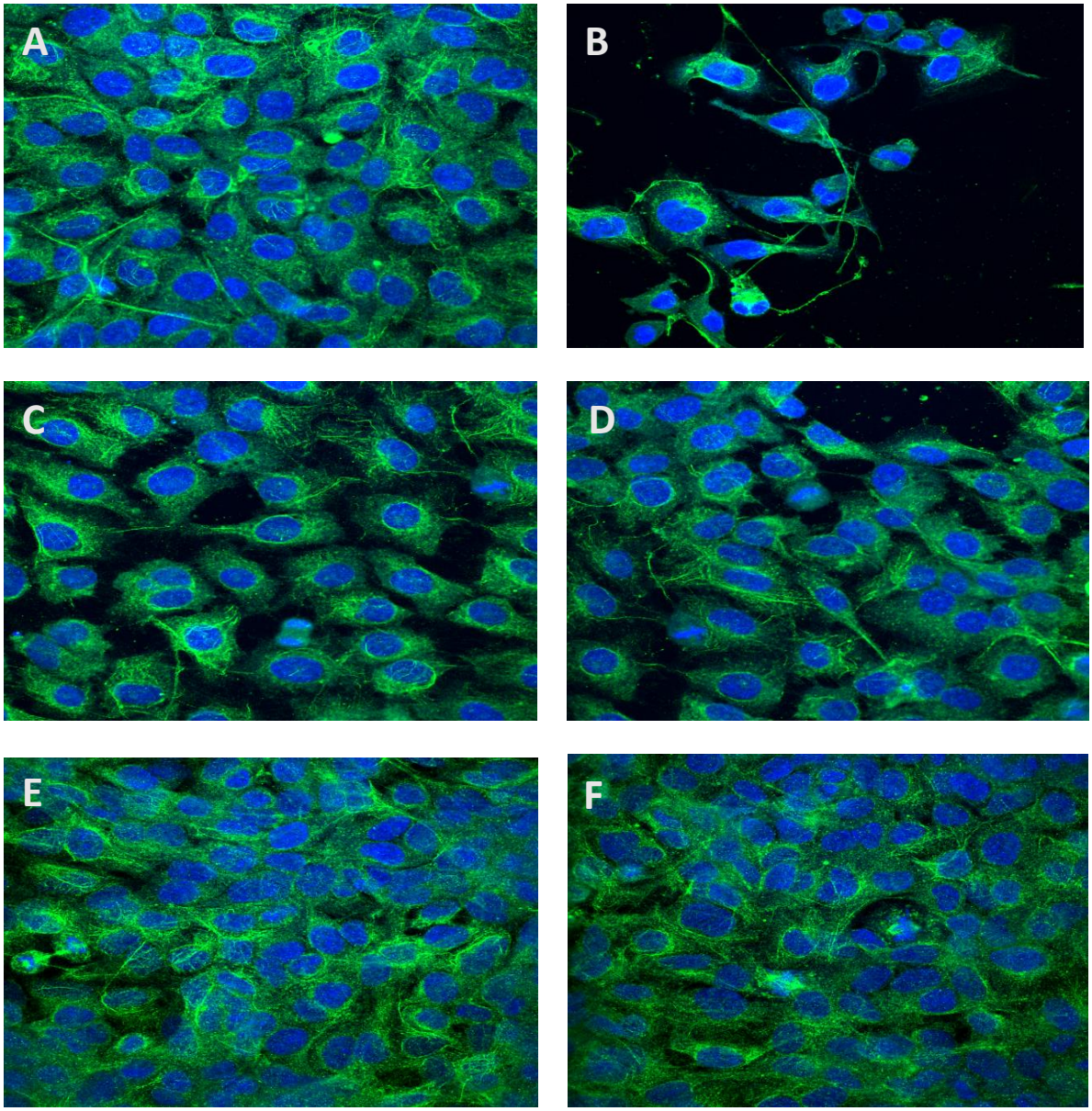


Figure (5-46): (A) The expression of CNP protein was determined by western blotting of protein lysates obtained from membrane of OVCAR-8 cell lines 24 hours after pitavastatin treatment (IC_{50} 0.82µM) with/without isoprenoids (GGOH (5 µM) and Farnesol(5 µM)). (B)Membrane CNP expression in Ovcar-8 were measured by western blot. The results (n=3, mean±S.D.) are expressed as a fraction of that measured in treated cells IC_{50} of pitavastatin alone and with/without GGOH and farnesol for 24 hours then normalized with GAPDH. The results were significantly different from the expression measured in Untreated cell as a control where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

5.2.3. Immunostaining of CNP expression in OVCAR-8 ovarian cancer cell treated with pitavastatin

The effects of pitavastatin on the CNP was measured by western blotting were confirmed by immunohistochemistry studies.

Pitavastatin induced the loss of CNP from the cytosolic and cell membrane fractions and this could be reversed by either farnesol or geranylgeraniol supplementation which confirmed by immunostaining images below (fig. (5-47)).



Figures (5-47): CNP Immunostaining of Ovcar-8 (Green staining), and nuclei (DAPI, blue) after 24 hours of pitavastatin treatment with IC_{50} (0.82µM) (A) drug solvent, (B) pitavastatin (C) pitavastatin and geranylgeranyl, (D) pitavastatin and farnesol € geranylgeranio alone (F) farnesol alone. The results are representative of three experiments and the images have taken by confocal microscope with 50 µm scale.

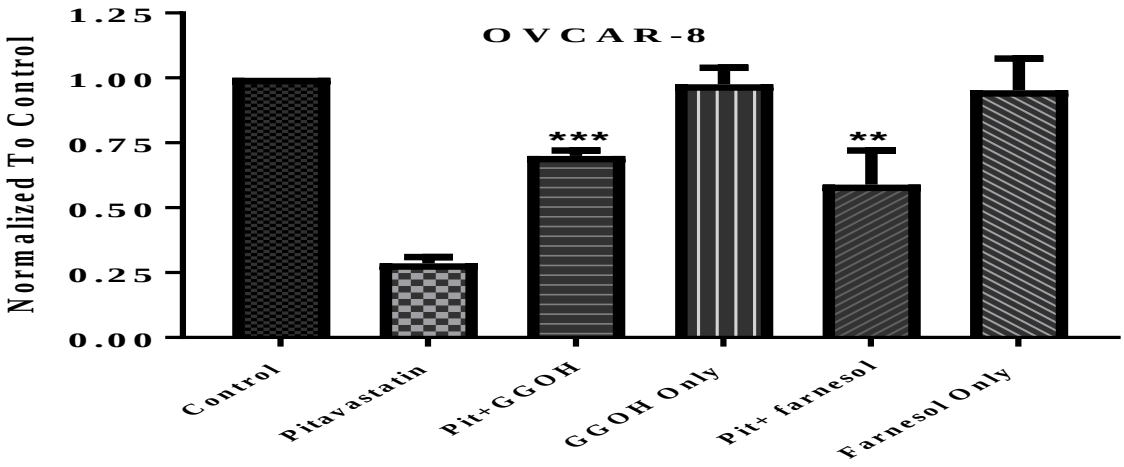


Figure (5-48): Quantitate the CNP expression in Ovcar-8 counted by Fiji (computer program). Cells were grown on a slide chamber for 24 hours then treated with IC_{50} of pitavastatin with/ without GGOH(5µm) and farnesol (5µm) for 24 hours and an anti-CNP monoclonal antibody and secondary antibody applied for two hours for each after fixation the cells with formaldehyde 10%, mounting the cells after removing the chambers and imaging by confocal microscope. The results were significantly different from the expression measured in untreated cell as a control where indicated (paired *t*-test; *, $P< 0.05$; **, $P< 0.01$; *** $P< 0.001$).

5.3. Discussion

A critical problem in designing personalised cancer medicine is the understanding of how to make the most of a patient's genomic knowledge while determining treatment options. Over the past decade, comprehensive efforts have been made to sequence genomes of cancer in large patient populations[486], sparking hopes of identify new targets for more successful and targeted treatment opportunities. Such sequencing efforts have uncovered a remarkable degree of genetic heterogeneity between and within different cancers types, which explains in part why the typical "one-size-fits-all" therapeutic strategies for cancer frequently provided disappointing results in clinical trials[487].

Different cell lines show different sensitivities to pitavastatin and the same may be true of patient's tumours. It is, therefore, important to identify biomarkers which predict a patient's response to pitavastatin. Although E-cadherin membrane expression has been reported to be both a marker and resistance mechanism for atorvastatin-mediated growth suppression of cancer cells [407], a transcriptome-based biomarker signature for statin sensitivity would also facilitate clinical treatment. Some progress has already been made to achieve that with other cancers [407], [488]. The goal of this study was to identify markers that predicted the statin sensitivity of ovarian cancer cells. We have attempted to confirm published gene expression data from the Broad Institutes "Cancer Therapeutics Response portal. This provided a resource to identify a correlation with the sensitivity of cancer cells to statins. Although the original data was published on a broad panel of cell lines from different cancer types, we attempted to replicate those findings using ovarian cancer cell lines.

The expression of these genes that were selected for investigation was assessed by both qPCR and western blotting in the panel of 6 ovarian cancer cell lines and compared to the sensitivity of these cells to pitavastatin. Both these methods were employed to avoid any bias introduced

by using just one technique. Some of the genes investigated were only highly single expressed in a single cell line when assessed by western blotting. A thorough analysis of more than 2 million manually annotated, high-resolution, images based on immunohistochemistry found a large fraction, of proteins are expressed proteins in most cells and tissues, with very few proteins, about 2 percent, were unique to a single cell type.[489]. It is therefore surprising that some genes appear to be only expressed one cell line of six, all of which were derived from the same tissue. This cell- line specific" expression, therefore, likely to reflect genetic abnormalities in cancer cells and these abnormalities affect drug sensitivity. It is possible that there are several mechanisms of drug resistance/sensitivity and that some cell lines make use of only one of these. Alternatively, is possible that some of the genes that were selected for investigation in fact do not contribute to drug sensitivity. It is also possible that the mechanisms that confer drug sensitivity to ovarian cancer cells are different from the mechanisms in other cancer types. Further investigation would be necessary to investigate this, for example, the expression of these genes could be altered in ovarian cancer cells and the effect on sensitivity determined. It would also be desirable confirm the results reported by the Cancer Therapeutics response portal, testing the sensitivity of the statins investigated there (which did not include pitavsastatin) in exactly the cell lines used by those investigators; it would also be worthwhile to alter the expression of these genes in those cell lines and test the impact on the sensitivity to the statins those investigators examined.

Some genes displayed high expression in all of the ovarian cancer cell lines used in both experiments (western blotting and qPCR). This was surprising considering that the ovarian cancer cells showed differences in sensitivity to pitavastatin. Again, it would be worthwhile to reproduce the results of from the Broad Institute using their experimental conditions and also manipulating the expression of these genes.

There was also some inconsistency between the qPCR and western blotting data. However, some genes showed strong protein expression in a single cell when measured by western blotting, while less expression was evident by the qPCR technique This may reflect that mRNA and protein levels are regulated by different mechanisms and suggests that most of mRNA is efficiently translated into its corresponding protein or that the mRNA has a short half-life. By comparison, few genes showed poor expression when measured by blotting but at the same time showed high expression by qPCR. In these cases, the mRNA may not be efficiently transcribed into the corresponding protein.

Arguably the most encouraging results from this analysis was that, among all genes used in this experiment, MAP7 was highly expressed in ovarian cancer sensitive cells to pitavastatin (OVCAR-8 and OVSAHO). This raised the possibility that this gene was involved in setting the sensitivity to pitavastatin. Although the data gave some encouragement that MAP7 may play a role ovarian cells' sensitivity to pitavastatin treatment. this was only a correlation. Only two sensitive cell lines were used so it is possible that the correlation of this gene with drug sensitivity arose by l pure chance. It would have been preferable to have a larger panel of statin sensitive and statin resistant cells to increase the power of the observations. However, as an alternative, RNAi experiments were performed to assess whether altering the expression of MAP7 changed the sensitivity to pitavastatin.

MAP7 expression was repressed by RNAi in the two cell lines where it was highly expressed. Preliminary experiments were performed to identify at least two different siRNA that decreased the protein levels of MAP7 without being overtly toxic. Multiple siRNA was used to give confidence that any affect of the siRNA did indeed result from an affect on MAP7 and not an off-target effect of the siRNA. Once appropriate conditions were identified, the effect of knockdown on pitavastatin sensitivity was evaluated. Unfortunately, this led to mixed results.

In OVCAR8 cells knockdown of MAP7 with two separate siRNA had little effect on the sensitivity to pitavastatin. In contrast, in OVSAHO cells, knockdown of MAP7 by siRNA led to a decrease in the sensitivity to pitavastatin. Furthermore, this was only observed in one cell line and with one of the two siRNA tested. This raised further concerns whether MAP7 really played a role in the effect of pitavastatin on cancer cells. It is possible that MAP7 plays role in the sensitivity to pitavastatin in one of the cell lines tested (OVSAHO). Alternatively, the rapid growth rate of OVCAR-8 cells after knocking down may somehow compensate for the effect of the final results of knockdown.

To explore further whether MAP7 may play a role in the sensitivity of cells to pitavastatin, the expression of one of its binding partners, KIF5B was investigated. KIF5B is the major MT plus-end motor involved in a wide range of transport processes[490], This motor is well known for being controlled by various MAPs. The MAP7 protein N-terminal domain interacts strongly with MTs, while the C-terminal domain binds to the kinesin-1 stalk region[432], [436], [437], and KIF5B may have a role in determining the sensitivity of drugs targeting microtubules. Mixed results were also obtained following knockdown of KIF5B. Again in OVCAR8, knockdown had little effect on the sensitivity of the cells to pitavastatin. However, in OVSAHO cells, one of the two siRNA tested reduced sensitivity to pitavastatin. This again made it difficult to draw definitive conclusions about the role of these proteins. It is striking however, that positive results were again observed with OVSAHO cells and not OVCAR-8 cells, possibly pointing to a cell-specific mechanism of drug sensitivity.

Considering the inconclusive nature of the data obtained to the is point and that MAP7 binds to microtubules, the effect of pitavastatin on the microtubule cytoskeleton was investigated. Here, at least, the results were more convincing. Pitavastatin caused a clear and transient decrease in the amount of tubulin measured by two separate methods. Both methods (western blots and

immunostaining) showed very significant changes after different periods of exposure to pitavastatin especially after 24, 48 hours with a slight recovery after 72 hours. The transient nature of this effect may be due to loss of feedback inhibition of the mevalonate pathway. Pitavastatin also had an effect on the actin cytoskeleton and if anything the results were even more transient and returned to normal after 24 hours of treatment with pitavastatin. However, it is not clear whether the effects of pitavastatin on microtubules depend on MAP7 or KIF5B.

To explore how pitavastatin may affect microtubules, further studies were performed. Some MAPs are known to bind microtubules to actin and/or intermediate filaments, and are called cytoskeletal cross-linkers. For a variety of cellular functions such as lateral cell migration and neuronal pathfinding, the interaction of these various cytoskeletal components is necessary[430]. Other MAPS help anchor microtubules to the cell membrane. Tubulin strongly binds CNP. Significantly, CNP is associated with membranes by virtue of its isoprenylation [380], making a potential link to pitavastatin. CNP can bind tubulin to membranes and control the distribution of cytoplasmic microtubules [491]. This suggests that the binding of microtubules to cell membranes may require a prenylated protein. Tubulin itself does not have a C-terminal CAAX box for prenylation [453]. Thus, CNP may make a linker between cytoskeleton protein (tubulin) and isoprenoids membranous proteins. Bifulco and colleagues have obtained compelling data at that such an interaction takes place and suggest that CNP has MAP-like properties. They find a strong interaction in brain tissue and FRTL-5 cells in two separate locations: cytoplasm and membranes. CNP promotes the polymerisation of pure rat brain tubulin at low molar ratios as determined by both protein pelleting and light dispersion [451], They concluded that normal microtubular distribution depends on CNP. In addition, CNP was reportedly bound to cytoskeletal elements based on actin [381] providing a potential explanation for the effects of pitavastatin on the actin cytoskeleton. This led to the hypothesis

that CNP is a linker between microtubules and the cell membrane and that loss of its prenylation results in the effects of pitavastatin one microtubule cytoskeleton.

The effect of pitavastatin on CNP was therefore evaluated. Cell fractionation experiments and immunofluorescence studies both revealed that pitavastatin causes a loss of CNP from cell membranes and cytosol. This could be blocked by supplementation with geranylgeraniol and surprisingly also by farnesol. In previous experiments evaluating the ability of these isoprenoids to block the effects of pitavastatin on cell growth or apoptosis only geranylgeraniol, and not farnesol, were able to suppress the effects of pitavastatin. This suggests that although CNP may be affected by pitavastatin and this may contribute to the cytotoxic activity of pitavastatin, it is unlikely to be the sole mechanism by which pitavastatin is cytotoxic. Interestingly, another study reported that both prenyl groups, farnesyl and geranylgeranyl, modify CNP [432], which support our findings.

There are a number of issues with the approach taken in this study. The use of a relatively small number of cell lines may have meant that this study lacked sufficient power to identify genes which do indeed determine drug sensitivity. As discussed above, MAP7 may have been identified by pure chance. In addition, using single-targeting siRNAs to knockdown gene expression is problematic. Apart from the potential for off-target affects, any on-target effects may vary according to the duration of the experiment and the rate of growth of the cells. Moreover, the cell types in the NCI-60 cell line panel of the National Cancer Institute show that (i) there is not the same broad range of refractoriness (resisting ordinary medical methods) as clinically found in tumours in patients, and (ii) the susceptibility spectrum in culture for these cells is in no way similar to the solid tumours. Studies with cultured cells may therefore only investigate a portion of a tumour's response to potential drugs[492]. Cancers are heterogeneous, and even if a predictor of drug sensitivity were to be identified, it may only be relevant for a

fraction of the cells present in a tumour. Another issue is how well ovarian cancer cell lines reflect clinical tumours[493].

Lastly, the role of the prenylated CNP as a microtubule membrane anchor can provide more insight into how novel anti-cancer agents may be discovered. Drugs targeting the microtubule cytoskeleton are already widely used in the treatment of cancer. *In vitro* studies and multiple *in vivo* correlative findings on the expression and vulnerability of various cancers to microtubule-targeting drugs argue that MAPs may provide alternative drug targets or predictors of sensitivity to existing drugs which target microtubules. While currently restricted, further information on the tumour MAP expression profile and the mechanisms regulating it could encourage combination therapies with agents resulting in a change in the expression of appropriate MAP in the desired direction (either up-or down-regulation). In addition, techniques to enhance MAP-tubulin interaction could be explored. High-throughput screening of small molecule libraries is an appealing technique for detecting compounds that interfere with MAP-tubulin interactions, altered expression of MAPs and their effect on sensitivity to microtubule-targeting drugs [447]. This provides an opportunity to control MAP-tubulin interactions influencing tubulin-drug interactions and clinical outcomes in cancer therapy [447].

Chapter 6

Anti-cancer activity of Ivermectin in combination with pitavastatin

6. Introduction

Cancer chemotherapy's primary objective is to eliminate malignant tissues. After the failure of single agents, combining drugs is a plausible approach for cancer treatment [494]. The fundamental rationale for cytotoxic chemotherapy drug combinations is that this simultaneously targets several cellular pathways[494], [495]. This provides several benefits including increased tumour cell death and therapeutic efficacy, reduced resistance and possibly reduced toxicity connected with high-dose chemotherapy[495]. Combinations involving pitavastatin are also plausible. Pitavastatin and prednisolone, or pitavastatin and bisphosphonates, show significant synergies in ovarian cancer cells [242], pitavastatin and dacarbazine combination therapy induces apoptosis and autophagy leading to synergistic cytotoxicity in melanoma cells [496]. Thus, the work in this chapter describes the exploration of a novel pitavastatin drug combination.

There are several reasons to believe that drug combinations can be particularly useful in treating cancer. First, tumours are a heterogeneous group of diseases whose development involves different pathological mechanisms [497]. As a result, drug combinations that inhibit different fundamental pathways could be potentially more effective than single agents because drugs could simultaneously influence different cell populations [498]. Second, drug combinations can simultaneously affect different signalling pathways in individual cancer cells. Such medications may act synergistically to increase the effectiveness of the treatment above that of the single agents. Third, mutation and epigenetic changes may trigger numerous compensatory pathways in cancer cells during cancer pharmacotherapy, leading to the development of drug-resistant subpopulations. Therefore, drug combinations or the use of multi-targeted drugs can provide an opportunity to achieve an improved clinical response [499], [500]. Finally, the use of combinations of drugs has a historical precedent, and many conventional chemotherapy regimens include several distinct drugs[494].

The elevated price of new molecularly targeted agents in most nations is likely to place a significant burden on healthcare budgets [501]. It takes a long time and a significant cost to develop a new drug and translate it to the clinic. It is presently estimated that the discovery, development and marketing of a fresh medication requires roughly $2 billion expenditure. This results in an exceptionally elevated price for the new targeted therapy, with annual treatment expenses often ranging from $50,000 to $100,000 per treatment course [502]. Since 1950, every decade the amount of newly licensed drugs has dropped by half. A recent study defined the drug development process as a crisis of efficiency. The crisis is especially difficult for the discovery of oncology drugs. Between 2003 and 2011, the number of non-oncological drugs authorized by the FDA was twice the number of oncological drugs that were approved [502]. At the same time, the price of developing cancer drugs has risen dramatically from 1990 to 2011[501]. Furthermore, there is significant attrition during drug development, with approximately only 10% of new cancer drugs that enter clinic trials eventually receiving marketing authorization. The cost of these failures must be covered by the profit derived from successful drugs, further elevating the cost of the latter[501]. Therefore, drug repurposing is an attractive alternative approach to developing new treatment options.

Drug repurposing, also referred to as redeployment, could be described as the process to validate and market new indications for a drug beyond the scope of the original medical use [503], [504]. It provides a strong clinical impact with low development costs relative to *de novo* drugs [504]. The goal of oncology pharmaceutical companies is to gain market acceptance by demonstrating the efficacy and superiority of their drugs. Pharmaceutical companies may lack incentives to invest in drug combinations, as this may entail negotiating with competing pharmaceutical companies. In the case of drug repositioning, there may be only a limited period of patent protection for their product, and as a result economic returns are reduced [505].

Indeed, the cost of clinical trials may not be covered by the brief period of financial returns for the repurposed drugs. Nonetheless, drug repositioning could benefit from safer, cheaper and faster validation protocols [501]. Repurposing accepted medication with a history of clinical application often provides a wealth of easily accessible data that includes guidelines of pharmacokinetics, pharmacodynamics, toxicology and dosage. The availability of such information may reduce the duration of clinical development of prescription products which is otherwise usually 5 to 7 years [506]. Another major benefit to healthcare systems of repurposing drugs is the use of low-cost generic drugs. Once the period of patent protection ends, drugs are often sold in a "generic" format at greatly reduced cost. This decreases the potential high costs of care that can put a significant burden on the government health finances of advanced countries and can be cheap for poor and middle-income countries[502]. However, it does raise the question of who would pay for clinical trials of generic drugs.

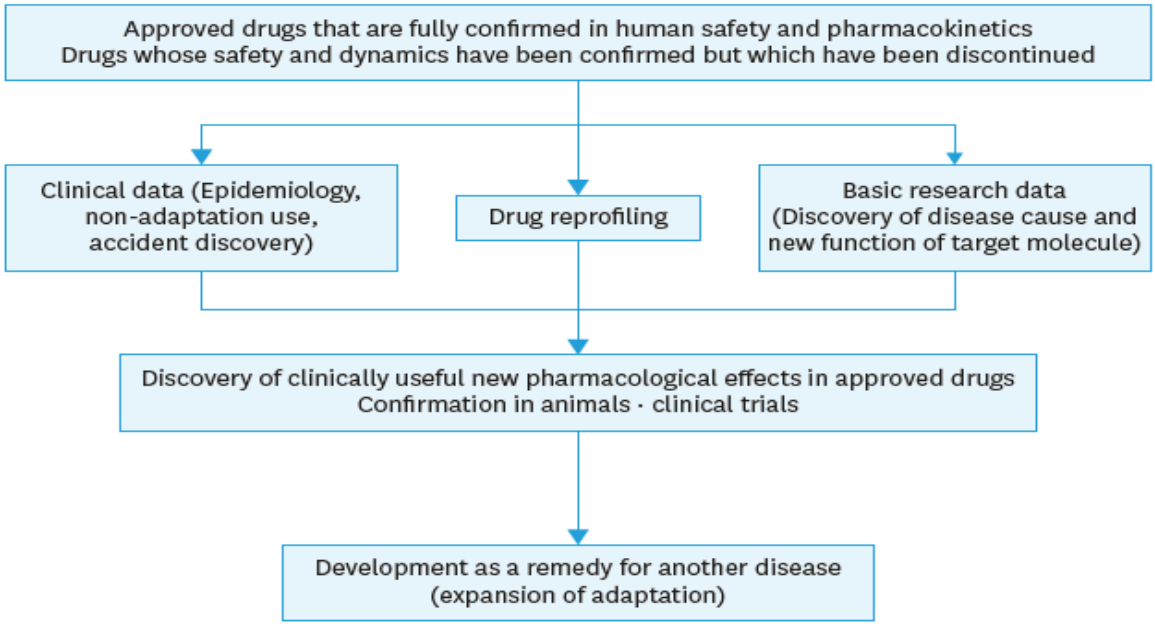


Figure (6-1): Shows the principle of drug repurposing [507].

There are many examples of successful drug repositioning which circumvent the obstacles associated with new drug development and speed up therapeutic discovery [508]. One well known example of drug repositioning is "The fall and rise of thalidomide," a drug that was originally prescribed for pregnant women to manage morning sickness in 1957 in Germany and

England [509]. At least 15,000 infants were born to mothers who had used thalidomide during the first trimester of pregnancy. Unfortunately, the medication resulted in serious deafness, blindness and deficiencies in the growth of limbs. However, it was later accidentally found that thalidomide was effective in the treatment of leprosy inflammatory disease` erythema nodosum laprosum (ENL). The medication was administered to relieve the pain of critically ill patients with ENL at the University Hospital in Marseille. Not only did the drug ease the pain, but it also healed the patient's sores [506]. As a result, a follow-up study funded by the WHO of over 4000 ENL patients reported full remission of disease within less than two weeks [510]. Thalidomide was found to inhibit the development of fresh blood vessels in the corneal model of the rabbit, which explains the teratogenic effects of this drug [511]. In 1994, the antiangiogenic properties of thalidomide allowed it to be redeployed as a candidate for oncology at the Children's Hospital in Boston and this opened the way for its use in cancer therapies, including for the treatment of multiple myeloma and breast cancer [506]. Several other effective drug repositioning attempts have also been successful, such as aspirin, an antagonist of cyclooxygenase, for the treatment of coronary artery disease, sildenafil, a phosphodiesterase blocker first formulated to treat hypertension and angina and then repurposed to treat erectile dysfunction, and antibiotic erythromycin now used in increasing of stomach motility [512], [513]. Several studies have shown that statins hinder cell growth and induce apoptosis *in vitro* in cell lines from a number of cancers [364], [514], [515]. Statins have also been found to inhibit the growth of xenograft tumours in mice [516]. Recently, our laboratory data has shown that pitavastatin induces tumour regression in mice with a controlled diet [308]. The work described in earlier chapters was designed to support the repurposing of pitavastatin to treat cancer. Nonetheless, for an adequate plasma concentration of the drug in patients, comparatively high doses of statins are likely to be needed [517], [518]. This raises concerns about the potential danger of myopathy, which is often associated with statin use [519]. One possible solution to this is to identify drugs that synergize with statins and possibly

reduce the statin dose required for patients. This could potentially reduce the chance of patients developing myopathy. It is particularly appealing to repurpose other drugs to combine with pitavastatin to achieve this.

6.1. Ivermectin

Ivermectin is a major anti-parasitic drug first recognized in the 1970s. Ivermectin's composition is that of a macrocyclic lactone, and *Streptomyces avermitilis* naturally produces the chemical in soil[520]. This compound was first isolated from a soil sample from a Japanese golf course[521]. Although originally considered solely for veterinary uses, the research team rapidly evaluated its potential for human use and in 1987 this led to authorization of the drug by the French regulatory authorities. Since then, ivermectin has been recognized as an extremely safe and dramatically effective drug in onchocerciasis therapy[522]. Case studies and some formal studies have also demonstrated the usefulness of the drug in the treatment of numerous systemic and cutaneous parasite diseases, including filariasis, skin larvae, scabies and pediculosis[520]. Ivermectin was approved by the United States Federal Administration of Food and Drugs (FDA) for onchocerciasis and strongyloidiasis in 1996 [523]. Ivermectin has also been used to treat livestock and a trial in 1981 by Dr. Mohamed Aziz in Dakar, Senegal[524] demonstrated its effectiveness in treating river blindness in animals[525].

6.1.1. Ivermectin Uses in Veterinary and Human Medicine

Ivermectin is one of the most effective medicines for the prevention of parasitic infection in veterinary and human medicine and was its discoverer was the recipient of the Nobel Prize in Physiology or Medicine 2015, some 35 years after its remarkable discovery[526]. Ivermectin’s effectiveness against both endoparasites and ectoparasites led to the creation of the word 'endectocide' and in 1981 Merck & Co. launched this first drug of its kind to the animal health industry [527]. The global community mobilised in the mid-1970s to combat the major

problems of neglected tropical diseases. The UN-based Special Program for Research & Training in Tropical Diseases (TDR) was established in 1975 after the establishment of the OCP in 1974 [520]. Onchocerciasis, one of two filarial infections among the eight target diseases of the TDR, was a major public health issue that affected 20-40 million people in endemic areas. Ironically, ivermectin has proved to be even more of a' wonder drug' in human health, nutritional improvement, overall health and well-being of billions of people around the world since it was first used to human use in 1988 to treat onchocerciasis[527]. Furthermore, it has proven to be ideal in many respects, highly effective and with a broad range of activity, well tolerated and easy to administer (a single annual oral dose). It is used to treat a number of internal nematode infections, including Onchocerciasis, Strongyloidiasis, Ascariasis, Cutaneous Migrants, Filariasis, Gnathostomiasis and Trichuriasis, as well as oral treatment of ectoparasitic infections such as Pediculosis (lice infestation) and Scabies (mite infestation)[520], [524], [527].

6.1.2. Mechanism of action of Ivermectin

The antiparasitic effect of ivermectin is thought to depend on it acting as an agonist of peripheral chloride ion-channels. The literature contains conflicting information whether its effects are mediated by glutamate or glycine-gated chloride channels of parasites [528] [529]. In either case, ivermectin simulates the ligand by ' opening the door ' and allowing an efflux of chloride ions to release the associated neurotransmitter, γ-aminobutyric acid (GABA) (hyper polarization mimicks GABA) of parasite [529]. Continuous neuronal discharge at high doses is supposed to fully paralyze the parasite; nevertheless, this is not certain to be the mode of action in parasites susceptible to ivermectin. Other authors believe that ivermectin primarily interferes with the gastrointestinal tract in the parasite [530], causing the parasite to starve to death. Ivermectin is a powerful antiviral drugs, ivermectin is a potent inhibitor of flaviviral replication by targeting the activity of NS3 helicase [531]. Ivermectin, approved by the FDA, inhibits

SARS-CoV-2 replication *in vitro* [532]. It has also been proposed as an anticancer drugs [533]–[535]. Ivermectin interacts with many cancer pathways and these are summarizedin figure (6-2) [534].

1. Ivermectin inhibits the cancer cell's P-glycoprotein pump, which causes a multidrug resistance phenotype. 2. Ivermectinopens chloride channels to induce apoptosis which death of osmotic cells. 3. By decreasing the mitochondrial complex I function, ivermectin restricts the electronic movement of the oxidative phosphorylation pathway, which stimulates the rate of oxygen consumption to produce ATP for the cell. Concomitantly, Akt's phosphorylation levels are reduced, impacting in the biogenesis process of the mitochondrial. In addition, changes in the mitochondrial machinery are associated with increased levels of reactive oxygen species, which damage DNA. 4. Ivermectin stimulates immunogenic cell death (ICD) by enhancing a microenvironment enriched with ATP-and HMGB1 which promotes inflammation. This drug also increases the sensitivity of ATP and calcium signals in membrane receptors P2X, especially P2X4 and P2X7, to induce immune responses based on ATP. 5. Ivermectin encourages the kinase PAK1 poly-ubiquitination, which leads it to proteasome degradation. In effect, defective PAK1 inhibits the Akt / mTOR pathway, at the same time, ivermectin activates Beclin1 and Atg5 expression, both linked to autophagy induction, and decreases the activity of the negative apoptosis regulator Bcl-2. It causes autophagy and apoptosis, combined. 6. Ivermectin suppresses AXIN2, LGR5 and ASCL2, all of which are positive WNT-TCF regulators thus supporting the WNT signal repressor FILIP1L. As a result, ivermectin promotes the expression of many genes linked to IFN, such as IFIT1, IFIT2, IF144, ISG20, IRF9 and OASL. 7. Ivermectin alters the epigenetic characteristic and self-renewal process in the malignant cell because of the need to to mimic the SIN3 interaction binding to the PAH2 pattern of cancer-associated deregulators SIN3A and SIN3B. SIN3A naturally activates NANOG and SOX2 which are pluripotence stimulants for stem cells. 8. Ivermectin restricts the activity of the NS3 and DDX23 RNA helicases, both relevant to ribosome biogenesis and post-

transcriptional modifications, as well as to mRNA degradation. DDX23 functions as a miR-21 promoter and is a well-recognized tumour progression stimulator. 9. Ivermectin inhibits the CSC population ideally, and up-regulates the NANOG, SOX2 and OCT4 genes for pluripotency and self-renewal. IVM: ivermectin; ATP: triphosphate adenosine; OCR: intake rate of oxygen; ROS: reactive oxygen species.

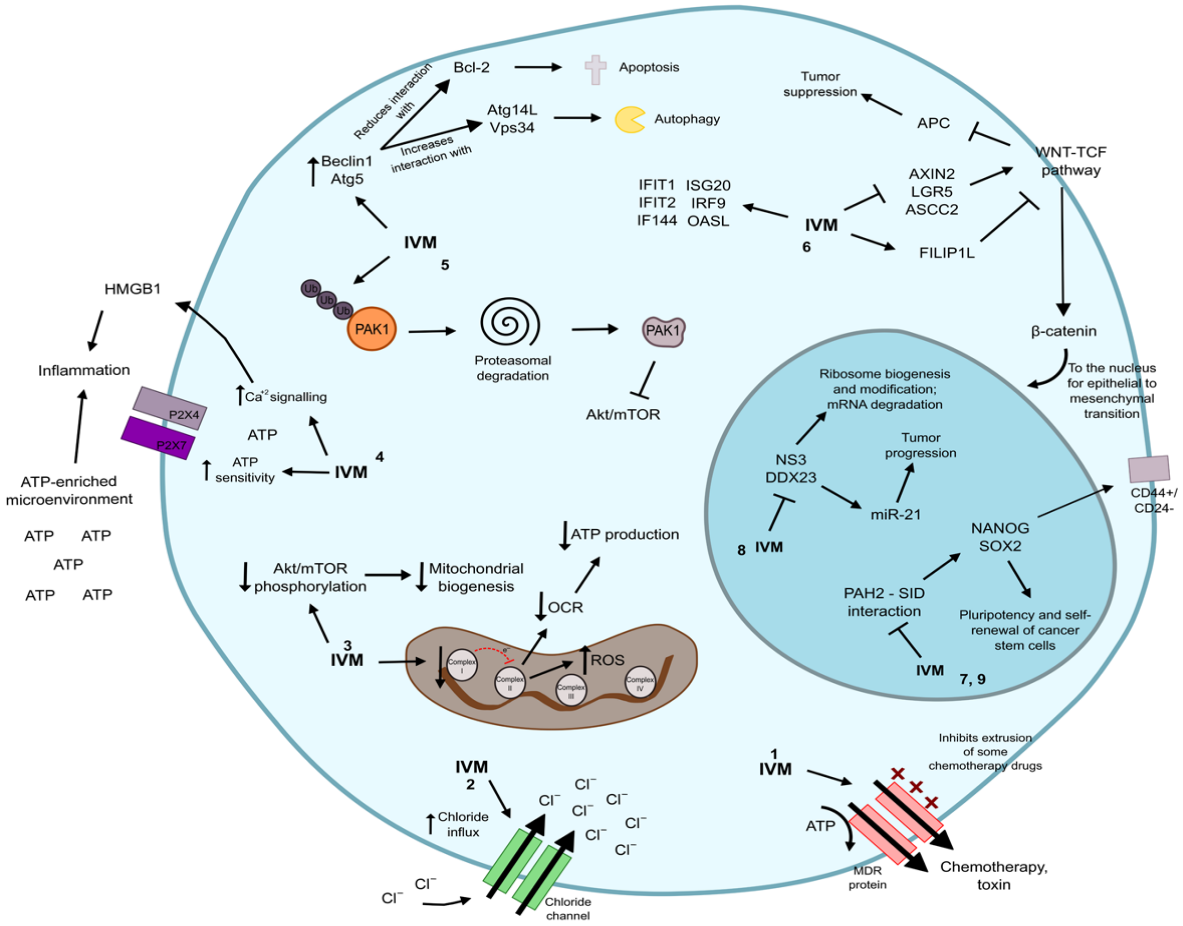


Figure (6-2): Mechanisms of repositioning antitumour for ivermectin [534]

Considering the potential for ivermectin to act as an anti-cancer agent prompted consideration whether it would synergize with pitavastatin and the research described in this chapter set out to test that hypothesis.

6.2. Results

6.2.1. Testing the activity of Ivermectin on the ovarian cancer cell lines

Six ovarian cancer cell lines (COV318, FUOV-1, COV362, OVCAR-4, OVCAR-8 and OVSAHO) were used to evaluate the anticancer activity of ivermectin. When tested as a single agent ivermectin inhibited the growth of all six cell lines (table 1) with comparable potencies, IC_{50}s varying from 13 to 16 μM, table (6-1) and fig. (6-1).

Table (6-1): Potency of ivermectin in cell growth assays with ovarian cancer cells. The IC_{50}s of ivermectin as a single agent for inhibition of the growth of six ovarian cancer cell lines are reported (mean ± S.D., n=3).

Cell line	Ivermectin IC_{50}s (μM)
Cov318	**14. 6 ± 2**
Fuov-1	**12.9 ± 2.2**
Cov362	**14.6 ± 1.3**
Ovcar-4	**11.9 ± 0.9**
Ovcar-8	**15.8 ± 1.2**
Ovsaho	**14.5 ± 1.9**

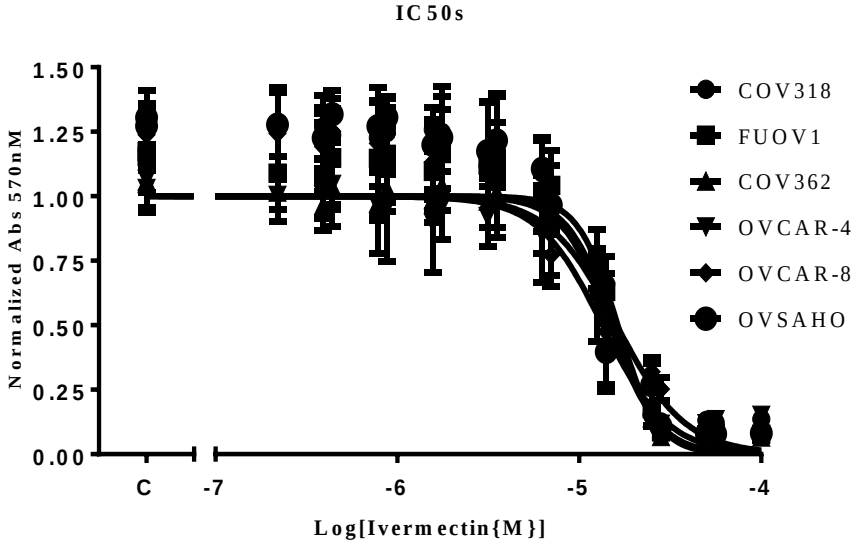
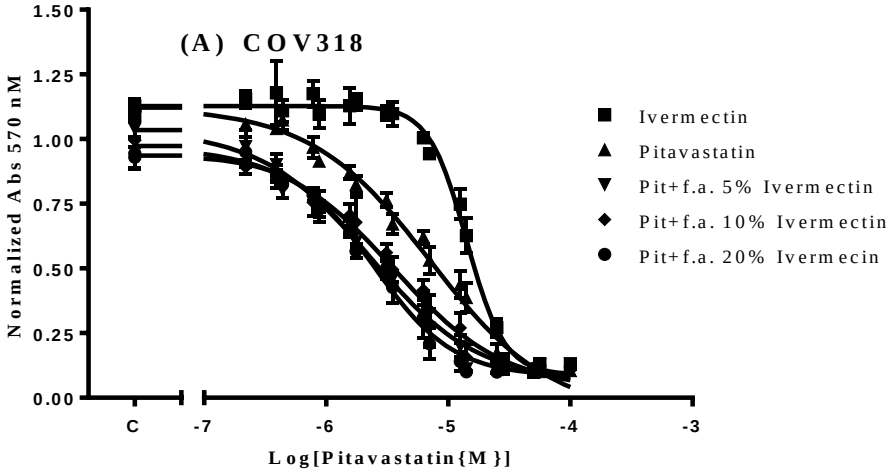
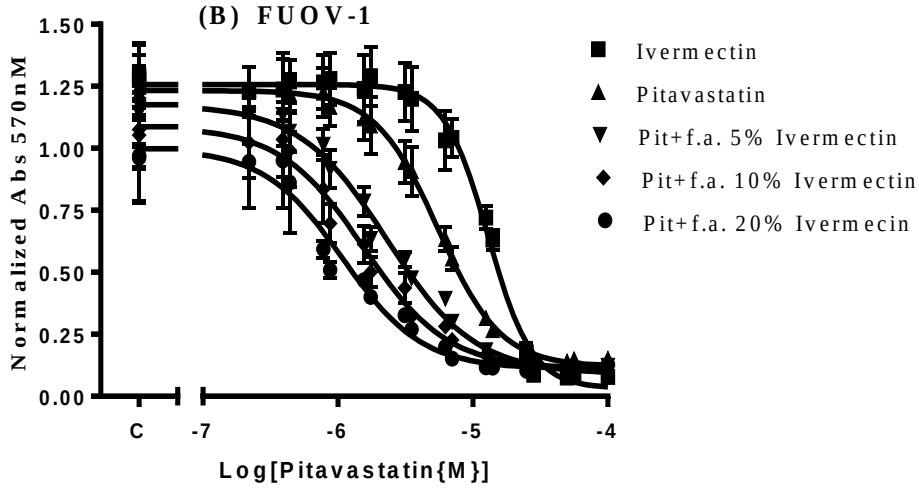


Figure (6-3): Effect of ivermectin on ovarian cancer cell lines. Cells were exposed to a range of concentrations of pitavastatin for 72 hours, except for the slow growing cell lines Cov-318 and Cov-362 which were exposed for 120 hours. The numbers of surviving cells were estimated using SRB assay. Dose response curve generated by curve fitting curve fitting and the results are expressed (mean ± SD, n ≥ 3) as a fraction of the top of the curve in each experiment. "C" on the x-axis indicates control samples measured in the absence of the drug.

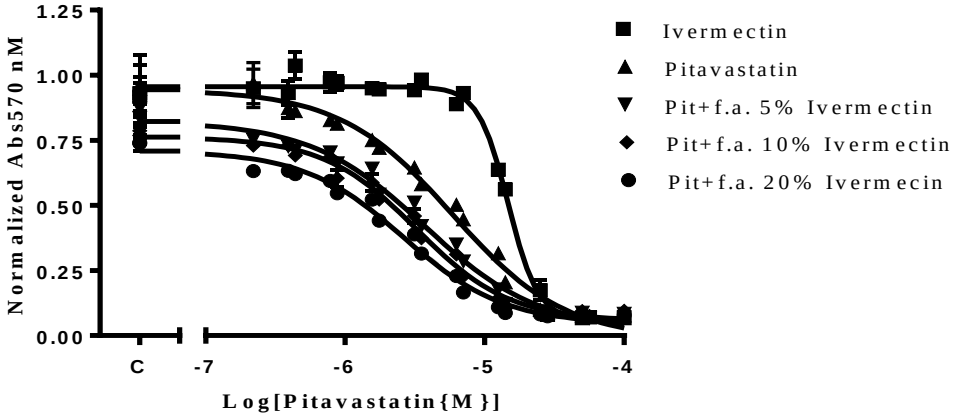
6.2.2. Confirmation of antiproliferative combination effect of pitavastatin with fixed doses of Ivermectin

To measure the activity of pitavastatin in combination with ivermectin the ovarian cancer cells were exposed to the indicated concentration of pitavastatin alone and in combination with fixed concentration of ivermectin for 72 hours or 120 hours (for COV318 and COV362) and the cells were stained using SRB. The concentrations of ivermectin used were those which on their own had 5%, 10% and 20% effect in the cell growth assays (referred to as f.a. (fraction affected) = 5%, 10%, or 20%).

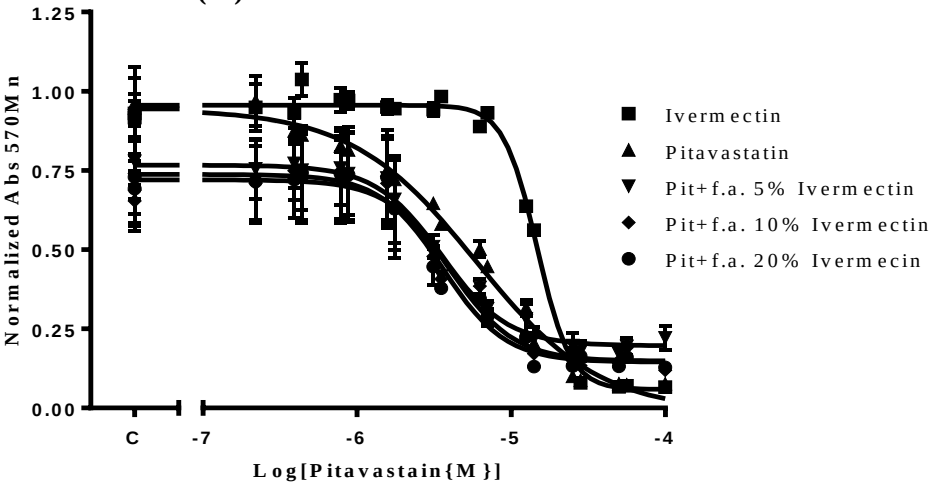




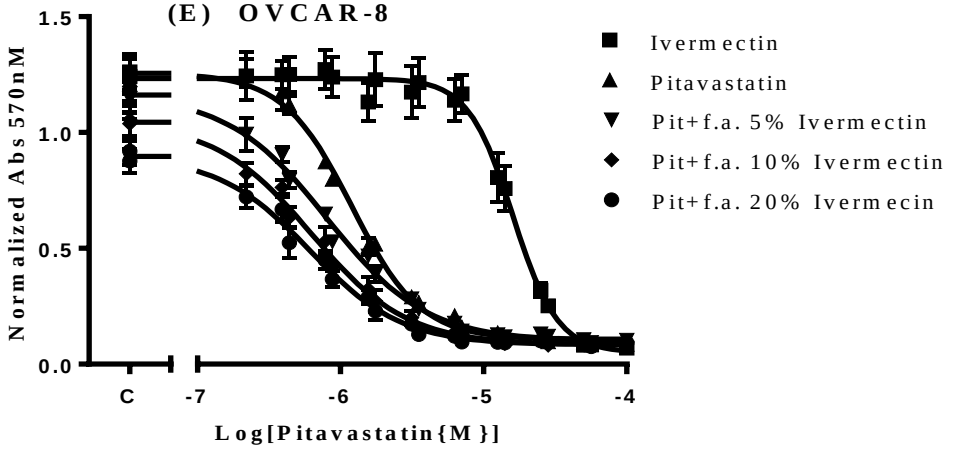
(C) COV362

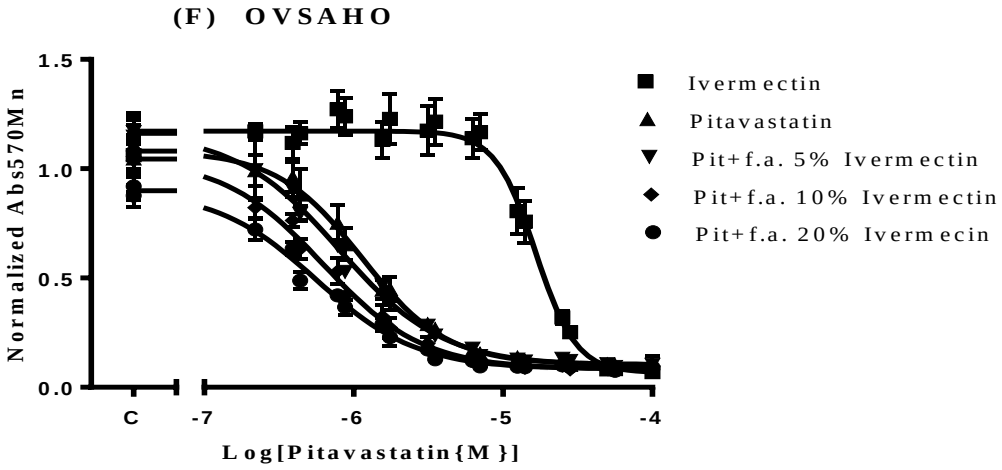


(D) OVCAR-4



(E) OVCAR-8





Figures (6-4): Effect of ivermectin combined with pitavastatin in ovarian cancer cell lines. (A) COV318, 5-3 (B) FUOV-1, 5-4 (C) COV362, 5-5 (D) OVCAR-4, 5-6 (E) OVCAR-8, 5-7 (F) OVSAHO). Cells were exposed to a range of concentrations of pitavastatin for 72 hours, except for the slow growing cell lines Cov-318 and Cov-362 which were exposed for 120 hours. The numbers of surviving cells were estimated using SRB assay. Dose response curves were generated by curve fitting curve and the results are expressed (mean ± SD, n ≥ 3) as a fraction of the top of the curve in each experiment. "C" on the x-axis indicates control samples measured in the absence of the drug. The concentration of ivermectin is denoted by 5%, 10%, 20%, referring to the fraction of cells affected and is summarized in table (6-2).

Pitavastatin combined with ivermection showed synergistic activity in 3 out of 6 cell lines tested (COV318, COV362 and FOUV1) at all concentration fraction of ivermection tested, Additive activity was observed in the three other cell lines (OVCAR-4, OVSAHO and OVCAR-8) at the same effective ivermectin concentrations (figures (6-5), (6-6) and (6-7)). These data indicate that ivermectin could be an effective choice for combining with pitavastatin, and led to further studies focusing on this combination.

Table (6-2): Ivermectin concentrations which had 5%, 10% and 20% effect on their (i.e. the fraction affected was 5%, 10%, 20%)

Cell lines	f.a. = 5%	f.a. = 10%	f.a. =20%
Cov318	4.94 µM	6.61 µM	8.97 µM
Fuov-1	4.35 µM	6.15 µM	8.81 µM
Cov362	4.5 µM	5.97 µM	8.01 µM
Ovcar-4	4.16 µM	5.88 µM	8.43 µM
Ovcar-8	4.56 µM	6.1 µM	8.3 µM
Ovsaho	4.55 µM	6.09 µM	8.25 µM

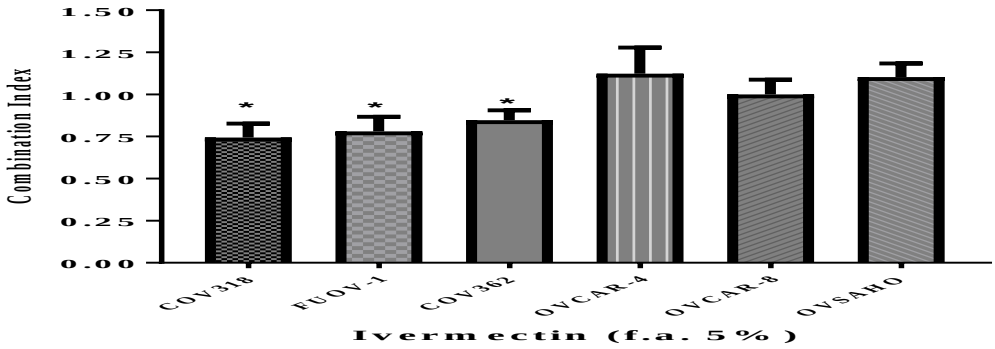


Figure (6-5): Evaluation of the combination of pitavastatin and ivermectin in cell growth assays. The indicated cells were simultaneously exposed to a range of pitavastatin concentrations with a fixed concentration with of ivermectin which, on its own, inhibited cell growth by 5% (denoted as fa 5%).Combination indices (CI) (mean ± S.D., n=3-4) are quoted at a fraction affected of 0.5 and differed significantly from unity where indicated (*, $P \leq 0.05$; paired *t*-test).

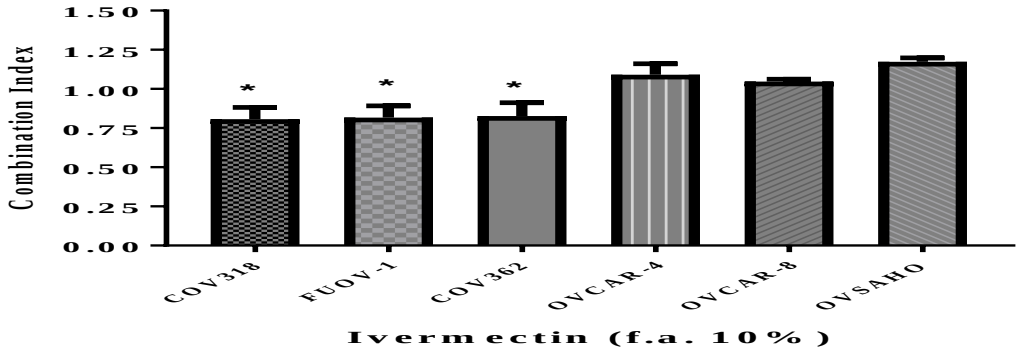


Figure (6-6): Evaluation of the combination of pitavastatin and ivermectin in cell growth assays. The indicated cells were simultaneously exposed to a range of pitavastatin concentrations with a fixed concentration with of ivermectin which, on its own, inhibited cell growth by 10% (denoted as fa 10%).Combination indices (CI) (mean ± S.D., n=3-4) are quoted at a fraction affected of 0.5 and differed significantly from unity where indicated (*, $P \leq 0.05$; paired *t*-test).

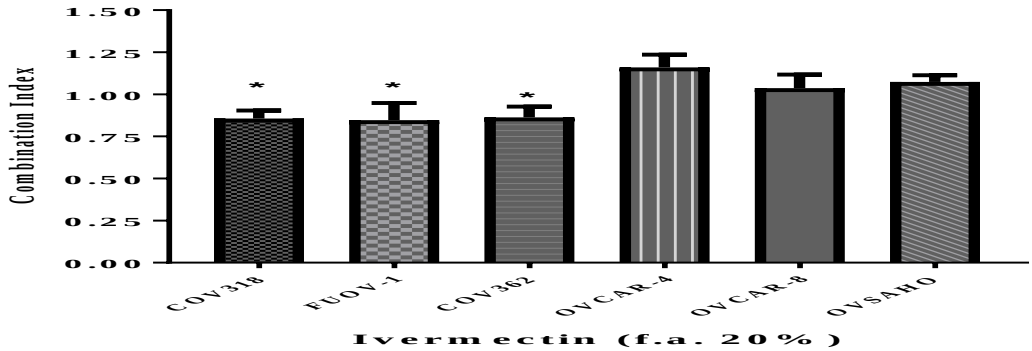


Figure (6-7): Evaluation of the combination of pitavastatin and ivermectin in cell growth assays. The indicated cells were simultaneously exposed to a range of pitavastatin concentrations with a fixed concentration with of ivermectin which, on its own, inhibited cell growth by 20% (denoted as fa 20%).Combination indices (CI) (mean ± S.D., n=3-4) are quoted at a fraction affected of 0.5 and differed significantly from unity where indicated (*, $P \leq 0.05$; paired *t*-test).

6.2.3. Cell viability assay after combination treatment

The effect of the drug combinations on cell viability was further assessed by trypan blue staining (figures (6-8, 6-9, 6-10)) to validate the previous results and to confirm that the drug combination induced cell death. These experiments were performed in the cells in which synergy was observed in the cell growth assays. The findings were generally consistent with those obtained in cell growth assays. Synergy was observed in COV318, COV362, and FUOV-1 cells with all the concentrations of ivermectin that were tested. The results showed significantly more cells death than the expected effect if the drugs were additive; the effect if the drugs combined additively was estimated by the Bliss independence criterion.

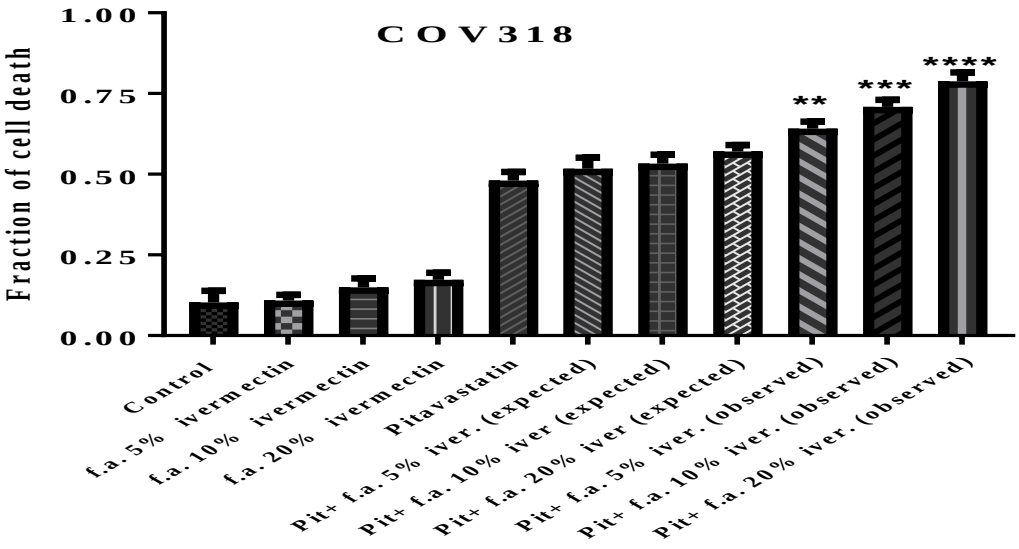


Figure (6-8): The effect of ivermectin in combination with pitavastatin on cell toxicity in COV318 cell lines were exposed to pitavastatin at a concentration equal to its IC50 (6.2 μM) with/without ivermectin at a concentration which affected 5% (4.94 μM), 10% (6.61 μM) or 20% of the cells The number of dead cells (mean ± S.D., n=3) was determined by staining with trypan blue and the percentage of viable cells calculated. There were significantly more dead cells in samples exposed to the combination than would be expected if the drugs acted additively (estimated using the Bliss independence criterion) (paired t- test, *, $P < 0.05$).

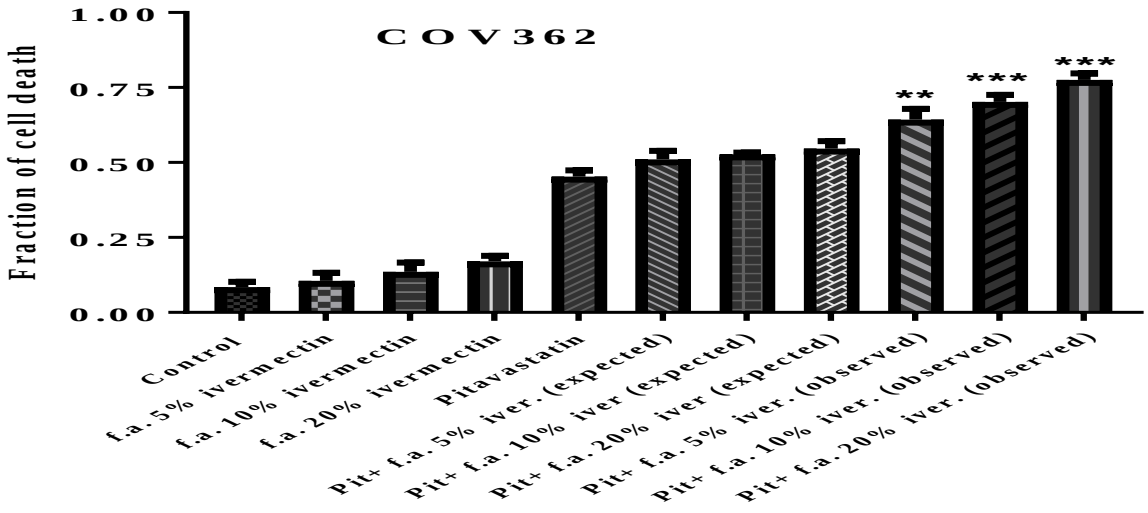


Figure (6-9): The effect of ivermectin in combination with pitavastatin on cell toxicity in COV362 cell lines COV362 cell line were exposed to the IC50 of pitavastatin (6.2 μM) with/without 5% (4.94 μM), 10% (6.61 μM) from IC50 of ivermectin after 72 hours the dead cells (mean ± S.D., n=3) was determined by staining with trypan blue and the percentage of viable cells. There were significantly less viable cells in cells treated with pitavastatin alone compared to the cells treated with 5% (4.94 μM), 10% (6.61 μM) from IC50 of ivermectin (paired t- test, *, $P < 0.05$).

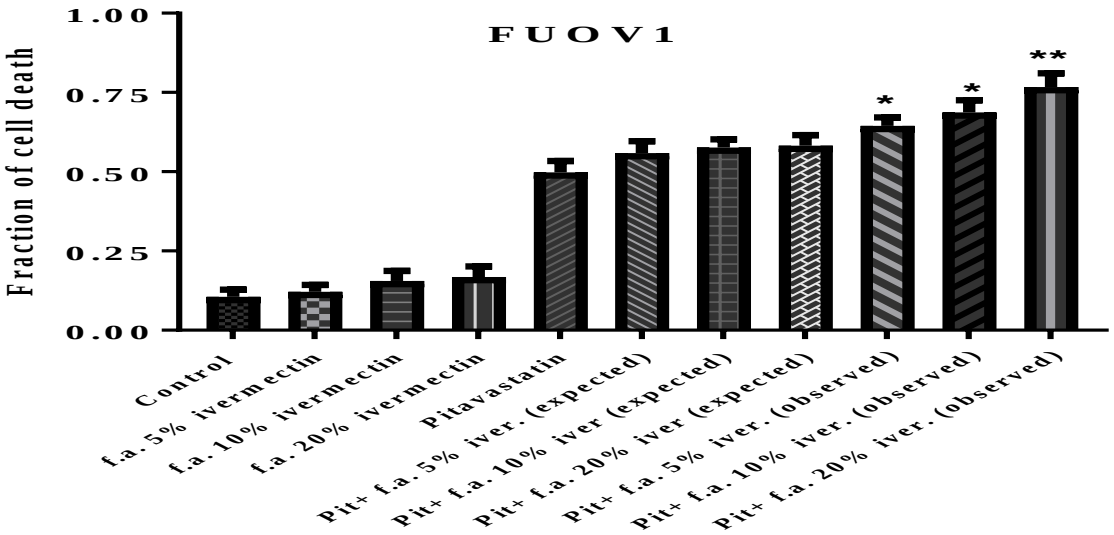


Figure (6-10): The effect of ivermectin in combination with pitavastatin on cell toxicity in FOUV-1 cell lines, FOUV-1 cell line were exposed to the IC50 of pitavastatin (6.2 μM) with/without 5% (4.94 μM), 10% (6.61 μM) from IC50 of ivermectin after 72 hours the dead cells (mean ± S.D., n=3) was determined by staining with trypan blue and the percentage of viable cells. There were significantly less viable cells in cells treated with pitavastatin alone compared to the cells treated with 5% (4.94 μM), 10% (6.61 μM) from Ic50 of ivermectin (paired t- test, *, $P < 0.05$).

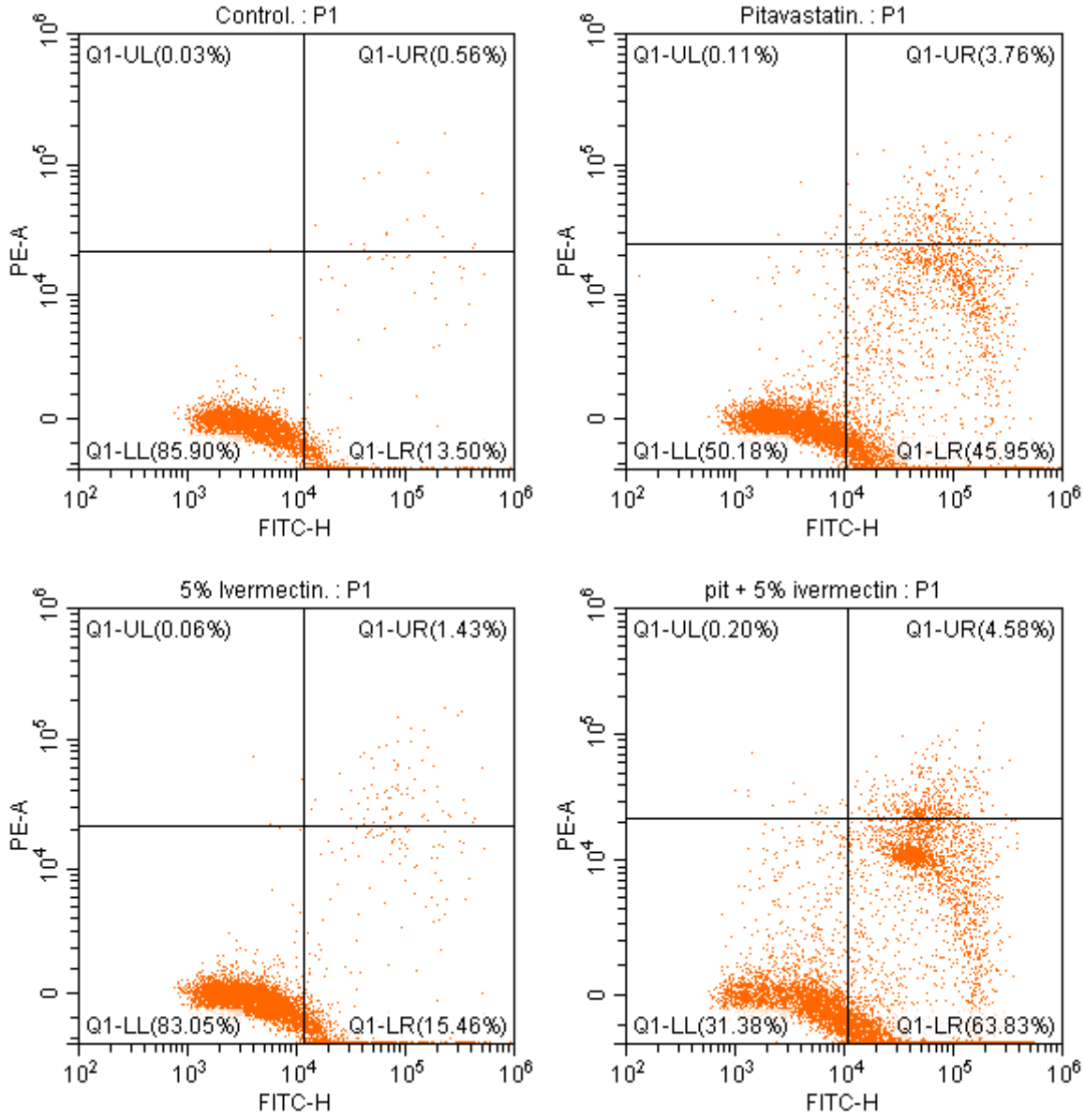
6.2.4. Caspase 3/7 activity assay

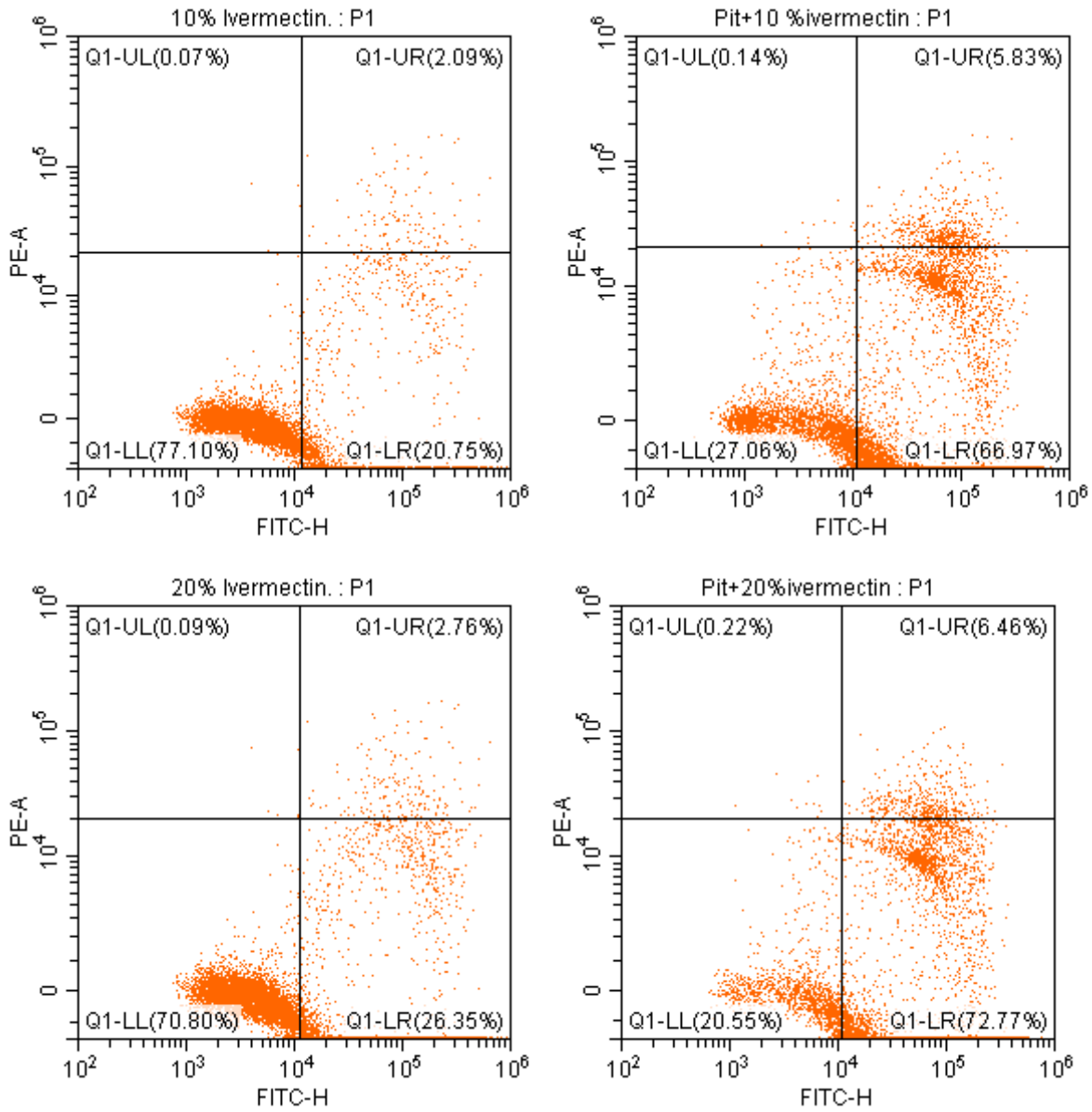
Previous work has shown that pitavastatin causes apoptosis. To determine if the combination of pitavastatin and ivermectin causes apoptosis, the effect of the combination on caspase-3/7 activity was determined. Activation of caspase 3/7 by the combination was reminiscent of the effect measured in the trypan blue assay, and was greater than that of the effect of pitavastatin alone. In these experiments, the additive effect could not be estimated by the Bliss independence criterion because the maximum caspase activity is difficult to determine (it is transient and the time at which peak activity is observed is not predictable). Consequently, the effect of the combination was compared to the effect of pitavastatin alone. This seems reasonable considering that ivermectin on its own had no effect on caspase-3/7 activity (fig 6-11). In COV318 cells, the combinations of ivermectin with pitavastatin showed significantly increased caspase-3/7 activity.

Figure (6-11): Relative caspase 3/7 activity in COV318 cells. Cov318 cells were exposed to pitavastatin (1&2 µM) with/without 5% (4.94 µM), 10% (6.61 µM) from IC50 of ivermectin and after 72 hours' caspase 3/7 activity was measured. To control for potential effects of the drugs on cell number after 72 hours, Caspase 3/7 activity was normalised to the number of surviving cells estimated at the same time by staining a separate plate with SRB. The results are expressed as a fraction of that measured in untreated cells (mean ± SD, n=3) alone. The results were significantly different from those in cells treated with pitavastatin alone where indicated (*, P< 0.05, **, P<0.01, paired t-test).

6.2.5. Confirmation of the synergy between Ivermectin and Pitavastatin by flow cytometry

To confirm the effect of the drug combination on apoptosis, the effect of the combination was also measured by flow cytometry to measure apoptosis by Annexin V/propidium iodide staining. In these experiments the results were again compared to the effect of pitavastatin alone because of the difficulty of knowing how to apply the Bliss criterion when there are four potential outcomes (live, early apoptotic, late apoptotic or necrotic cells). The drug combination resulted in significantly more early and late apoptotic cells, and significantly less cells alive, than in the samples in which the cells were exposed to pitavastatin alone (figure (6-13)).





Figures (6-12): The effect of ivermectin combinations on annexin V/propidium iodide staining Cov318 cells were treated with the indicated concentration of ivermectin with/ or without pitavastatin (8.2 μM) for 48 hours. The cells were labelled with annexin V and propidium iodide and analysed by flow cytometry, (Q1-UL= necrotic cells, Q1-LL = live cells, Q1-LR= pre – apoptotic cells, Q1-UR= apoptotic cells). The results shown in the figures are representative of 3 experiments. The results are quantified in figure (5-5).

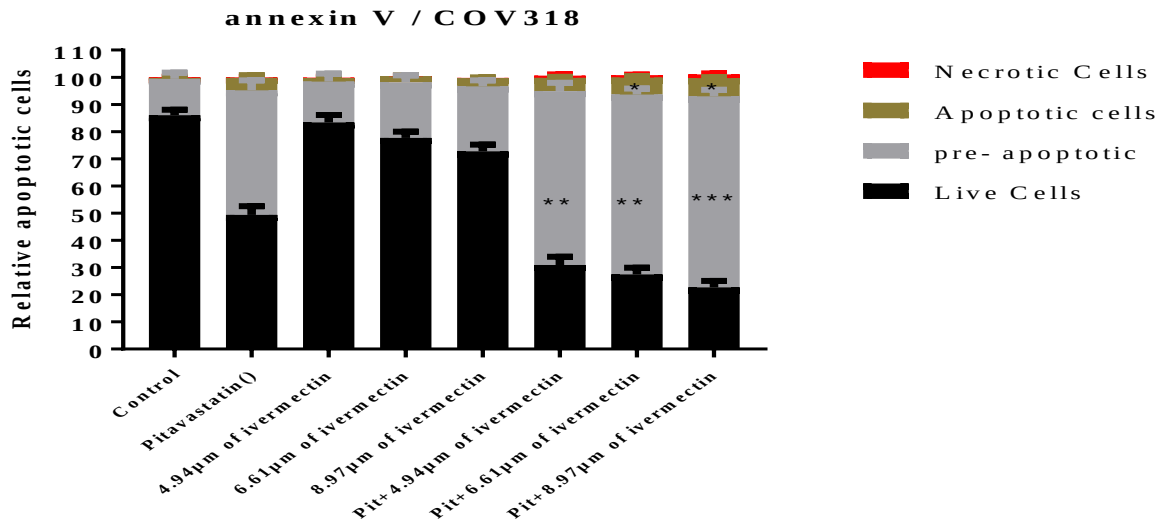


Figure (6-13): The effect of ivermectin combinations on annexin V/propidium iodide staining Cov318 cells were treated with the indicated concentration of ivermectin, with/without pitavastatin (8.2 μM) for 48 hours, the cells were labelled with annexin V and propidium iodide and analysed by flow cytometry, the results are expressed as a fraction of that measured in untreated cells (mean ± SD, n=3) alone. The results were significantly different from those in cells treated with pitavastatin alone where indicated (*, P< 0.05, **, P<0.01paired t-test). Note: the necrotic cells not be reduced but it's very little numbers.

6.3. Discussion

Failure to treat cancer poses a major challenge for modern medicine[191]. Sadly, the efficacy of chemotherapy is limited by side effects, damage to normal tissue and drug resistance. Multi-drug regimens for cancer are therefore used to boost effectiveness and the need for large doses of drugs [536]. Simultaneously, it is well recognized that cancer is a heterogeneous group of diseases with different pathogenesis molecular mechanisms, it is therefore advantageous to target multiple pathways to inhibit tumour growth and enhance survival [537], [538]. This study indicated that one way to potentiate the cytotoxic activity of statins in OC is to use a combination of pitavastatin and ivermectin. While repurposing statins is attractive, legitimate concerns have been raised about the potential for causing myopathy [519], [539]. This makes it important to identify drugs that may reduce the dose of pitavastatin used in patients to reduce the risk of myopathy.

Although ivermectin was investigated as a potential addition to pitavastatin, like pitavastatin, ivermectin as a single agent inhibited the growth of ovarian cancer cell lines but it was slightly less potent than pitavastatin. Ivermectin showed comparable potency as a single agent in all the cell lines tested. According our evidence, the concentration of ivermectin that we used in table (6-1) to get synergy is achievable in patient's plasma or within a normal dose to use it as a single agent even though the wide margin of safety of ivermectin[540] therefore, it could achieve the IC50s concentration in patients and higher doses is possible.

In this study, ivermectin was observed to act synergistically with pitavastatin using several different assays using three different cell lines, namely COV318, COV362, FUOV1. In contrast, there was an apparently additive interaction observed in cell growth assays using OVCAR-4, OVCAR-8 and OVSAHO cells. Synergy between pitavastatin and ivermectin was also observed using two other assays in COV362, FUOV1 and COV318 cells. Ivermectin potentiated the activity of pitavastatin in inducing apoptosis, assed by measuring caspase-3/7 activity and annexin V/propidium iodide labelling. These later studies were only performed with COV318 cells because significant synergy was observed with these cells in previous experiments. Tremendous morphological changes of all treated cells were also evident under the light microscope. The fact that synergy was observed between pitavastatin and ivermectin using several different assays give confidence that this observation is robust. It would have been helpful to test the combination in OVCAR-4, OVCAR-8 and OVSAHO cells as well, but time did not permit this. It would also be worthwhile to investigate different periods of drug exposure in case different cells exhibit different kinetics of cell death.

The studies presented here did not address the mechanistic explanation for synergy between pitavastatin and ivermectin. Pitavastatin inhibits HMGCR, thus reducing the supply of mevalonate used for isoprenoid synthesis. Ivermectin has not been reported to directly affect

the mevalonate pathway. Thus, this is unlikely to be the mechanism underlying the synergy between these two drugs. However, this is significant because it gives a good indication that ivermectin is unlikely to increase the risk of myopathy.

Ivermectin inhibits multiple pathways which contribute to cancer cells growth figure (6-2). One of these includes YAP-1, and this is particularly important because it is inhibited by both lipophilic statins [541] and ivermectin [542], [543]. A library screen for YAP1 targeting compounds demonstrated that ivermectin has antitumour activity through dysregulated signalling of YAP1/TAZ and TGF-β which mediates hepatocarcinogenesis in Mob1a/1b deficient mice[542], [543]. Thus, this may be one pathway that is inhibited by two drugs and which leads to synergy.

It has also been shown that ivermectin has anti-ovarian cancer KPNB1-dependent gene properties, KPNB1 in a nuclear transporter and its inhibitors exert antitumor activity through the arrest and activation of multiphase cell cycles and apoptosis [544]. Ivermectin was observed to block KPNB1 function. potentially providing a further mechanism for the anti-cancer activity of ivermectin[544]. Interestingly, ivermectin showed antitumor effects on epithelial ovarian cancer via KPNB1-dependent, acting as an alternative treatment for patients with higher rates of KPNB1 expression with earlier recurrence and better prognosis than those with lower levels of KPNB1 expression [544]. It was found that the combination of ivermectin and paclitaxel had a greater anti-tumour effect on OC *in vitro* and *in vivo* than any single drug[507], [544], Thus, it is possible that inhibition of KPNB1 by ivermectin contributes to the synergy observed with paclitaxel.

Ivermectin has already been explored as an anti-cancer agent by others and this may help understand the basis for synergy between pitavastatin and ivermectin. For example, ivermectin has been shown to suppress the development of human melanoma and several other cancer

xenographs in mice with no adverse effects [545]. However, the mechanism by which ivermectin sensitizes cells to pitavastatin is unclear, research to explore new indications for ivermectin as an antiviral and or an anticancer agent has shed light on this [546]. Multiple diseases, including intestinal and lung cancers, require constitutive activation of canonical WNT-TCF signalling, but there are no WNT-TCF antagonists in clinical usage. The repositioning of WNT-TCF blockers response aim to recapitulate the genetic blockade that dominant-negative TCF has afforded. A drug repositioning screen for WNT-TCF blockers showed that ivermectin inhibits WNT-TCF target expression and blocks proliferation and increases apoptosis in different types of human cancer[547]. In colon and lung cancer cells, macrocyclic lactones that are structurally related to ivermectin have also been identified as effective WNT-TCF response blockers and confirmed in primary tumours obtained from patients in colorectal cancer (CRC) preclinical tumour growth models [548]. It is possible that the effects of ivermectin on the WNT pathway synergize with the anti-cancer effects of pitavastatin.

It is also possible that there are multiple mechanisms underlying the synergy between ivermectin and pitavastatin. Ivermectin may have multiple modes of action like most, if not all, of the drugs. Its function as an anti-parastic drug is believed to rely on its ability to activate glutamate-chloride channels, thereby altering chloride flux and preventing neuronal excitement, resulting in parasite paralysis and death [528]. It was proposed that the same mode of action could underlie the toxicity of ivermectin to liquid tumour cells and its ability to sensitise cancer cells to classical chemotherapy[545]. Research to redeploy ivermectin to treat skin, breast, lung, ovary and colon cancers [549], could identify other possible targets for ivermectin that can inhibit tumourigenesis.

In addition, in these studies it was noticed that the cells grown in DMEM growth media (COV318, COV362 and FUOV1) showed significant synergy while others grown in RPMI growth medium did not show synergy. It would be worthwhile swapping media for all these cells and measuring the impact on synergy between pitavastatin and ivermectin. Comparison of the different constituents of these media might also provide insight into the mechanism underlying the synergy.

These data suggest that it may be worthwhile to explore further the ivermectin and pitavastatin combination in further studies. The use of ivermectin as single agent for cancer treatment may also be possible even though, numerous targeted drugs have entered clinical trials but have shown limited effectiveness so far, often due to variability in treatment responses and sometimes rapidly increasing resistance. For more successful treatment options, multi-target drugs or drug combinations may be preferred to avoid drug resistance escape mechanisms. Interestingly, ivermectin's *in vitro* and *in vivo* anticancer activities are reported at concentrations that can be reached clinically, based on the human pharmacokinetic trials in healthy and patients infested with parasite. Existing knowledge on ivermectin may thus allow its rapid transition into clinical trials with cancer patients[534]. Ivermectin's pharmacokinetic characteristics and safety profile for exploring novel uses for further expanding its use through strategies for mass drug administration to boost coverage levels. Additionally, in healthy subjects, ivermectin binds strongly to plasma proteins (93.2%) with a specific serum albumin binding, there were two binding sites at the primary site, with a persistent interaction of 2×10^8 mol^{-1}. This is of considerable significance as the medication is administered in worldwide areas where malnutrition and hypoalbuminemia are prevalent, so a decrease in plasma proteins may be anticipated in such patients, and thus a higher free fraction of ivermectin [550]. It will be important to consider this when designing trials of ivermectin.

In conclusion, these findings show that ivermectin in combination with pitavastatin limits the growth of ovarian cancer cells; his may help decrease the dose of pitavastatin for cancer treatment and correspondingly decrease the likelihood of myopathy.

Chapter 7

Conclusion

7. Conclusion

Every year, ovarian cancer accounts for about 240,000 new cases worldwide with 152,000 deaths. Europe has the highest rates of new cases (11.4 per 100,000) and deaths (6.0 per 100,000) [551]. There is a very poor prognosis for advanced ovarian cancer patients [552]. Advanced ovarian cancer treatment currently includes surgery followed by adjuvant chemotherapy consisting of three weekly cycles of carboplatin and paclitaxel, while most patients respond to initial chemotherapy, drug resistance frequently occurs with few remaining treatment options and only about 40% of patients survive 5 years after initial diagnosis of advanced ovarian cancer. Drug resistance is one of the major obstacles to improving the prognosis of a patient and substantially leads to the low survival rate of OC patients [553]. Therefore, new strategies of ovarian cancer therapies are urgently needed. This thesis has attempted to make a contribution to the development of these new therapies.

Targeting metabolic pathways in malignant disease care provides an exciting new potential intervention. Inhibiting the mevalonate pathway may provide one new therapeutic approaches [227]. A recent study from our laboratory has identified the need for consideration of a patient's diet for the effective use of pitavastatin in clinical trials. It was observed that geranylgeraniol in mouse food can reverse the cytotoxic activity of pitavastatin and feeding mice with controlled diet resulted in an increase in statin efficacy [308]. It has also been reported that the introduction of high-fat diet decreases statin activity and increases the growth of renal tumour cells in mice [554], [555].The work reported in Chapter 4 evaluated which foods could suppress the cytotoxic effects of pitavastatin (e.g. sunflower oil) and which did not. These observations suggest that dietary sources of geranylgeraniol may interfere with cytotoxic/cytostatic activity of statins and should be controlled during clinical trials of statins in oncology. Our data identify potential foods which patient can eat while being treated with pitavastatin for cancer therapy. Further analysis is desirable to determine which factors can interfere with pitavastatin’s

anticancer activity like mevalonate or other factors not yet discovered that could interfere with pitavastatin action. In addition, it is likely that the food constituents differ according to source (differ from country to country, perhaps depending on soil compositions) and a particular species of fruit, or vegetable that is consumed. It may be advisable to test foods in each country to allow pitavastatin to be used as an anticancer therapy.

Currently there is a need for the production of reliable, non-invasive biomarkers that can be used in cancer patients to predict response to statin therapy. We have attempted to identify biomarkers which predict patients that are sensitive or resistant to pitavastatin. We identified 18 genes whose expression correlated with sensitivity to pitavastatin from publically available databases reporting gene expression and drug sensitivity The expression of these candidate biomarkers was assessed in a panel of ovarian cancer cell lines by western blotting and qPCR. MAP-7 was found to be highly expressed in cells sensitive to pitavastatin. MAP7 is a member of a family of cellular proteins associated with, and altering the dynamics of, microtubules. The growing family of microtubule-associated proteins (MAP) includes products from oncogenes apoptosis regulators[447]. It is perhaps not surprising then that microtubule function impacts the activity of other classes of drugs, in this case a statin, and can have anti-cancer effects. However, it is unclear to what extent the effect statins have on microtubules contributes to the drug's anticancer activity compared to effects on other pathways. And in particular, CNP does not appear to be a "key target" affected by statins because farnesol blocks the effect of pitavastatin on CNP subcellular localization, but it does not suppress the anti-cancer effects of statins. In addition, our hypothesis that KIF5B might play a role in setting sensitivity to pitavastatin, based on its association with MAP7, did not appear to be supported by the experimental data. Further research into MAP7 and KIF5B is desirable to understand the contribution of these proteins to the activity of pitavastatin. The role of the prenylated CNP as a MAP7 and KIF5B membrane can provide more insight into how isoperinoid compounds

and/or pitavastatin can affect microtubulesact as an anti-cancer agent, these insights may give us an indication how to select other anticancer which anticipated make synergistic effect with pitavastatin and decreasing the incidence of resistance.

Preclinical studies have shown that *in vivo* and *in vitr*o, inhibitors of the MP are have anti-cancer efficacy [462], [556]–[560]. The most significant advantage of the antitumour effects of statins is that they maintain their effectiveness against OC cell line that is fairly resistant to chemotherapy[412]. Previous studies found that the induction of cell death required continuous blockage of HMGCR with long half-life statins [412] and potentially using a high dose of statin. This brings with it the risk of myopathy. Myopathy, and rhabdomyolysis in particular, remains one of the most devastating consequences of high statin dose [561]. There are a range of pharmacologically tractable targets that could be tested to identify possible combinations to enhance the sensitivity of OC cells to pitavastatin. Although it has been documented that statin inhibition of prenyltransferases may not be the cause of muscle cytotoxicity [218]. One strategy to achieve this is to find other drugs which inhibit the mevalonate pathway. The results in Chapter 6 showed that Ivermectin was synergistic with pitavastatin in half of the cell lines tested. Ivermectin may be a worthy option to obtain a synergistic effect in clinical treatment, especially because ivermectin showed a good synergetic effect those cells less sensitive cells to pitavastatin. Animal studies to confirm this observation using ovarian cancer xenografts are desirable to justify subsequent clinical trials. Ivermectin may also be useful in the treatment of ovarian cancer independent of its role in sensitizing cells to pitavaststin. Several potential new therapeutic target of pitavastatin have been identified including YAP1 [493], and KPNB1 [455]. It may be worth exploring ivermectin's activity as a single agent against ovarian cancer.

Another major challenge in the field of exploring the role of MP inhibitors is to identify the substrates of prenyl transferase that are responsible for mediating antitumour activity in different types of cancer[562]. There are more than 100 experimentally confirmed 100 proteins

that are prenylated and several hundred other proteins are also holding the CAXX box whose prenylation is waiting to be confirmed [563], [564]. Drugs inhibiting these prenylated proteins may also be synergistic with pitavastatin and deserve investigation. The role of prenylated proteins in malignant transformation has also stimulated interest in developing prenyl transferase enzyme inhibitors as an anticancer agent. These may also be synergistic with pitavastatin, although work from our group has alsready shown that both geranylgeranyl transferase 1 and II must be inhibited and inhibition of one isoform is inadequate [242].

It is also possible that drugs could be used with statins which have no anti-cancer effect but which minimize the risk of myopathy. The work presented here suggests that conenzyme Q10, which is already used to reduce statin-induced myopathy, does not interfere with pitavastatin's anti-cancer activity. It was also recently suggested that curcumin, a natural dietary polyphenol, could be added in patients with muscle problems as an alternative to statin therapy [565].

Chapter 8

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8. References

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