# Investigation of the Effects of Genistein and Fenretinide on Ovarian Cancer Cells

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A thesis submitted to Faculty of Graduate and postdoctoral studies, University of Ottawa
in partial fulfillment of the requirements of the degree of

**Master of Science**

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**To my parents Mrs. Rokhsar Poormojib and Mr. Abolghasem Azadi**

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# Abstract

The effects of the fenretinide and genistein as single or combined drugs on ovarian cancer proliferation and viability were investigated.

***Hypothesis:*** Co-treatment with genistein will enable a lower dose of fenretinide to be effective in inhibiting the proliferation and survival of ovarian cancer cells.

***Methods:*** Low and high doses of genistein and fenretinide were tested on A2780s and A2780cp cells using trypan blue viable cell count, MTS assay.

***Results and conclusions:*** Unlike low doses of fenretinide, genistein had anti-proliferative effects on both cell lines. There were no additive or synergistic effects of the two compounds. Higher dose treatments induced anti-proliferative effects and apoptotic cell death in both A2780s and A2780cp cells, with a greater sensitivity of A2780s cells to both test compounds**.**

***Overall Conclusion:*** Genistein and higher doses of fenretinide similarly impair cell cycle progression and induce apoptosis. The anti-proliferative effects of genistein can be affected by co-treatment with fenretinide.

# ACKNOWLEDGMENTS

I would like to thank both of my supervisors, Dr. Barbara Vanderhyden and Dr. Stan Kubow for their financial and scientific support throughout the Master of Science program. Without their trust, fairness and help I would not have been able to finish my graduate studies. I would like also to acknowledge Dr. Ben Tsang and Dr. Gerard Cooke for their advice and comments all along my graduate studies.

I would also like to thank Dr. Ken Garson, Elizabeth Macdonald and Olga Collins for their technical and scientific support.

Special thanks go to my best friend Elena Gheorghiu for her kind support and assisting me with Stats.

# STATEMENT OF ORIGINALITY

Ovarian cancer has the worst prognosis among gynecologic cancers because it is rarely diagnosed at the early stages and it progresses aggressively. There will be an estimated 2,500 new cases of ovarian cancer in Canada in 2011 and the life-time probability of a woman developing ovarian cancer in this country is 1 in 71. Approximately 1,750 women diagnosed with ovarian cancer die of that disease each year. Thus, the development of novel effective therapeutic agents is needed to improve patient outcome. One major accomplishment of this research was the successful demonstration that, at physiological concentrations, the phytoestrogen genistein exerted anti-proliferative effects on cisplatin-resistant (A2780cp) human ovarian cancer cell lines, which have not been previously tested with this compound. No adverse effect on cellular metabolism, apoptosis or cell cycling was observed with either genistein or fenretinide. At low doses of genistein and fenretinide, that reflect *in vivo* physiological systemic concentrations observed following their intake, A2780cp cells were more sensitive to the anti-proliferative effects of genistein. Conversely, A2780s cells were more sensitive than A2780cp cells to the pro-apoptotic effects of exposure to high concentrations of both compounds. At both the lower and higher than physiologic concentrations of genistein and fenretinide, genistein treatment was associated with a greater susceptibility of tumor cells to cytotoxicity and cell cycle arrest than observed following exposure to fenretinide. At both high and low concentrations of genistein, apoptosis occurs in both cell lines that was enhanced by co-exposure to fenretinide, which suggests future directions regarding exploration of possible cellular mechanisms of action of the anti-proliferative properties of genistein and fenretinide. Also, the research findings indicate paradoxical dose-

dependent differences regarding the sensitivity of cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines to the antiproliferative and cytotoxic effects of genistein. The results generated by this study provide promising results towards future research regarding the use of genistein as an adjuvant therapy to potentiate ovarian anti-cancer agents such as fenretinide to prevent growth and/or survival of ovarian cancer cells. The results also suggest the need for further research regarding the possible benefits of genistein or genistein-rich soy foods for the prevention of ovarian cancer, or as candidate agents for anticancer therapy.

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# LIST OF ABBREVIATIONS

1DF: 1 day fermentation

A2780cp: Human epithelial ovarian cancer cells resistant to cisplatin

A2780s: Human epithelial ovarian cancer cells sensitive to cisplatin

ANOVA: Analysis of variance

DMSO: Dimethyl-sulfoxide

Ein: Genistein

eNOS: Endothelial nitric oxide synthase

ER: Estrogen receptor

ES2: Ovarian clear cell carcinoma cell line

FBS: Fetal bovine serum

Fen: Fenretinide

FSC: Forward scatter

FACS: Flow cytometry

GlcCer: Glucosylceramide

IC50: Half maximal inhibitory concentration

LTF: Long term fermentation

μM: Micromolar

MEM-α: Minimum essential media

MTS: Cell viability titration assay using tetrazolium salts

NF-kB: Nuclear factor-kappa B

nM: Nanomolar (Molarity)

nM: Nanometer (Wavelength)

OD: Optic density

PBS: Phosphate buffer solution

PI: Propidium iodide

PLAB genes: Placental Bone morphogenetic

PTEN: Phosphatase and tensin homolog

ROS: Reactive oxygen species

Rpm: Revolutions per minute

SKOV-3: Ovarian cancer cell line

SSC: Side scatter

Sub-G1: Populations of apoptotic cells

SM/NFS: Non-fermented soy milk

SK: Soy kefir

# Chapter 1: Introduction

## Soybean Products

Soybean is a legume that is native to East Asia for over five thousand years. As soybeans mature in the shell, they develop into a hard, dry bean, which is consumed by millions of Asians every day as a source of protein, making the soybean the world's most widely grown legume (the world healthiest foods web link, 2011). Soybeans can be grown in a wide variety of soils and climates, ranging from tropical Brazil to the snowy island of Hokkaido in the north of Japan. Soybean consumption was initiated in the Western world in the early $20^{th}$ century.

Soy milk is a water extract of whole soybeans and made from soaking soybeans in water, grinding the beans and filtering the liquid. It is an off-white emulsion/suspension containing the water soluble proteins and carbohydrates, and most of the oil of the soybeans. Heat treatment is applied to soy milk to inactivate possible anti-nutritional factors, such as trypsin inhibitors, and to ensure safety by adequate pasteurization. Vegetable oils, sweeteners, salt, seasonings and/or other functional or flavoring ingredients may be added to soy milk. Resulting products should have a soy protein and fat content in accordance with the criteria stipulated for the specified classification as soy milk, soymilk drink, soymilk powder, or soymilk concentrate. According to the *Soymilk Standards of the Soy Foods Association of America,* soy milk "shall contain no less than 3.0% soy protein, no less than 1.0% soybean fat and no less than 7.0% total solids".

## Anti-Cancer Actions of Soy Foods

Epidemiological studies have shown that the consumption of soy products in

Asian populations is associated with a lower incidence of breast, prostate and ovarian cancer in China as compared to the U.S. population (Korde et al 2009, Butler et al, 2010, Wu et al, 2008, 2009, Sakaucki et al, 2007). Epidemiological studies have also shown that Asian women have much lower breast cancer rates than their Western counterparts, unless they move to Europe or North America when the cancer incidence in these women begins to match local norms (Raloff et al, 2001). Data from a large number of epidemiological studies demonstrate reductions in cancer risk in various population groups consuming soy and soy products (Korde et al 2009). These epidemiologic data, when considered with the expanding data set demonstrating cancer chemoprevention by soy components in animal models, clearly support the development of soy constituents for possible use in human cancer prevention. Zhang et al. (2004, 2011) compared two diet patterns in a cohort study and reported a 74% decrease in breast cancer risk in Chinese women who had a high soy milk diet pattern as part of their daily intake. These latter findings support other data showing that a higher consumption of soy products in Asian populations may be partly responsible for their lower incidence of breast cancer (Korde et al, 2009; Butler et al, 2010; Wu et al, 2008, 2009). Hence, epidemiologic data clearly support the potential of soy constituents for possible use in human cancer prevention and treatment. Also, the above observations suggest that diet could be involved in promoting cancer in western countries and inhibiting cancer in populations of the Far East.

Bioactive components found naturally in soybeans are currently being studied for the relief of menopausal symptoms such as hot flashes, the maintenance of healthy bones, and the prevention of prostate, breast, ovarian and colorectal cancer. Previous studies

have shown that several bioactive soy compounds have anti-mutagenic and anti-carcinogenic properties in ovarian cancer cells, including: isoflavones (genistein, genistin, daidzein, daidzin, equol, O-desmethylangolensin, glycitein, glycitin) (Kennedy et al, 1995; Nhan et al, 2005; Park et al, 2004; Vallachovicova et al, 2004; Solomon et al, 2008), peptides (Lunasin (Dia et al, 2010, 2011)); trypsin inhibitors (Furukawa et al, 1992a, 1992b) and sphingolipids (Maurer et al, 1999; Geley et al, 1997; Dbaibo et al, 1997).

The components of soy products that might have the most potent anti-proliferative effects on cancer cells are controversial due to conflicting reports regarding the bioavailability, pharmacokinetics and plasma levels of each soy component when consumed in physiologically relevant intakes as found in typical soy-based diets. Thus, to determine the exact role of various bioactive compounds of soy products, the bioavailability and pharmacokinetics of each individual bioactive compound in soy foods must be understood. To date, there has been no comprehensive study regarding the bioavailability of bioactive compounds of soy foods or soy milk. Controversial reports, however, exist regarding bioavailability of soy components when provided individually, which are most likely in part due to variabilities in the gut micro-flora of individuals that can exert a significant impact on the structure and bioactivity of soy components such as isoflavones (Xia Xu et al, 1995). There are several components of soy foods that exert anti-proliferative effects on cancer cells, which are described below.

***Saponin***

It has been shown that saponin extracted from ginseng inhibits proliferation of

ovarian cancer cells, but the effect of saponin extracted from soy products on ovarian cancer cells has not been investigated (Xu et al, 2007; Shibata et al, 2001; Nakata et al, 1998; Kikuchi et al, 1991). Soya-sapogenol B is a metabolite of saponin that is produced *in vivo* by the action of human intestinal microorganisms and is excreted in the feces (Hu Jiang et al, 2004). Due to the low bioavailability of saponin from ingested soy (Hu Jiang et al, 2004); it is not considered to be a potent bioactive soy compound.

***Phytosterols***

Although the structure of phytosterols (plant sterols) has similarity with cholesterol, their pharmacological behaviors (intestinal absorption and metabolic fate) differ. As compared to cholesterol, plant sterols have poor intestinal absorption. Despite this poor absorption, there is evidence for beneficial effects of plant sterols on disorders such as cutaneous xanthomatosis, colon cancer and prostate hyperplasia (Rubis et al, 2010). More pharmacokinetic data is needed to understand the metabolic fate of plant sterols and their interactions with other nutraceutical/pharmaceutical agents as well as possible anti-cancer effects as there is some suggestive evidence for protective effects against ovarian cancer (Woyengo et al, 2009).

***Protease inhibitors***

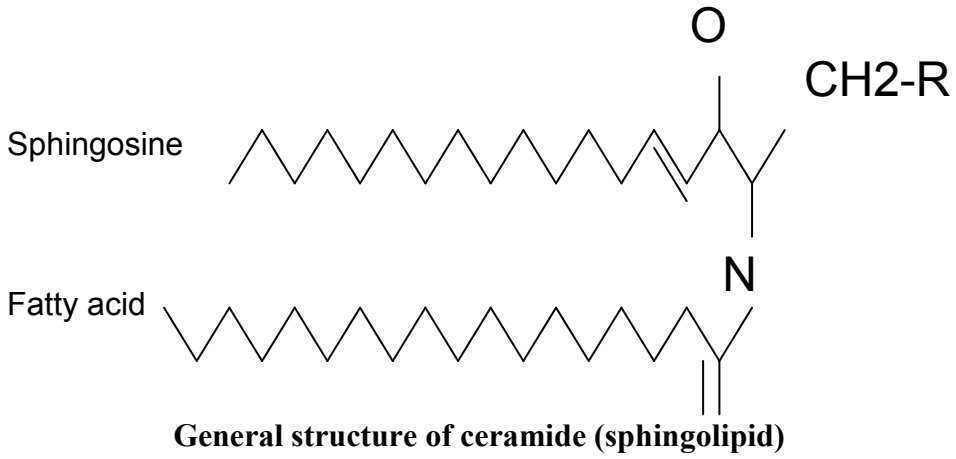
Proteolytic proteases are products of normal and tumor cells. They are important for invasiveness and metastasis of tumors. The balance between proteolytic activity and inhibition is crucial in determining the metastatic behavior of cancer cells (Reuning et al, 1995). Protease inhibitors are naturally found in plants such as soybeans, which contain

several protease inhibitors including trypsin-chymotrypsin inhibitors. These latter compounds potentially bring a new approach to anti-cancer therapy by manipulating or suppressing the signal transduction of the plasminogen activator system in malignant cells (Brandon et al, 1991). Bowman-Birk is a protease inhibitor found in soy milk, which has efficacy in cancer chemoprevention (Johnson et al, 2006). Not all enzyme inhibitors have chemopreventive and anti-invasiveness activity, but the Bowman-Birk inhibitor has a broad range of chemopreventive activities in animal carcinogenesis models (Kobayashi et al, 2004). It has been reported that soybean trypsin inhibitor has both cancer inhibitory and potential cancer promoting effects in pancreatic cancers in animal models (Furukawa et al, 1992a, 1992b).

***Sphingolipids (ceramides)***

Glucosylceramides are one of the bioactive compounds from soy that has anti-tumorigenic activity in mammals. Dietary supplementation with soy milk sphingolipids inhibits colon tumorigenesis in CF1 mice treated with a colon carcinogen (1,2-dimethylhydrazine) and in multiple intestinal neoplasia (Min) mice, which develop intestinal tumors spontaneously (Symolon et al, 2004). Sphingolipids from soybeans primarily include glucosylceramide (GlcCer), which consists predominantly of a 4,8-sphingadiene backbone and hydroxy-palmitic acid (Figure 1). Glucosylceramide is poorly absorbed from the gut of mammals and shows the lowest bioavailability among ceramides (Symolon et al, 2004). Although sphingolipids can modulate both cell growth and cell differentiation and can induce apoptosis *in vitro* and *in vivo* (Ginis et al, 1999; Segui et al, 2006, Lee et al, 2001), the poor absorption of soy glucosylceramides.

**A**




**General structure of ceramide (sphingolipid)**

**B**

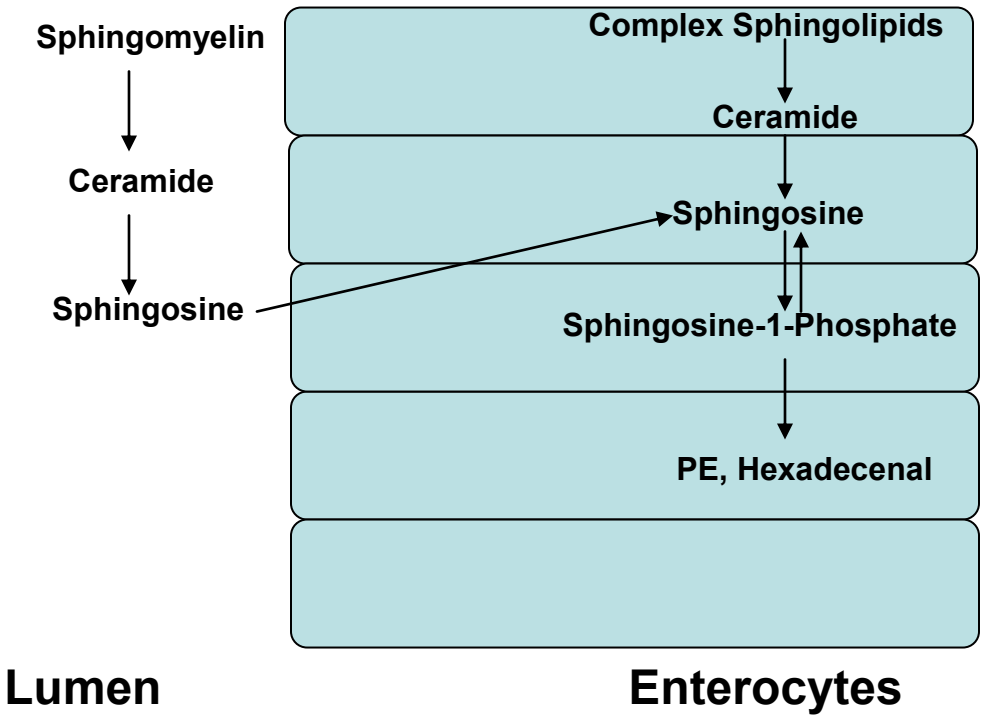



**Figure 1. A:** General Sphingolipid structure B**:** Scheme for intestinal digestion of sphingomyelin.

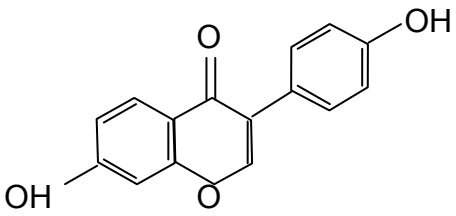
suggests that it is unlikely that these compounds can directly affect ovarian cell function or ovarian cancer risk.

***Isoflavones***

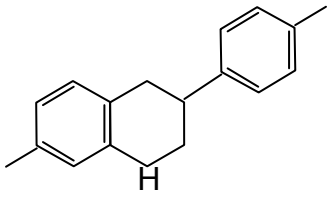
Isoflavones (phytoestrogens) are other important bioactive compounds present in high concentrations in purified soy proteins, which has brought the most attention to the nutritional benefits of soy foods (Murphy et al, 1982). Isoflavones are a subclass of glucosidic flavonoids (including diadzin and genistin; Figure 2) that occur naturally in soy milk. They are exclusively conjugated to sugars, which results in their low bioavailability after oral administration. Glucosidic flavonoids do not readily enter into intestinal enterocytes to reach the systemic circulation and thus plasma levels of the glucosidic form of isoflavones are significantly lower than their non-glucosidic form (Coward et al, 1993). On the other hand, the aglycone form of isoflavones (including genistein and diadzein; Figure 2) can be produced by the action of bacterial β-glucuronidase in the intestinal mucosa in the gut, and the aglycone form results in significantly higher plasma levels after oral administration (Setchell et al, 2002, 2003; McMahon et al, 1997).

Numerous studies have examined the effect of isoflavones in ovarian cancer and other types of cancer; however, whether they have any anti-proliferative effects remains controversial. While some human studies have shown high bioavailability of aglycone isoflavones such as equol following oral administration (Setchell et al, 2005), other studies have reported a lower production among certain test subjects resulting in low equol bioavailability (Vergne et al, 2002), which might be dependent partly on the differential action of intestinal microflora of the tested subjects.

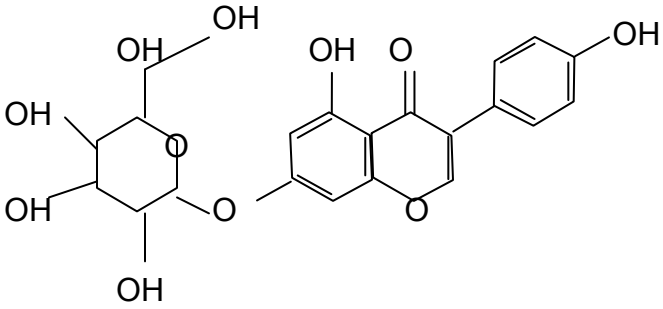
**Daidzein**



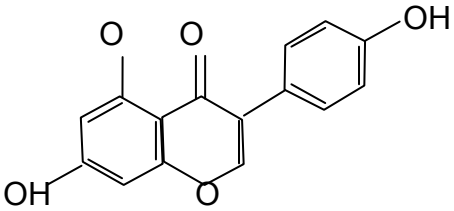

**Equol**




**Genistin**




**Genistein**




**Fenretinide**

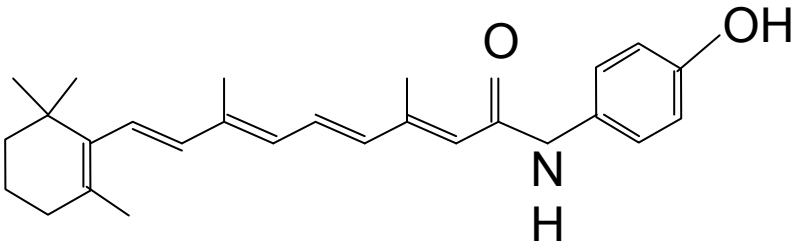



**Figure 2:** Structures of daidzein, equol genistin, genistein and fenretinide

Isoflavone aglycones (genistein) are substances found in traditionally fermented soybean products. However, in non-fermented soy products such as tofu and soy milk, these isoflavones are present in an altered form as beta-glycoside conjugates (genistin), which have lower bioavailability after oral administration (Setchell et al, 2005). Genistein is a non-glycosidic isoflavone that can cross the gut barrier to achieve total genistein concentrations in human plasma of 0.5-5 μmol after oral consumption of a soy rich diet (Allred et al, 2004). Equol (Figure 2) is a metabolite with estrogenic properties produced *in vivo* from the soy phytoestrogen daidzein by the action of gut microflora. The blood concentrations of equol, however, appear to depend greatly upon the gut microflora, which differs greatly among individuals.

Among all glycosidic and aglycone isoflavones, there is recent evidence that equol was the most effective isoflavone with anti-proliferative effects on various cancers tested *in vitro* and *in vivo* (Lampe et al, 2010, Brown et al, 2010, Ko et al, 2010). Brown et al (2010) investigated the effect of dietary equol in Sprague-Dawley rats with dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinoma. Rats fed a diet containing 250 mg/kg of equol for 155 days exhibited a significantly lower number of palpable tumors, lower tumor weight, increased tumor latency and decreased tumor invasiveness than rats fed a control diet. A recent case-control study in Korea reported that higher plasma concentrations of equol, genistein, and daidzein were associated with a decreased risk of gastric cancer, which suggests a beneficial effect of a high soybean diet against gastric cancer risk (Ko et al, 2010) after controlling for confounding factors including sex, age (±5), year of recruitment and residential area. Studies performed by Choi et al. (2007) concluded that both genistein and genistin at doses of 5 – 100 μM

significantly inhibit the proliferation of human SKOV-3 ovarian cancer cells and caused cell cycle arrest at the G2/M phase and increased caspase-3 activity although genistin required higher concentrations or a more prolonged exposure to exert a significant effect. As only a relatively small fraction of individuals show significant elevations of plasma equol concentrations after soy intake, it can be argued that the more consistent bioavailability of genistein from soy products makes the study of anti-proliferative properties of genistein more applicable for the general population (Aedin et al, 2006).

**Mechanisms of anti-cancer action of genistein**

The *in vitro* and *in vivo* anti-carcinogenic activity of soy isoflavones has been related to their anti-estrogenic activities, effects on growth signaling pathways and the cell cycle, the induction of apoptosis and anti-angiogenic activities (Park et al, 2004; Kennedy et al, 1995). Soy isoflavones have a similar structure to estrogens, which allow them to bind to estrogen receptors (ER) (Vallachovicova et al, 2004) with higher affinity to ER-beta than ER-alpha, and with binding affinity comparable to 17 β-estradiol (Morito et al, 2001). Because of their weak estrogenic properties, they are considered as estrogen receptor modulators. Studies involving genistein have also focused on specific target molecules and signaling pathways involved in cell proliferation and differentiation, cell cycle regulation, apoptosis (Choi et al, 2007; Gossner et al, 2007), angiogenesis (Yu et al, 2010), cell adhesion and migration (Chen et al, 2011), metastasis and the activity of different enzymes (Arabinda et al, 2006; Swami et al, 2005; Vera et al, 1996). Mechanisms of cytotoxicity and anti-neoplastic activity of genistein have been reported that include: inhibition of glucose uptake and autophagocytosis, production of endothelial

nitric oxide synthase (eNOS), reactive oxygen species (ROS) generation in tumor tissue, and increasing mitochondrial permeability transition.

There are controversial reports regarding the importance of isoflavone uptake via ER for the anti-proliferative effects of isoflavones on tumor cells. Chen et al, (2001) reported that only 63% of primary epithelial ovarian carcinomas are ER+ and they suggested that proliferation inhibition by isoflavones is an ER-dependent event based on the ovarian cancer cell lines they tested in vitro. On the other hand, Lim et al. (2006) showed that genistein was associated with inhibition of glucose uptake in both ER-positive (ER+ MCF-7) and ER-negative (ER- MDA-MB-231) cells by up-regulating the expression of glucose-regulated protein 78 (GRP78) in a time- and dose-dependent manner. It thus appears that the effect of genistein on glucose transporting function is cell line- and ER-independent, which might indicate that ER-negative ovarian tumor cells such as A2780 (Ferlini et al. 1999) might also be negatively affected by genistein exposure. One of the mechanism in which isoflavones including genistein induce apoptosis in ER- mammary cancer cells is reported by Vanden Bergh et al. (2006). They reported that isoflavones inhibit NF-kB transduction via down-regulating the target genes, *Erk* and *Mek*. It has been also reported that genistein down-regulates AKT via stimulation of the protein expression of tumor suppressor PTEN, which inhibits AKT kinase in MCF-7 cells (Waite et al. 2005). Genistein has also been noted to adversely affect glucose uptake in other tumor cell types as Vera et al. (1996) reported a dose-dependent inhibition by genistein of uptake of deoxyglucose and dehydroascorbic acid and methylglucose in Chinese hamster ovary cells with overexpression of GLUT1, a mammalian facilitative hexose transporter that appeared to involve inhibition of the

activity of protein tyrosine kinases. They also reported that genistein inhibited the uptake of deoxyglucose in human erythrocytes.

Gossner et al. (2006) reported cytotoxicity of genistein on three ovarian cancer cells, A2780, CaOV3 and ES2. They showed that both apoptosis and autophagy are two mechanisms of genistein-induced cell death in ovarian cancer cells. Ovarian cancer cells treated with genistein resulted in a caspase-independent cell death. It is possible that genistein could reduce glucose uptake and induce a starvation-like signaling response, which can lead to production of methyl pyruvate that can trigger autophagocytosis. Gossner et al. (2006) also reported that genistein treatment is associated with reduced levels of phosphorylated AKT, which may contribute towards a mechanism to limit glucose utilization.

Nitric oxide (NO) is an oxidative agent produced by endothelial cells in response to any stress or injuries (inflammatory conditions, carcinogenesis and cardiovascular diseases) to eliminate injured cells. Shin et al. (2007) reported a high production of NO after exposing mammalian cancer cells to genistein.

White et al. (2010) reported that plasma concentration of genistein increased to a maximum of 39.85 μmol while testing the effects of high doses of genistein-rich soy extract on serum concentrations of prostate-specific antigen (PSA) in a group of 53 prostate cancer patients who consumed 450 mg genistein daily for 6 months. They reported that plasma concentration of genistein increased to 39.85 μmol at the end of 6 months but concentrations of PSA did not decrease after 6 and 12 months of treatment. Their findings demonstrated, however, that high plasma concentrations of genistein are achievable after oral consumption of genistein-rich products. To date there is no evidence

to support that PSA is a specific biomarker for early detection of ovarian cancer..

Das et al. (2006) examined the effect of genistein against neuroblastoma SH-SY5Y cell lines and reported a decrease of viable cells after 24 hour treatment with 100 μmol genistein. They showed that apoptosis is a predominant event that is associated with an increase in Bax:Bcl2 ratio, mitochondrial release of cytochrome c, intracellular free [Ca‡], and activation of calpain (activation of mitochondrial-mediated pathway), caspase-3, caspase-9, and caspase-12.

In rat mitochondria, Salvi et al. (2002) showed that genistein caused an increased mitochondrial permeability transition (MPT) and consequent generation of ROS due to the interaction of genistein with the respiratory chain at the level of mitochondrial complex III. Swamiet al. (2004) examined the effect of low doses of genistein on mitochondrial function of DU-145 human prostate cancer cells. 1, 25-$(OH)_2$-$D_3$ is a growth inhibitory agent, which can be catabolized with the action of CYP24 enzyme. They showed that genistein at low doses (0.05-0.1 μM) can act as a growth inhibitory agent via two mechanisms: i) down-regulation of the CYP24 enzyme resulting in an increased half life of 1,25-$(OH)_2$-$D_3$; and ii) up-regulation of the Vitamin D receptors. Lim et al (2006) introduced the idea that genistein can act differently according to the concentrations that are tested. They suggested that genistein used at low doses *in vitro* (<10 μmol) might act as estrogen agonists whereas high doses of genistein (>10 μmol) can exert multiple biological effects on cells, in addition to their effects on estrogenic activity.

There are several mechanisms reported for genistein-induced cytotoxicity towards cancer cells, including both autophagy (due to starvation by blocking glucose uptake) and

apoptosis. For example, after 24 and 48 h of genistein treatment at doses ranging from 0.1-100 μM on A2780, CaOV3 and ES2 cells, western blot analysis demonstrated cleavage/activation of caspases-9 and -3 showing that 25% of cells were apoptotic at the highest dose (Gossner et al, 2007). Other studies showed that genistein at 111 μM inhibited cell proliferation of Caco-2BBe cells by causing G2/M cell cycle arrest (Chen et al, 2004). Studies performed by Choi et al. (2007) examined the anti-proliferation effect of genistein and genistin alone or in combination on the SKOV-3 ovarian cancer cell line and concluded that both genistein and genistin at a dose of 100 μM can have cytotoxic effects on SKOV-3 cells and inhibit cell proliferation by disrupting the cell cycle, but the effects of genistein were more pronounced. They assayed the proliferation of SKOV-3 cells after 24 and 48 hours of treatment with genistin and genistein. Genistein significantly decreased cell proliferation of SKOV-3 cells in a dose- and time-dependent manner. Genistin did not decrease cell proliferation of SKOV-3 cells after 24 hours but there was a slight decrease in proliferation after 48 hours.

Shin et al (2007) reported a high production of nitric oxide and prostaglandin E2, and elevated expression of cyclooxygenase-2 (*COX-2*) after exposing mammalian MCF-7 breast cancer cells to genistein. *COX-2* is a central enzyme in prostanoid biosynthesis from arachidonic acid, which leads to the production of prostaglandin E2 (PGE2), leading to NO production. They also showed that over-expression of COX-2 was followed by the increased expression of ERK-1 and -2 and P38. They explained the possible mechanism of the effects of genistein on MCF-7 cells by down-regulation of COX-2 and possibly by the activation of AMP-activated protein kinase (AMPK).

In addition to the examination of the anti-proliferative effects of genistein and

other isoflavones on ovarian cancer cell lines, recent studies have shown that genistein can enhance the anti-proliferative effects of chemotherapeutic agents. Solomon et al. (2008) reported the sensitization of platinum-resistant ovarian cancer C200 cells and platinum-sensitive ovarian cancer A2780 cells to chemotherapeutic agents such as cisplatin, taxotere or gemcitabine following pretreatment with genistein. They reported that pre-exposure of A2780 platinum-sensitive ovarian cancer cells to genistein sensitized these cells to combination treatment of any two chemotherapeutic agents.

**Normal Ovarian Physiology**

The ovaries are the female reproductive organs which produce eggs (ova). They are almond-shaped and approximately 3.5 cm long. The ovaries are located deep in a woman's pelvic cavity, on either side of the uterus, close to the end of the fallopian tubes. They are made up of at least five different cell types: (a) epithelial cells that make up the outer layer or covering of the ovary (epithelium); (b) germ cells, which are the egg cells found inside the ovarian follicles; (c) granulosa and theca cells that are the predominant steroid hormone-producing cells; (d) and stromal cells that make up the supportive or connective tissues.

The two major functions of ovaries are to store and develop eggs and to produce two female sex hormones that regulate reproduction and sexual development. The two female sex hormones are estrogen, which is responsible for the development of secondary sex characteristics, such as the growth of breast tissue, and progesterone, which prepares the body for conception by stimulating the thickening of the uterine lining among many other physiological functions. The ovaries are the main source of estrogen in sexually

mature women.

**Ovarian Cancer**

Ovarian cancer is a disease in which normal ovarian cells start to grow in an uncontrolled, abnormal manner producing tumors in one or both ovaries. The mortality rate of women who are diagnosed with ovarian cancer in Canada is about 1,750 deaths each year (Canadian Cancer Society)

There are three types of ovarian cancer: i) epithelial ovarian cancers, which arise from the epithelial cells that line or cover the ovaries; ii) germ cell tumors, which start in the cells that will produce the egg cell; and iii) sex cord-stromal tumors that develop from the connective tissue of the ovary (Jacobs, J., 2002, A text book). The most common type of ovarian cancer initiates from the human ovarian surface epithelial cells, and comprises 90% of all ovarian cancers. Among ovarian cancers, approximately 50-60% are papillary serous epithelial cancers, which are the most common epithelial ovarian cancers. The less common ovarian cancers are endometrioid (4%), clear cell (4%) and mucinous (4%) (Bhoola et al, 2006). There are also rare types of ovarian tumors such as borderline ovarian tumors or low malignant potential tumors (10-15% of epithelial ovarian tumors), extra-ovarian primary peritoneal cancers that are mostly undifferentiated cancers and transitional cell cancers that are poorly differentiated but are very malignant cancers (Jacobs, J., 2002, A text book).

Although the main cause of ovarian tumors is unknown, many studies have characterized the genes that might explain the mechanistic basis of the development of ovarian tumors such as tumor suppressor genes. The Farley model shows the

development of human epithelial ovarian cancer after inclusion cyst formation (Farely J, et al., 2008). Based on this model, the highest risk of genomic material abnormalities in ovarian surface epithelial cells results from non-stop ovulation and wound repair, which lead to an increase in chronic inflammation that infiltrates immune cells, increases activated fibroblast formation and angiogenesis. Genetic abnormalities (such as mutation or inactivation of tumor suppressor genes) lead to dysplastic transformation in epithelial cells lining the inclusion cyst. Activated fibroblast formation, microvessel proliferation and growth factors contribute to dysplastic formation and malignancy.

**Cancer treatment**

Cancer treatment may be given for a number of reasons (prevention, cure, control or palliation) and sometimes the goal of treatment can be changed over the course of treatment. Strategies to cure cancer most commonly include surgery, chemotherapy and radiation therapy. Depending on the stage of cancer, ovarian cancer often initially responds well to chemotherapy. Healthy tissue, however, can be also adversely affected during chemotherapy treatment, so that patients may experience side effects like nausea, vomiting, loss of appetite, fatigue, hair loss and an increased risk of infection. Unfortunately, the disease frequently recurs after remission, at which point the cancer cells are often resistant to further drug treatment. Therefore, new chemotherapeutic approaches and drugs are needed to be developed with fewer propensities for the development of drug resistance and with less toxicity.

The standard of care for ovarian cancer patients involves debulking surgery followed by chemotherapy with a combination of a platinum drug (carboplatin or

cisplatin) and taxol. Lieberthal et al (1996) first reported the cancer cell killing properties of carboplatin in renal proximal tubular epithelial cells. They reported that high dose of carboplatin (800 μM) causes cell necrosis of proximal epithelial cells, which is associated with cytosolic swelling, early loss of plasma membrane integrity and random DNA degradation. On the other hand, lower doses of carboplatin (8 μM) led to apoptosis as loss of the cell monolayer, cell shrinkage, loss of attachment to the monolayer and nuclear chromatin condensation and fragmentation were observed. They also reported that DNA of apoptotic cells developed a ladder pattern resulting from internucleosomal DNA cleavage.

Taxol is used in combination with carboplatin to eliminate ovarian cancer cells. Torres et al. (1998) reported that the major mechanism of action of taxol relates to an alteration in specific intracellular transduction events including the activation of Raf-1 kinase. Raf-1 is known as an essential gene for drug-induced apoptosis. Torres et al. (1998) showed that taxol acts differently at low and high concentrations. At low concentrations (< 9 nM) taxol induces p53 and p21 production independently of the activation of Raf-1, which causes an aberrant mitosis. At high concentrations (≥ 9 nM), taxol can activate Raf-1 and cell death, which results from a terminal mitotic arrest occurring via a Raf-1 dependent pathway. Charles et al (2004) reported that higher than normal production of ceramide is an important event in the taxol-induced apoptosis in hormone-dependent (MCF7) and hormone-independent breast cancer cells (MDA-MB-468).

**Synthetic retinoids for cancer treatment**

Synthetic retinoids hold great promise as new agents for the prevention and treatment of cancer as the development of novel retinoid-like structures has resulted in several compounds with potent anti-tumor activity. The compound N-(4-hydroxylphenyl) retinamide (4-HPR; fenretinide; Figure 2) represents a synthetic retinoid that has been demonstrated to induce apoptosis in a wide variety of human cancer cell lines, including retinoid-resistant tumor cells (Delia et al, 1993). Acyclic retinoid, a novel synthetic retinoid, has been demonstrated by Suzui and colleagues (2006) to inhibit the *in vitro* growth of human hepatoma cells. This latter effect was associated with modification of the cell cycle control, suggesting that acyclic retinoids may be useful in the chemoprevention and therapy of various types of malignancies (Suzui et al, 2006). Wu and colleagues demonstrated that naturally occurring retinoids such as 9-cis retinoic acid can prevent breast cancer in animal models (Wu et al, 2005). Martin et al (2000) showed that retinoic acid induces production of endothelial nitric oxide synthase (eNOS) in ZR-75-1 breast cancer cells after treatment with 1 μM of retinoic acid. They also showed that retinoid induced growth inhibition of those cells, which was associated with an increased production of nitric oxide.

(E)-3-(4'-hydroxy-3'-adamantylbiphenyl-4-yl)acrylic acid (ST1926), 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437), all trans retinoic acid (ATRA) and 4-oxo-fenretinide are four recently discovered retinoids that all have anti-proliferative effect on tumor cells and could be promising drugs for ovarian cancer therapy. Thus far, however, only 4-oxo-fenretinide and fenretinide have been tested on ovarian cancer cells *in vitro*. 4-Oxo-fenretinide is a polar oxidized metabolite of

fenretinide that is formed from fenretinide at the tumor site and has shown anti-proliferative effects in ovarian cancer cells (Villani et al, 2004).

Addition of ceramide to isolated mitochondria induces an interruption in electron-chain transport, resulting in production of reactive oxygen species (ROS) (Garcia-Ruiz et al, 1997 and Degli Esposti et al, 1998). It is intriguing that fenretinide has been documented to trigger intracellular increases in ROS, and it has been suggested that ROS generation may be its chief mechanism of action to induce apoptosis (Tiberio et al, 2010; Lovat et al, 2004; Delia et al, 1993, 1995). Studies by Susin and Green (1998) showed that both ceramide and ROS are associated with cell death induction.

Green and colleagues (1998) showed that mitochondrial damage was likely a primary effect or at least an early event in apoptosis after exposing cancer cells to fenretinide. Delia et al (1995) demonstrated effects on the expression of certain *bcl-2* family genes *Bax* and *Bak* (Morales et al, 2007). The proteins encoded by these genes play a central role in apoptosis regulation, functioning as either inducers or blockers of cell death. Adams et al (1998) showed that anti-apoptotic *bcl-2* genes could be down-regulated by fenretinide. Most Bcl-2 family proteins reside in mitochondrial membranes, and anti-apoptotic members such as *Bax* and *Bak* can prevent cell death induced by ROS, ceramide, and anticancer drugs. Caspase inhibitors do not prevent cell death induction by either fenretinide (Maurer et al, 1999) or ceramide (Geley et al, 1997), whereas enforced expression of Bcl-2 reportedly does (Geley et al, 1997; Dbaibo et al, 1997). When taken together with the observation that fenretinide decreases the expression of bcl-2, at least in leukemia cell lines (Maurer et al, 1999), the above data suggest that fenretinide induces apoptosis through its suppression of bcl-2 expression.

Appierto et al (2004, 2007) identified *PLAB* genes (PLAcental Bone morphogenetic) that contribute to fenretinide-induced apoptotic activity in ovarian cancer cells. They added fenretinide to A2780 cells, a human ovarian carcinoma cell line sensitive to the retinoid, and found that *PLAB*, a pro-apoptotic gene, was the most highly induced by fenretinide which increased stability of *PLAB* mRNA. PLAB has been reported to be a retinoic acid target gene, which has therefore been implicated in retinoic acid signaling (Ma et al, 2003). Down-regulation of *PLAB* with small interfering RNAs reduced the ability of fenretinide to induce apoptosis. PLAB up-modulation by fenretinide was also observed *in vivo* via ascitic cells collected from patients with ovarian cancer before and after fenretinide treatment. *PLAB* was noted to be up-modulated in 2/4 patients, although this result was not tested statistically. The above results shown in certain ovarian cancer cell lines indicate a role for PLAB as a mediator of fenretinide-induced apoptosis (Appierto et al, 2007). Morales et al (2007) also reported that fenretinide increases ceramide levels in mitochondria that lead to ROS generation causing inactivation of *Bak* and *Bax*, loss of mitochondrial membrane permeabilization, and consequently cell death in leukemia cells. Appierto et al (2004) also found that c-Fos plays a role in mediating fenretinide-induced growth inhibition and apoptosis in ovarian cancer cells and suggested that c-Fos regulates these processes as a member of the AP-1 transcription factor. They also showed that ceramide, which is involved in fenretinide-induced apoptosis, was also involved in c-Fos induction because its upregulation by fenretinide was reduced by fumonisin B1, a ceramide synthase inhibitor in a variety of human tumor cell lines including ovarian tumor cells.

Kalli and colleagues (2003) showed that fenretinide treatment increased ceramide

levels equally in two ovarian carcinoma cells that do (OV177) and do not (OV202) rely on caspase-8 to initiate apoptosis. Their results indicate that synthetic retinoids can use caspase-8 as an initiating caspase. Parrella and colleagues (2006) studied the anti-tumor activity of the retinoid-related molecules (E)-3-(4'-hydroxy-3'-adamantylbiphenyl-4-yl)acrylic acid (ST1926) and 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) in F9 teratocarcinoma cells. Results of their study showed the role of retinoic acid receptor gamma and retinoid-independent pathways for these two newly discovered retinoids (Parrella et al, 2006).

**Mechanisms of anti-cancer action of fenretinide**

Villani et al (2004) showed that an over expression of CYP26A1 enzyme in ovarian tumors is associated with generating 4-oxo-fenretinide, a polar oxidized metabolite of fenretinide. These researchers also reported (2006) that 4-oxo-fenretinide induces G2-M cell cycle arrest and apoptosis in both fenretinide sensitive and fenretinide resistant cell lines. Appierto et al, (2009a) reported that 4-oxo-fenretinide induces ROS generation (Lovat et al, 2004), which activates an apoptotic cascade involving endoplasmic reticulum (ER) stress response and JNK activation that up-regulate the proapoptotic *PLAB* gene. Tiberio et al (2010) reported two independent mechanisms of 4-oxo-fenretinide-induced apoptosis: i) 4-oxo-fenretinide induces ROS generation (Lovat et al, 2004) that activates an apoptotic cascade involving endoplasmic reticulum (ER) stress response and JNK activation and up-regulation of the proapoptotic *PLAB* gene; ii) 4-oxo-4-fenretinide can inhibit microtubule generation via inhibition of tubulin polymerization and consequently mitotic arrest (Appierto et al, 2009b). Kalli et al, (2003) reported a role

of caspase-8 in fenretinide induced apoptosis in epithelial ovarian cancer cells. Holmes et al (2003) studied the effect of 10 μmol fenretinide on CA-OV-3 ovarian carcinoma cells. They showed that fenretinide induced both caspases-3 and caspase-9 enzyme activity and mitochondrial depolarization, which are essential for induction of apoptosis.

Nitrite (NO) is an oxidative agent produced by endothelial in response to any stress or injuries (i.e., inflammatory conditions, carcinogenesis and cardiovascular disease) to eliminate injured cells. Martin et al (2000) showed that retinoic acid induces the production of endothelial nitric oxide synthase (eNOS) on ZR-75-1 breast cancer cells after treatment with 1 μM of retinoic acid. NO is an oxidative agent that able to kill cancer cells and minimize the size of tumor.

## Rationale:

Fenretinide is a recently discovered retinoid that has anti-proliferative effects on tumor cells and could be a very promising drug for ovarian cancer therapy. Although this drug attempts to target only cancer cells, the side effects of fenretinide drugs include temporary night blindness due to decreased plasma levels of retinol (nyctalopia) in 21% of children (Garaventa, A., 2003; Gross, et al, 1992; Modiano, et al, 1990). Soy milk has bioactive compounds that can either induce apoptosis or decrease the proliferation rate of ovarian cancer cells *in vitro*, and genistein appears to be the most potent bioactive soy component tested for anti-cancer activity. Moreover, earlier cell culture work has suggested an increased sensitivity of cisplatin-sensitive and cisplatin-resistant ovarian tumor cells to the anti-proliferative effects of certain anti-neoplastic agents when they are combined with genistein. We proposed to optimize treatment of ovarian cancer cells *in vitro* by identifying effective combinations based on the lowest effective concentrations of both the synthetic drug fenretinide and genistein. The results generated by this study were aimed to provide a greater understanding of the possible mechanisms of anti-proliferative actions of fenretinide and genistein and to explore for potential additive or synergistic effects of these compounds to prevent growth and/or decrease survival of ovarian cancer cells since they both induce anti-proliferative effects and cell cycle arrest. It was anticipated that the outcomes of this study could, in the long term, lead to the ability to control the side effects of fenretinide therapy by lowering the effective dose of the synthetic drug used clinically.

## Hypotheses:

The following are the study hypotheses for this thesis:

1- Soy milk will inhibit proliferation of A2780 ovarian cancer cells.

2- Genistein and fenretinide individually will exert inhibitory effects on the proliferation and survival of ovarian cancer cells.

3- Genistein and fenretinide in combination will exert additive or synergistic anti-proliferative effects on ovarian cancer cells.

## Objectives:

1- To compare the effects of fresh and lyophilized soy milk and fermented soy (soy kefir) fractions on the proliferation and survival of ovarian cancer cells.

2- To determine whether genistein can inhibit the proliferation and/or survival of A2780s and A2780cp ovarian cancer cells and to identify the lowest effective concentration.

3- To determine whether fenretinide can inhibit the proliferation and/or survival of A2780s and A2780cp ovarian cancer cells and determine the lowest effective concentration.

4- To identify the lowest maximally effective concentrations of the combinations of genistein and fenretinide that can impair the proliferation and/or survival of ovarian cancer cells *in vitro*.

5- To explore for possible mechanisms of action in terms of apoptosis and cell cycle arrest regarding anti-proliferative effects of genistein and fenretinide both alone and in combination at a wide range of concentrations.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Ovarian cancer cell lines

In this study, two ER- ovarian cancer cell lines were used: ES2 (ovarian carcinoma clear cells), A2780cp (cisplatin-resistant epithelial ovarian cancer cells) and A2780s (cisplatin-sensitive epithelial ovarian cancer cells) (Ferlini et al. 1999), all of which are used routinely in Dr. Vanderhyden's laboratory. All cells were maintained in MEM-α supplemented with 10% fetal bovine serum (MEM/FBS) prior to treatment.

### 2.2 Soy milk and soy kefir preparation and cell count

Soy kefir was made freshly produced in one batch with 1:3 ratio of kefir grain/soy milk and was immediately used for all experiments involving soy kefir. Soy milk (Sonice) was purchased from a local supermarket and kefir grains were obtained from the laboratory of Dr. Stan Kubow and incubated for 20 hours (1 day fermentation-1DF) and for 5 days (long term fermentation-LTF) at room temperature. The liquid soy kefir was separated from the kefir grain using a plastic screen. Soy milk and soy kefir were centrifuged for 15 minutes at 2,000 x g and again ultra-centrifuged at 13,000 x g for one hour. The supernatants were collected, pH adjusted to 7.2 and filtered through both 0.45 μm and 0.22 μm pore size filters. Serial dilution of 10 to 0% of soy milk and kefir were made in MEM/FBS and 1 ml of treatment/media distributed to wells of 24 well plates containing ES2 and A2780cp ovarian cancer cell lines. The cells were cultured (5,000 cells/well) in MEM/FBS for 24 hours prior to treatment. Treatment/media were replenished after 48 hours and the cells were incubated for three more days. Once the cells in the control group became 90% confluent, the cell numbers were determined by Coulter counter.

### 2.3 Soy milk and soy kefir lyophilization and cell count

Soy milk was lyophilized at -60$^{o}$C in a freeze-drier and resuspended in PBS/0.5% DMSO at a concentration of 20% w/v. Serial dilutions of 2, 1, 0.5, 0.25, and 0.125% for each treatment were prepared in MEM/FBS + 0.5% DMSO. All treatments were pH adjusted to 7.2 and filtered through 0.45 and 0.22 μm filters, and 1 ml of this treatment/media distributed to a 24 well plate containing 5,000 cells/well A2780cp ovarian cancer cells. The cells were cultured in MEM/FBS for 24 hours prior to treatment. Treatment/media were replenished after 48 hours and the cells were incubated for three more days. Cell numbers were determined by Coulter counter after 5 days when the control cells became 90% confluent.

### 2.4 Assessment of cell viability by MTS assay

All compounds to be tested were purchased from Sigma-Aldrich, Canada (genistin G0897 from soybeans, genistein G6776 from soybeans and fenretinide H7779). In order to test the effects of drug concentration on cell viability, dose response experiments were performed simultaneously for either fenretinide and genistein alone and their combination. The assay layout is shown in Table 1. Two epithelial ovarian cancer cell lines (A2780cp and A2780s) were seeded at 5,000 cells/well in 100 μl of MEM/FBS in 96 well plates. After 24 hours, media were replaced with 200 μL of individual and combinations of genistein (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 μM) and fenretinide (FEN: 0, 1.25, 5, 10 μM) in MEM/FBS + 0.5% DMSO. Cells were treated for up to 72 hours, and cell number and viability was then determined by MTS assay according to the manufacturer's instructions (Promega, Madison, WI). The MTS cell viability assay is a colorimetric method that can identify the cytotoxic potential of any given drug. The assay

measures the formation of a soluble formazan product which is directly proportional to the number of live cells in the culture. MTS (tetrazolium compound) was added (10 µl/well) to the cell culture and incubated at 37 ˚C for 4 hours. MTS is reduced by mitochondria to a colored formazan product, which was then quantified using a spectrophotometer at 490 nm wavelength.

Data from each set of experiments (n=3) were pooled and the effect of two parameters (treatment and drug concentration) were evaluated using two-way analysis of variance (ANOVA) analysis with Bonferroni post-test correction (GraphPad Prism version 5). In each case if the P value was less than 0.05, the effect of that parameter was considered as significantly different from control (untreated).

### 2.5 Assessment of cell viability by trypan blue exclusion/viable cell count

To determine if genistin, genistein and fenretinide could alter the proliferation and/or survival of ovarian cancer cells *in vitro*, a direct viable cell count using a VI-cell coulter counter was carried out (Beckman Coulter counter, Miami, FL, USA). Viable cell counting measures the number of viable cells in a cell suspension, which are identified by trypan blue dye exclusion. Cells with intact membranes are able to exclude the dye while cells without an intact membrane take up the coloring agent. 5,000 cells were seeded in 500 µl of MEM/FBS in a 24 well plates for 24 hours. The media were replaced with serial dilutions of genistin (0, 1, 5, 10, 50 and 100 µM), genistein (0, 15.5, 31, 62, 125 and 250 µM), and fenretinide (0, 1.25, 2.5, 5, 10 and 20 µM) in MEM/FBS + 0.5% DMSO media. The numbers of total cells, viable cells, and dead cells were counted with the VI-cell counter after 48 and 72 hours of treatment.

**Table 1:** Tissue culture plate layout for MTS assays to perform dose response experiments testing the effects of fenretinide (Fen) and/or genistein (Ein) on ovarian cancer cells. Concentrations indicated are in μM.

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| **A** | 1.25 Fen | 1.25 Fen | 1.25 Fen | 2.5 Fen | 2.5 Fen | 2.5 Fen |
| **B** | 1.25 Fen + 1.55 Ein | 1.25 Fen + 1.55 Ein | 1.25 Fen + 1.55 Ein | 5.0 Fen + 1.55 Ein | 5.0 Fen + 1.55 Ein | 5.0 Fen + 1.55 Ein |
| **C** | 1.25 Fen + 3.1 Ein | 1.25 Fen + 3.1 Ein | 1.25 Fen + 3.1 Ein | 5.0 Fen + 3.1 Ein | 5.0 Fen + 3.1 Ein | 5.0 Fen + 3.1 Ein |
| **D** | 1.25 Fen + 6.2 Ein | 1.25 Fen + 6.2 Ein | 1.25 Fen + 6.2 Ein | 5.0 Fen + 6.2 Ein | 5.0 Fen + 6.2 Ein | 5.0 Fen + 6.2 Ein |
| **E** | 1.25 Fen + 12.5 Ein | 1.25 Fen + 12.5 Ein | 1.25 Fen + 12.5 Ein | 5.0 Fen + 12.5 Ein | 5.0 Fen + 12.5 Ein | 5.0 Fen + 12.5 Ein |
| **F** | 1.25 Fen + 25 Ein | 1.25 Fen + 25 Ein | 1.25 Fen + 25 Ein | 5.0 Fen + 25 Ein | 5.0 Fen + 25 Ein | 5.0 Fen + 25 Ein |
| **G** | 1.25 Fen + 100 Ein | 1.25 Fen + 100 Ein | 1.25 Fen + 100 Ein | 5.0 Fen + 100 Ein | 5.0 Fen + 100 Ein | 5.0 Fen + 100 Ein |
| **H** | Blank | Blank | Blank | Blank | Blank | Blank |

| 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|
| 5.0 Fen | 5.0 Fen | 5.0 Fen | 10 Fen | 10 Fen | 10 Fen |
| 10 Fen + 1.55 Ein | 10 Fen + 1.55 Ein | 10 Fen + 1.55 Ein | 1.55 Ein | 1.55 Ein | 1.55 Ein |
| 10 Fen + 3.1 Ein | 10 Fen + 3.1 Ein | 10 Fen + 3.1 Ein | 3.1 Ein | 3.1 Ein | 3.1 Ein |
| 10 Fen + 6.2 Ein | 10 Fen + 6.2 Ein | 10 Fen + 6.2 Ein | 6.2 Ein | 6.2 Ein | 6.2 Ein |
| 10 Fen + 12.5 Ein | 10 Fen + 12.5 Ein | 10 Fen + 12.5 Ein | 12.6 Ein | 12.6 Ein | 12.6 Ein |
| 10 Fen + 25 Ein | 10 Fen + 25 Ein | 10 Fen + 25 Ein | 25 Ein | 25 Ein | 25 Ein |
| 10 Fen + 100 Ein | 10 Fen + 100 Ein | 10 Fen + 100 Ein | 100 Ein | 100 Ein | 100 Ein |
| Cell Blank | Cell Blank | Cell Blank | Cell Blank | Cell Blank | Cell Blank |

## 2.6 Cell cycle analysis

Cell cycle analysis is a method which employs flow cytometry to distinguish cells in different phases of their cell cycle. Cells (150,000) were seeded in 12-well plates in MEM/FBS and after 24 hours, the media were replaced with fresh media containing serial dilutions of genistin (0, 1, 5, 10, 50 and 100 μM), genistein (0, 15.5, 31, 62, 125 and 250 μM), and fenretinide (0, 1.25, 2.5, 5, 10 and 20 μM), alone or in combination, in MEM/FBS + 0.5% DMSO media. Cells were harvested 24 hours after treatment and washed twice in PBS. Cells were then fixed in 70% ethanol for 30 minutes at -20˚C. Ethanol was discarded after spinning for 7 minutes at 1,200 rpm and cells were washed with PBS once. PBS was replaced with 50 μg/ml propidium iodide (PI) and cells stained for 30 minutes at 4˚C. Cell cycle was evaluated with a flow cytometer.

The amount of propidium iodide (PI)-stained DNA in each cell at certain wavelengths directly correlates with the fluorescence intensity of the stained cells. The DNA content of cells during the course of the cell cycle is doubled starting from synthesis phase (S-phase) until end of G-Mitosis (GM) which includes (S-phase, G2 phase, and GM phase) meaning that the relative amount of DNA at G0 and G1 phases is half of the amount of DNA at S-phase, G2/GM phases. The percentage of cells in each phase of the cell cycle was calculated using Modfit LT software (version 3.2.1, July 08, 2008, Topsham Maryland, USA).

# CHAPTER 3: RESULTS

## *Part 1: Examination of the effects of fresh and lyophilized soy milk and soy kefir on the proliferation and viability of ovarian cancer cell lines*

The growth inhibition and cytotoxic effects of fresh and lyophilized soy milk and soy kefir (fermented soy milk) fractions were studied on ES2 and A2780-cp ovarian cancer cell lines in three preliminary experiments. The anti-proliferative effects of all tested soy milk and soy kefir fractions in both cell lines were examined in a dose response manner. Both fresh and lyophilized soy milk and soy kefir fractions showed strong anti-proliferative effects on both ovarian cancer cell lines. (Figures 4-7). The anti-proliferative effect of soy milk was 8.1% more potent than the effect of soy kefir, suggesting that soy milk lost some of its bioactivity during the fermentation process. Lyophilized soy milk showed greater anti-proliferative bioactivity than either fresh soy milk or fresh and lyophilized kefir fermented soy milk suggesting that lyophilization most likely concentrated the bioactive components of soy milk.

In the first pilot experiment (Figure 3), the effect of fresh soy milk and soy kefir on the rate of proliferation of A2780cp and ES2 ovarian cancer cells was determined. This exploratory experiment (n=1) showed a possible anti-proliferative effect of fresh soy milk and soy kefir on ovarian cancer cells which can not be statistically interpreted. Figure 3A shows an 81% proliferation suppression of A2780cp cells after 5 days of treatment with a 10% (v/v) concentration of 1 day fermented (non-lyophilized) soy kefir (1DF) vs. 69% proliferation suppression from the 5 day fermented soy kefir (LTF). Figure 3B also indicates a 95% proliferation suppression of ES2 cells after 5 days of

treatment with a 5% (v/v) concentration with both 1 day and 5 day fermented (non-lyophilized) soy kefir. Firm conclusions from these 1- and 5-day fermentation experiments cannot be made since these were preliminary exploratory studies that need to be repeated.

To examine this anti-proliferative effect further in the soy milk and kefir fermented products, the soy products were concentrated by lyophilization and tested on the A2780cp cell lines (Figures 4 and 5). Although the isoflavone content of the tested soy products was not measured, the anti-proliferative effects of both lyophilized soy kefir and soy milk were tested in a dose response manner. Figure 4 shows a dramatic decrease in the number of A2780cp cells after 5 days of treatment with lyophilized soy milk (0-2% lyophilized soy milk-w/v) in a dose response manner. The amount of DMSO used on the cell lines in all experiments never exceeded 0.5%. Both tested cell lines were shown to tolerate concentrations of 1% DMSO without adverse effects on cell viability, which was tested prior to the initiation of the experiments (data not shown). Figure 5A indicates a 98% proliferation suppression of A2780cp cells after treatment with 1% (w/v) of lyophilized soy kefir. There was a similar effect with a 96% suppression of proliferation of A2780cp cells after treatment with 0.5 % (w/v) of lyophilized soy milk. As shown in Figure 5B, control cells receiving no treatment apart from exposure to 0.5% DMSO showed no inhibition on cell proliferation or effects on cell viability.

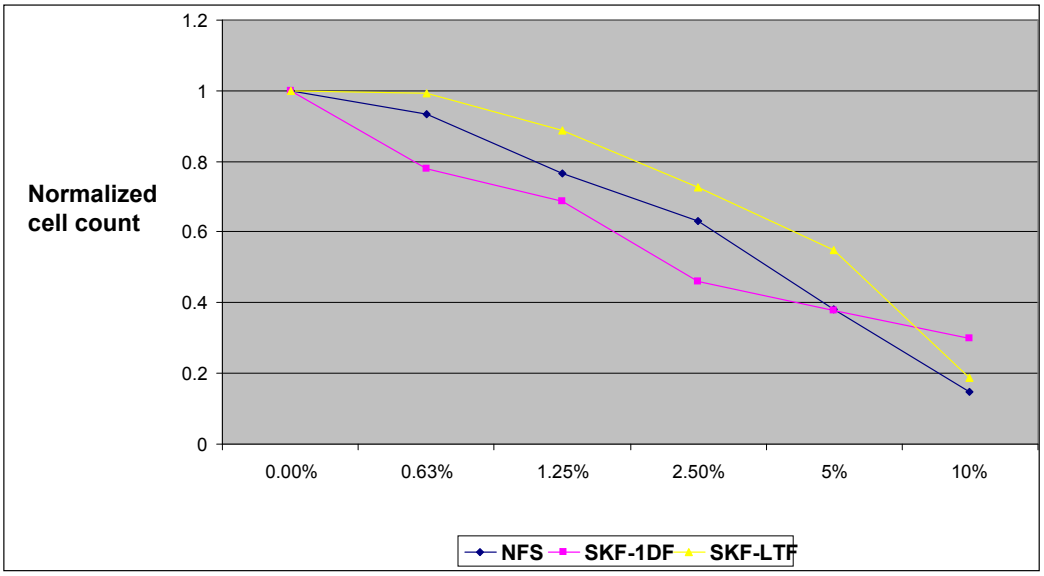
The IC50 of the two treatments (soy milk and soy kefir) were calculated at two time points (48 and 120 hours; Figure 6A). The 120 hours of treatment with soy milk showed the lowest IC50 (0.26 g/dl) in comparison to the 120 hours of treatment with soy kefir (IC50= 0.45 g/dl) and 48 hours treatment with either soy kefir (0.66 g/dl) or soy

milk (0.4 g/dl). Figure 6B showed a comparison of the percentage of anti-proliferative efficiency of soy milk vs. soy kefir, calculated according to the %IC50. The anti-proliferative efficiency of soy milk fraction was 8.1% higher than soy kefir group; however, it is possible such a minor difference in anti-proliferative activity between treatments would not show statistical significance if more experiments were performed due to inherent variability in treatment effects among experiments. Moreover, such relatively minor differences in anti-proliferative activities are unlikely to exhibit significant differences in studies performed to determine possible *in vivo* anti-proliferative effects of the two types of soy products.

[Drug efficiency=100-(SM IC50/SK IC50)*100].

The above findings have shown the strong anti-proliferative effects of soy milk fractions on A2780cp ovarian cancer cells, suggesting that some of the compounds present in soy milk can suppress proliferation of these cells.

**A**




**B**

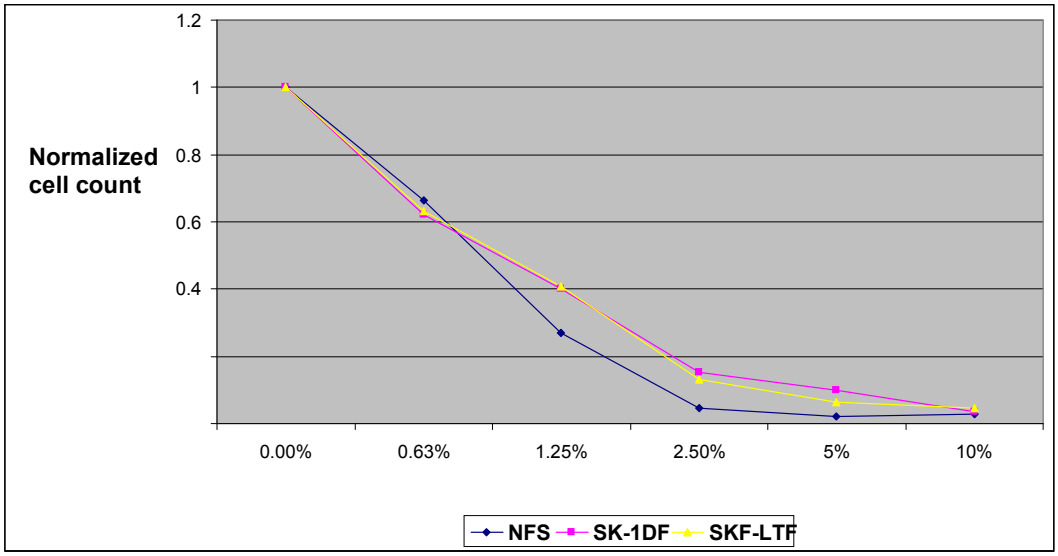



**Figure 3: Effect of soy milk and fermented soy milk (soy kefir) on A2780cp cells (A) and ES2 cells (B).** Cell numbers were determined using a Coulter counter after 3 days when the control cells became 90% confluent. This figure shows cell numbers after 5 days treatment with up to 10% (v/v) concentration of 1 day fresh (non-lyophilized) soy kefir fermented (1DF) vs. 5 days fresh (non-lyophilized) soy kefir fermented (LTF), respectively in a dose response manner. This graph shows cell counts normalized to the control non-treated group. One experiment was performed with 6 replicates (wells) per treatment. *NFS= Non-Fermented Soy, SKF-1DF= Soy Kefir Fermented-1 day Fermentation, SKF-LTF= Soy Kefir Fermented- Long Term Fermentation*

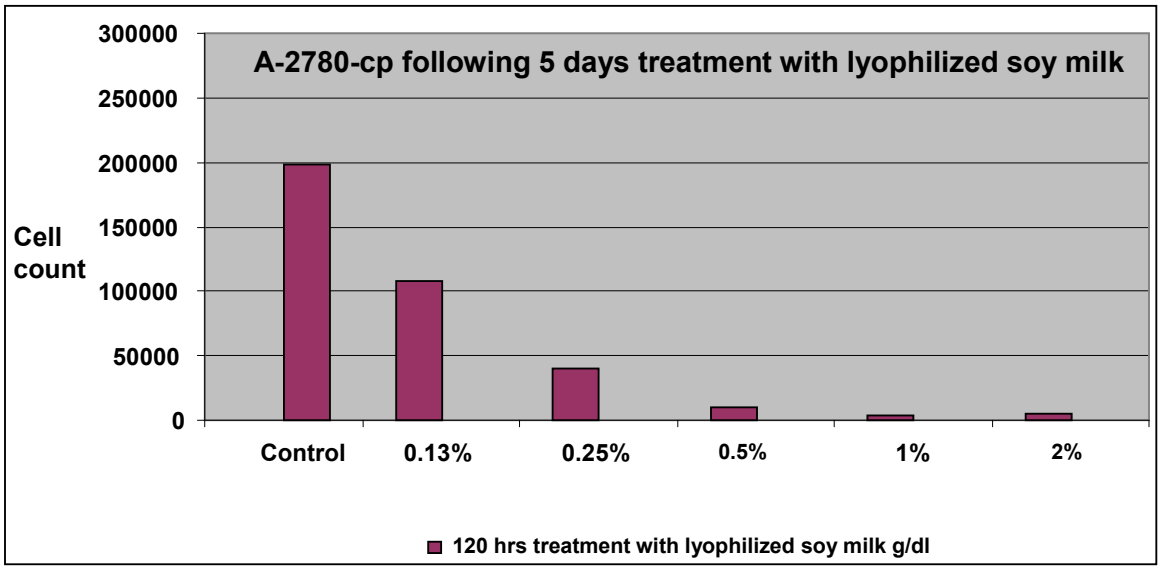
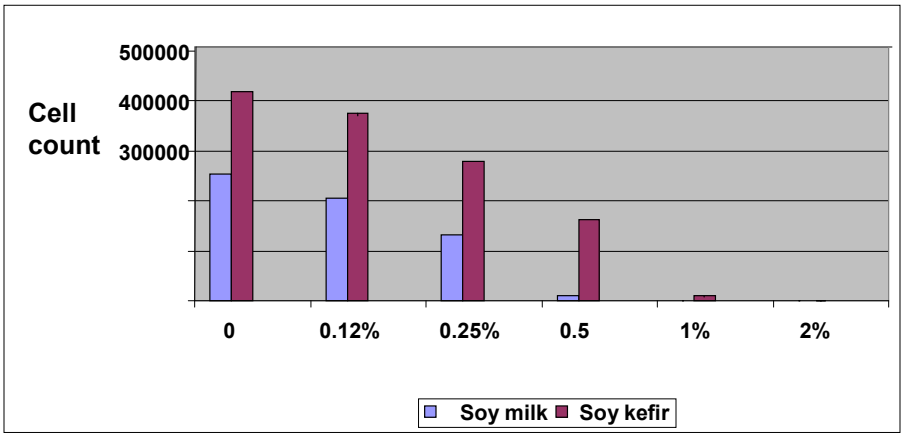



**Figure 4: Effect of lyophilized soy milk on A2780cp (w/v).** Cell numbers were determined by an automatic Coulter counter when cells became 90% confluent in the control wells. This figure shows a dramatic decrease in number of A2780cp cells after 5 days treatment with lyophilized soy milk (w/v) in a dose response manner. One experiment was performed with 6 replicates (wells) per treatment, *SM-120hrs= Soy Milk-120 hours treatment.*

**A**




**B**

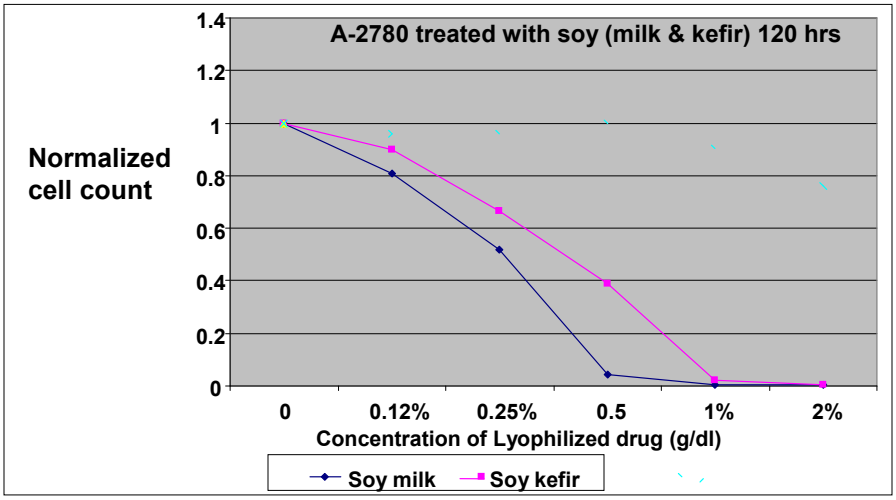
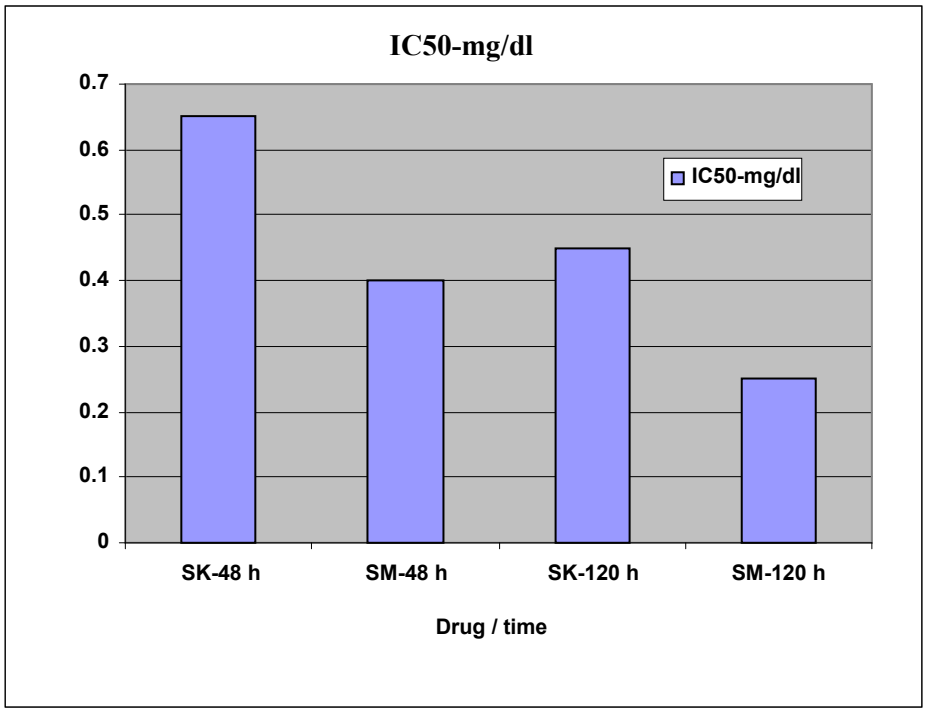



**Figure 5: Effect of lyophilized soy milk and soy kefir on A2780cp cells (w/v).** Cell number was determined by an automatic Coulter counter when cells became 90% confluent in the control wells. Figure 5A shows the mean cell number ± standard error of the mean for soy milk vs. soy kefir experiments. Cells were treated with 2% (w/v) of lyophilized soy kefir. Figure 5B shows the same data with cell counts normalized to the control non-treated group. One experiment was performed with 6 replicates (wells) per treatment. *SM-120hrs= Soy Milk- 120 hours treatment.*

**A**




**B**

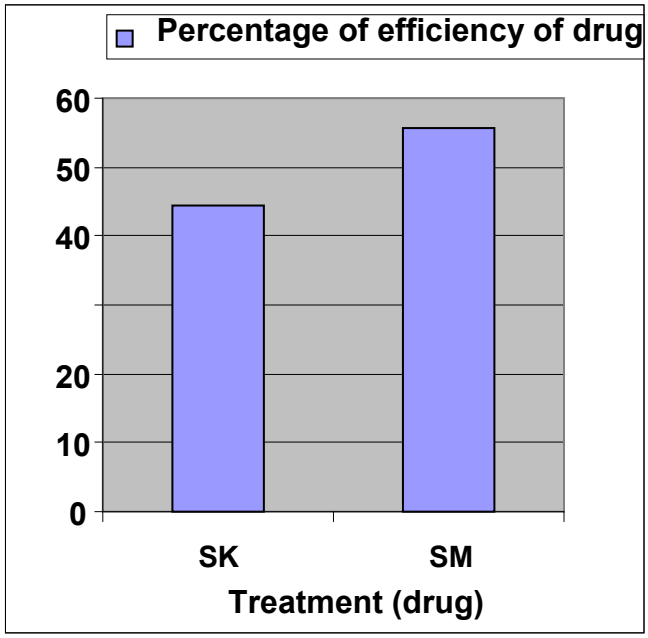



**Figure 6: (A) Calculated IC50 for two treatments (soy milk, SM, and soy kefir, SK) at two time points (48 and 120 hours).** Cells treated for 120 hours with soy milk showed the lowest IC50 (0.26 g/dl) compared to 120 hours treatment with soy kefir (IC50= 0.45). (**B) Comparison of percentage of drug efficiency of soy milk (SM) vs. soy kefir (SK).** *[Drug efficiency=100-(SM IC50/SK IC50)*100]*.

## *Part 2: Effects of genistein, genistin and fenretinide on ovarian cancer cells*

As described earlier, one of the main long-term goals of the research program is to reduce the side effects of fenretinide and improve the treatment regimen by lowering the effective dose of fenretinide when used in combination with natural bioactive isoflavones derived from soy milk. One of the side effects of fenretinide is nyctalopia (reversible night blindness), especially in children. Hence, it is proposed that the effective dose of fenretinide as an anti-proliferation drug can be minimized when administered in combination with an optimally effective dose of genistein, which could have potential to lower the side effects of fenretinide in a clinical setting.

To identify the lowest effective anti-proliferative and cytotoxic concentrations of fenretinide and the most bioactive isoflavone compounds present in soy milk, two specific isoflavones compounds genistein and genistin, were studied alone or in combination with fenretinide in the ovarian cancer cell culture model. These experiments were aimed to show the possible synergistic or additive effects of the lowest concentrations of three individual compounds (genistein, genistin and fenretinide) and their combinations to inhibit proliferation of two epithelial ovarian cancer cells (A2780s and A2780cp). The two methods used to determine cell viability were the MTS assay and trypan blue exclusion/viable cell counts.

### Part 2A: MTS Assay Results

To determine the effects of the various compounds on ovarian cancer cell proliferation and survival, MTS assays were performed on cells treated with individual

compounds and combinations of the tested compounds: (i) genistein, (ii) genistin, (iii) fenretinide: (iv) genistein + fenretinide, and (v) genistin + fenretinide. Cells (5,000) from three ovarian cancer cell lines (A2780cp, A2780s and ES-2) were seeded for 24 hours in 100 µl of MEM/FBS in 96 well plates. After 24 hours media were replaced with 200 µl of individual and combination drugs in MEM/FBS + %0.5 DMSO.

The results of the MTS experiments are shown in Figures 7-12 and indicate that none of the compounds, either alone or in combination, had any significant anti-proliferative or cytotoxic effect on any of the three ovarian cancer cell lines. Statistical analysis of the results from MTS assays confirmed that the effects of the compounds were not significantly different for any of the individual drugs or their combinations vs. the untreated control cells. The various concentrations of drugs had the same effect on the measured outcomes indicating that any variance in data for the MTS assay was not due to the concentrations used. Since the MTS assays were not successful in revealing any notable effects of the drugs, an alternative approach (Vi-Cell counts) was then used to confirm that the tested compounds were indeed inactive against ovarian cancer cells.

**Genistein and Fenretinide on A2780cp cells**

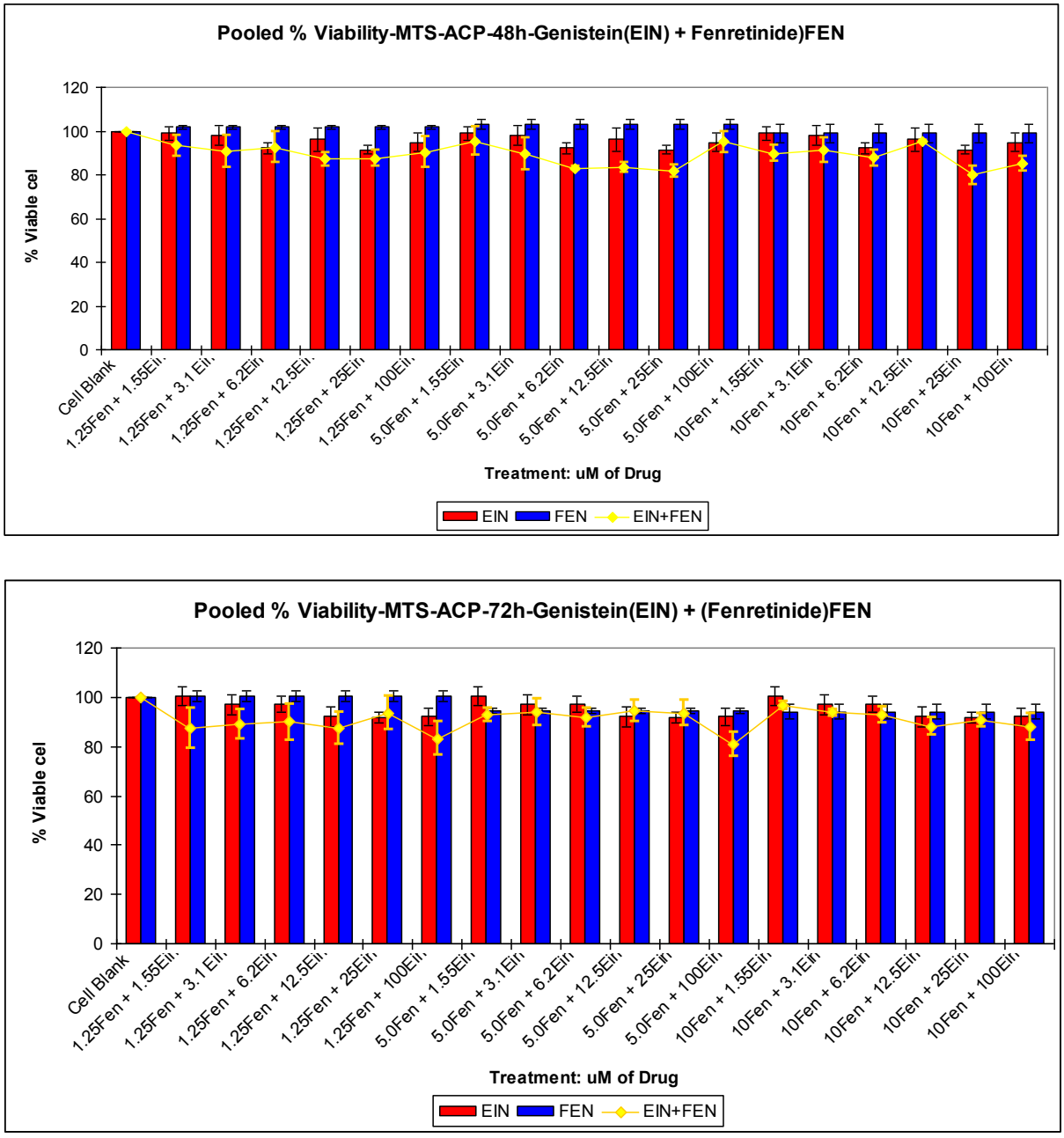



**Figure 7:** Percent viability determined by MTS assay (n=3) on A2780cp cell lines after 48 hours (top) and 72 hours (bottom) treatment with genistein (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM, red bars), fenretinide (FEN: 0, 1.25, 5, 10 µM, blue bars) and genistein + fenretinide (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM + FEN: 0, 1.25, 5, 10 µM cross concentration, yellow line). Data shown are the mean percentages and the standard error of the mean. Three experiments were performed with 6 replicates (wells) per treatment in each experiment.

**Genistin and Fenretinide on A2780cp cells**

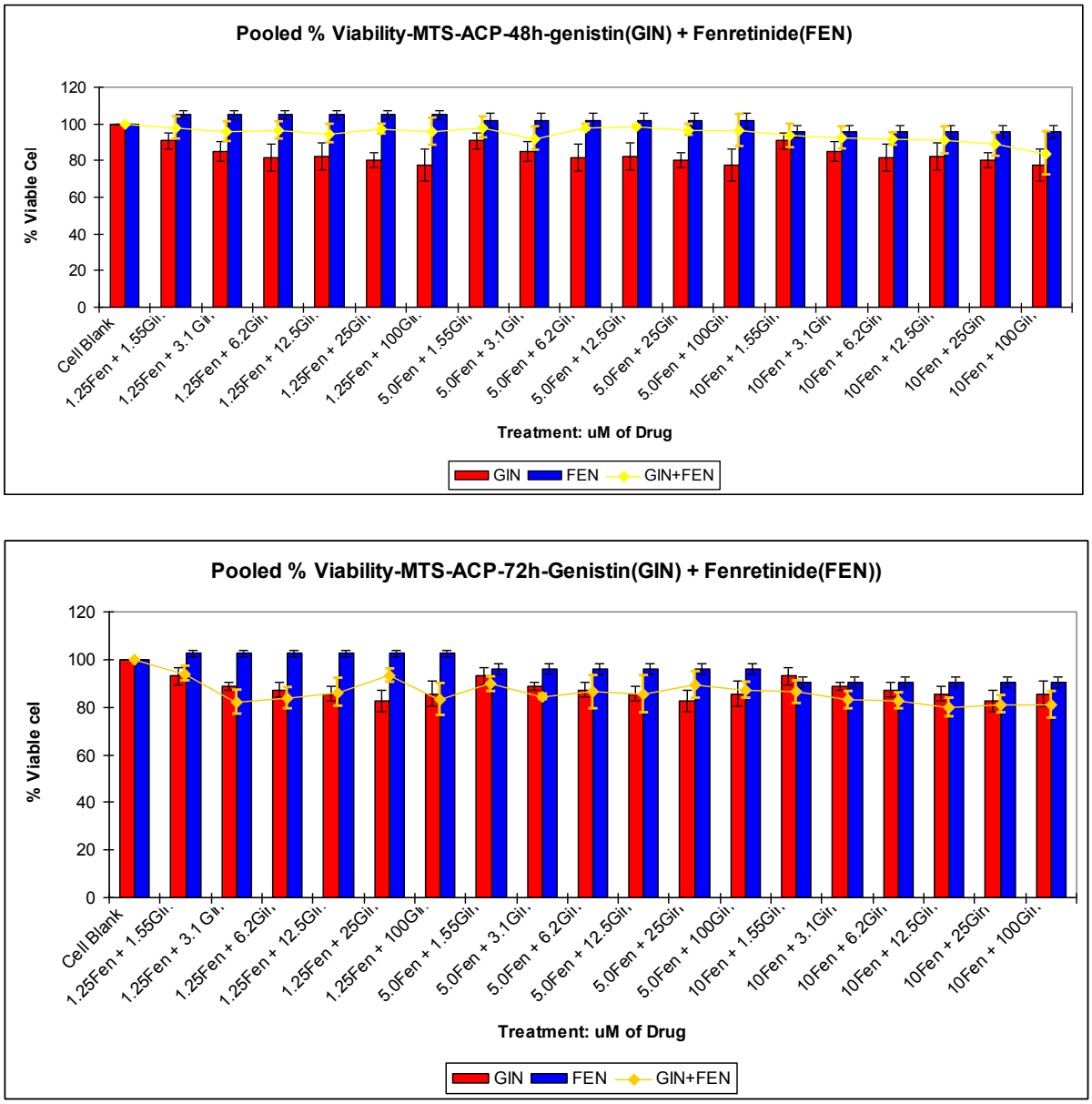



**Figure 8:** Percent viability determined by MTS assay (n=3) on A2780cp cell lines after 48 hours (top) and 72 hours (bottom) treatment with genistein (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM, red bars), fenretinide (FEN: 0, 1.25, 5, 10 µM, blue bars) and genistein + fenretinide (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM + FEN: 0, 1.25, 5, 10 µM cross concentration, yellow line). Data shown are the mean percentages and the standard error of the mean. Three experiments were performed with 6 replicates (wells) per treatment in each experiment.

## Genistein and Fenretinide on A2780s cells

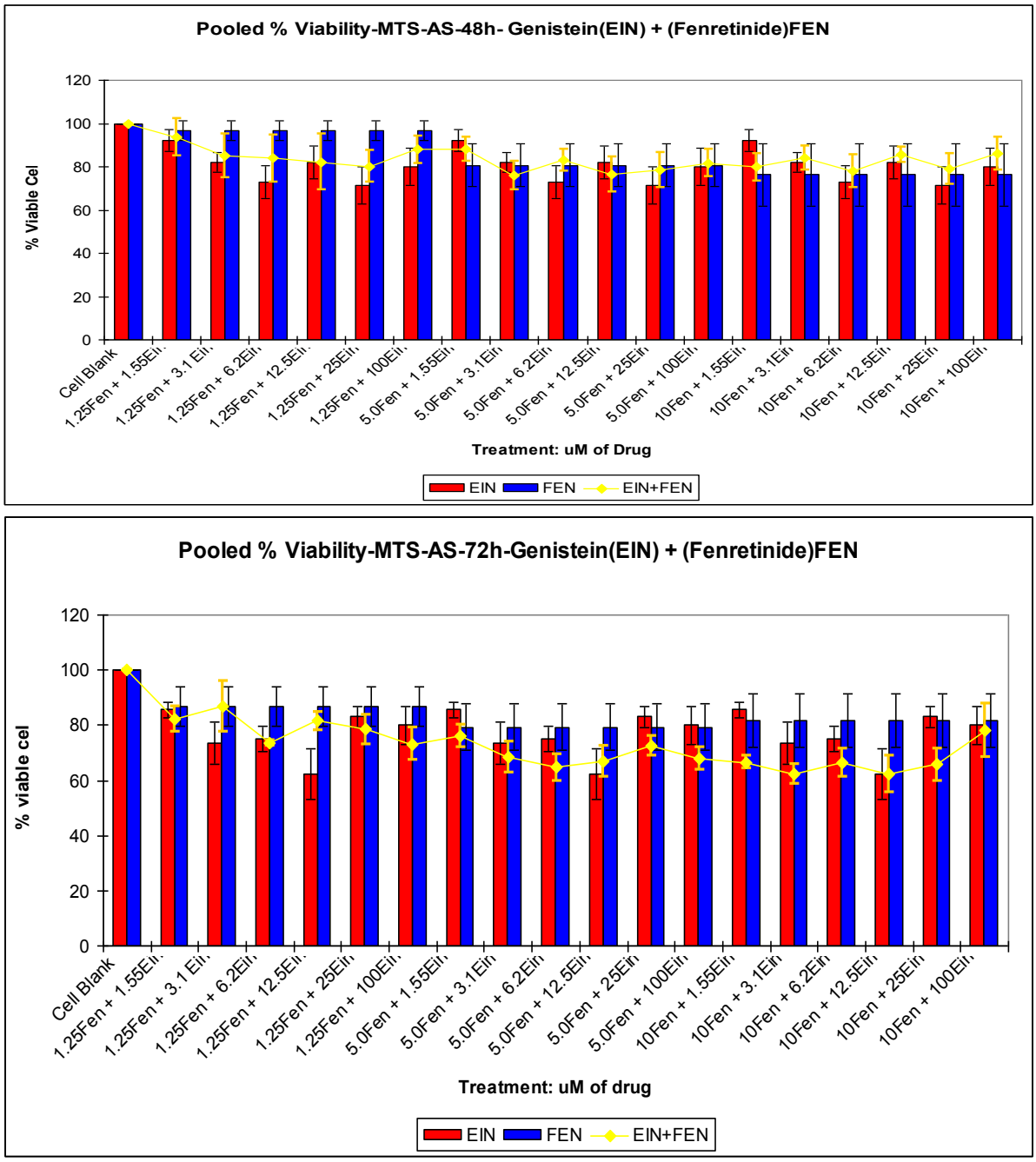



**Figure 9:** Percent viability determined by MTS assay (n=3) on A2780s cell lines after 48 hours (top) and 72 hours (bottom) treatment with genistein (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM, red bars), fenretinide (FEN: 0, 1.25, 5, 10 µM, blue bars) and genistein + fenretinide (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM + FEN: 0, 1.25, 5, 10 µM cross concentration, yellow line). Data shown are the mean percentages and the standard error of the mean. Three experiments were performed with 6 replicates (wells) per treatment in each experiment.

## Genistin and Fenretinide on A2780s cells

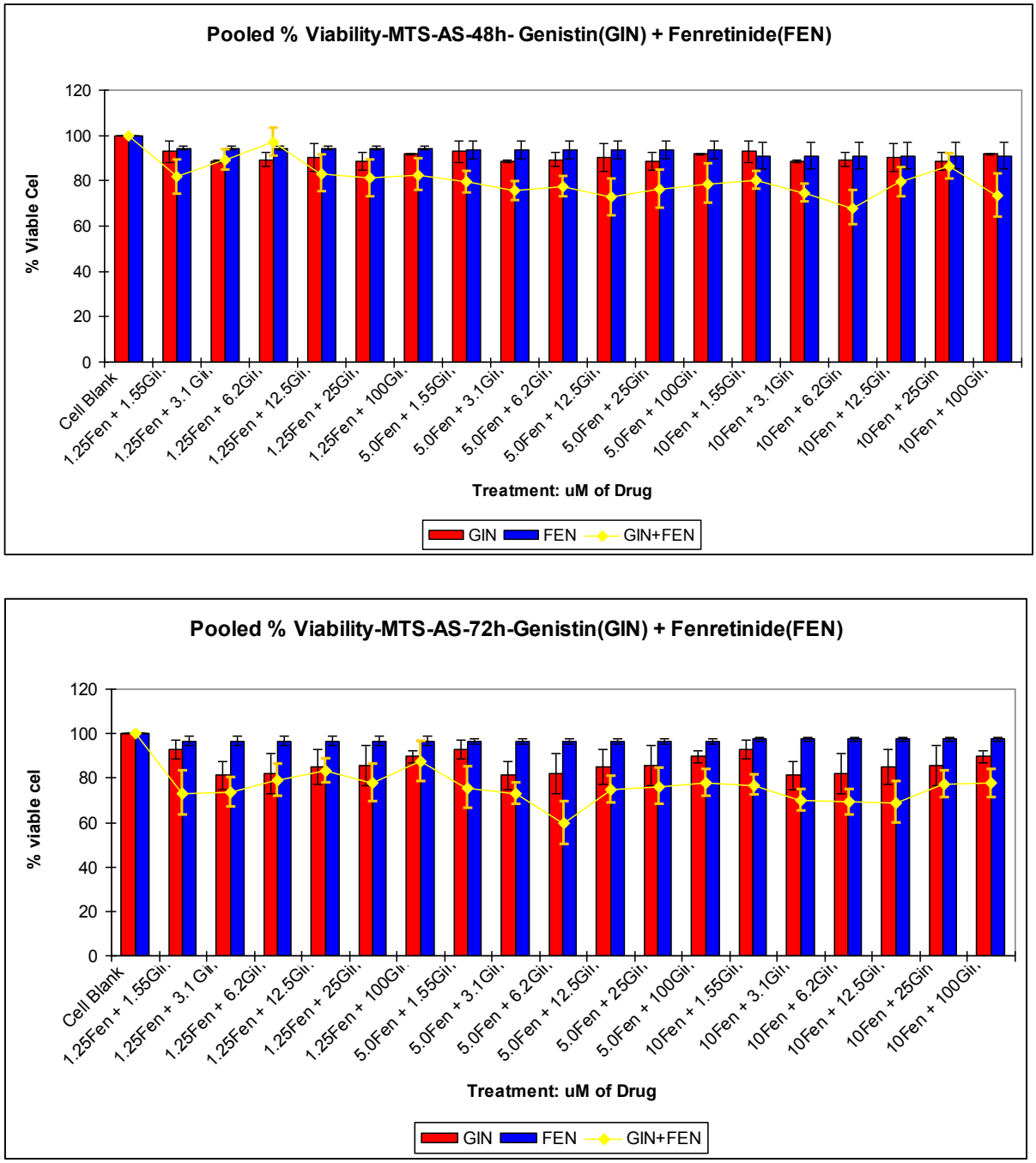



**Figure 10:** Percent viability determined by MTS assay (n=3) on A2780s cell lines after 48 hours (top) and 72 hours (bottom) treatment with genistein (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM, red bars), fenretinide (FEN: 0, 1.25, 5, 10 µM, blue bars) and genistein + fenretinide (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM + FEN: 0, 1.25, 5, 10 µM cross concentration, yellow line). Data shown are the mean percentages and the standard error of the mean. Three experiments were performed with 6 replicates (wells) per treatment in each experiment.

**Genistein and Fenretinide on ES-2 cells**

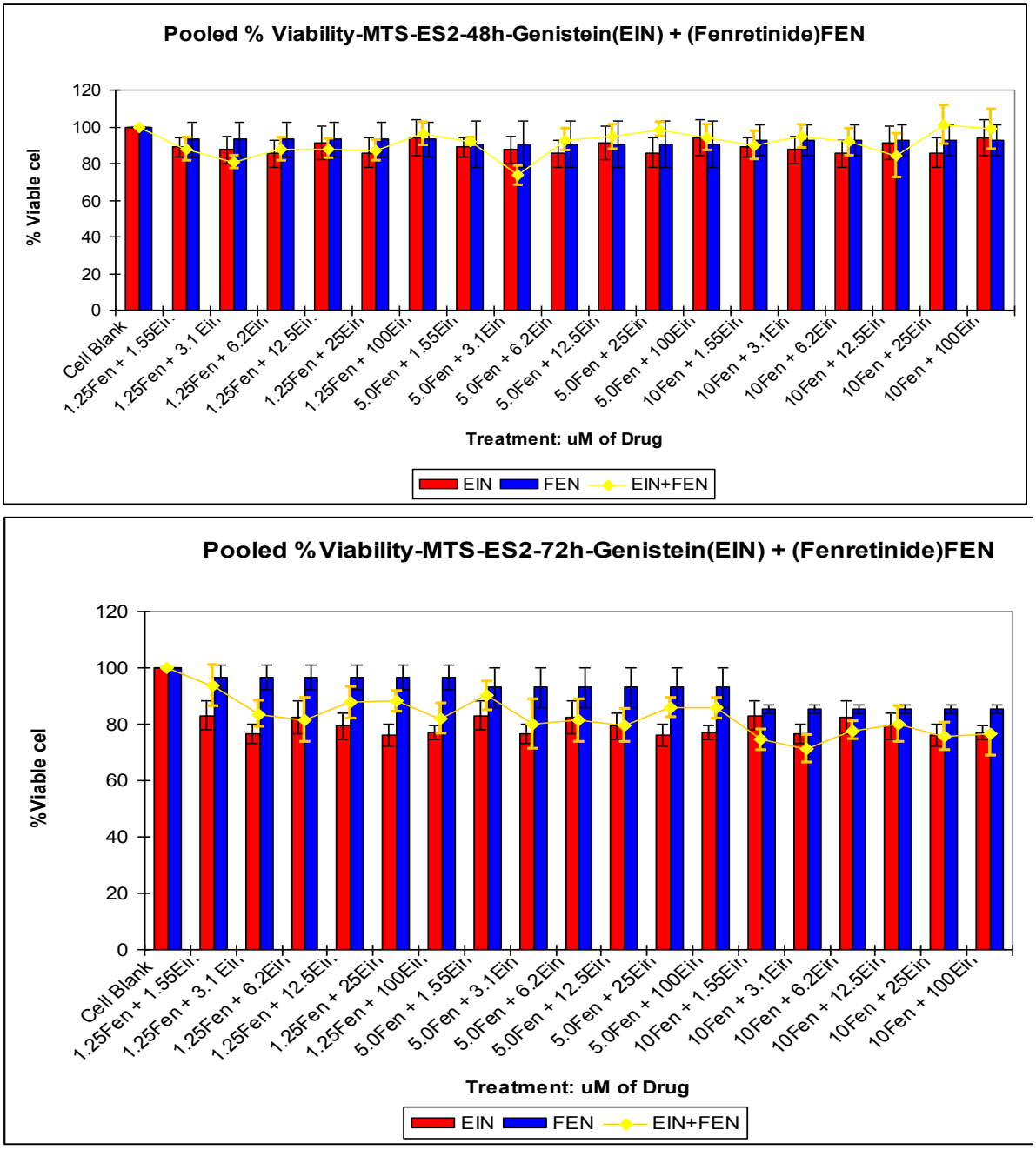



**Figure 11:** Percent viability determined by MTS assay (n=3) on ES2 cell lines after 48 hours (top) and 72 hours (bottom) treatment with genistein (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM, red bars), fenretinide (FEN: 0, 1.25, 5, 10 µM, blue bars) and genistein + fenretinide (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM + FEN: 0, 1.25, 5, 10 µM cross concentration, yellow line). Data shown are the mean percentages and the standard error of the mean. Three experiments were performed with 6 replicates (wells) per treatment in each experiment.

**Genistin and Fenretinide on ES-2 cells**

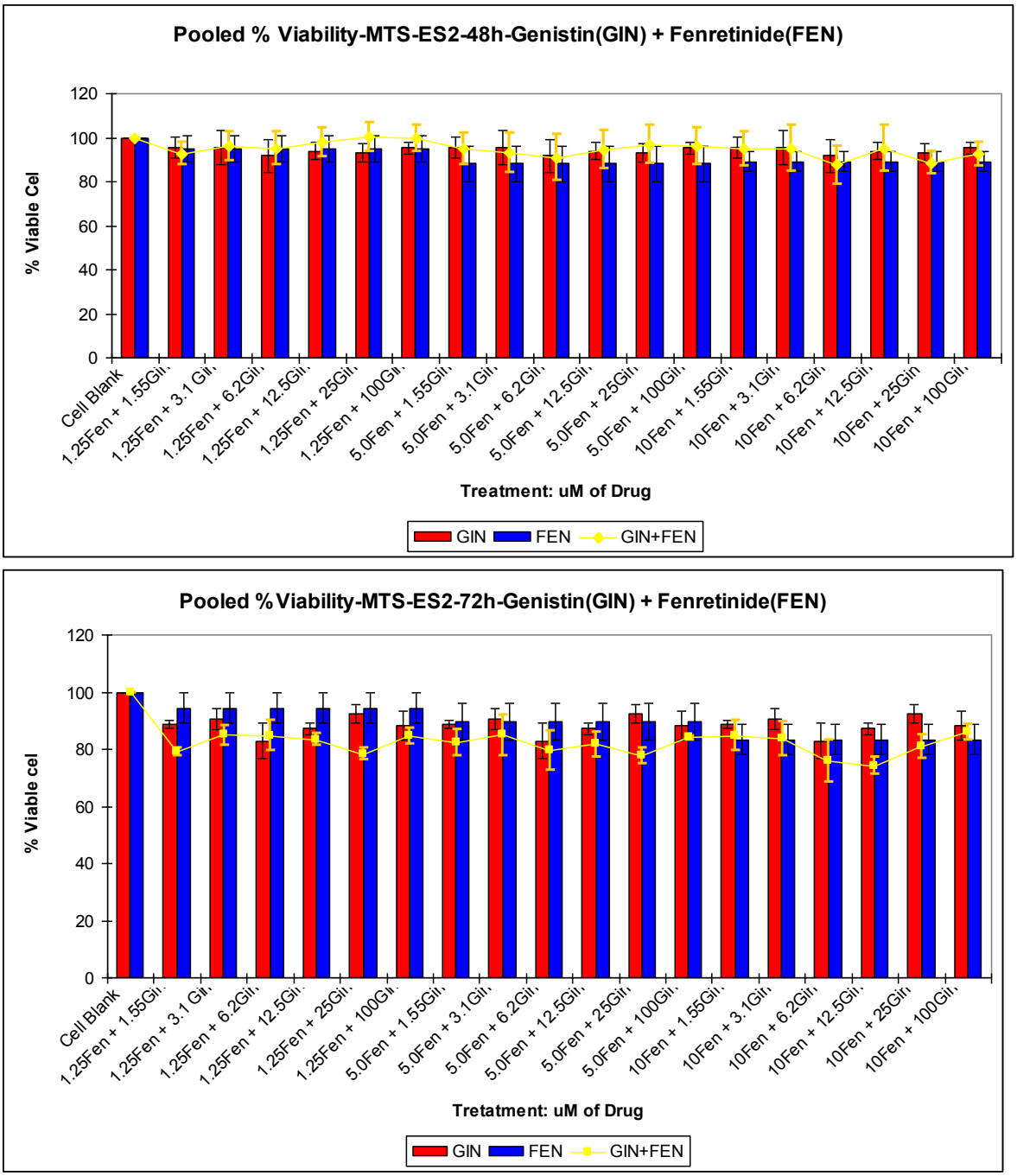



**Figure 12:** Percent viability determined by MTS assay (n=3) on ES2 cell lines after 48 hours (top) and 72 hours (bottom) treatment with genistein (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM, red bars), fenretinide (FEN: 0, 1.25, 5, 10 µM, blue bars) and genistein + fenretinide (EIN: 0, 1.55, 3.1, 6.2, 12.5, 25, 100 µM + FEN: 0, 1.25, 5, 10 µM cross concentration, yellow line). Data shown are the mean percentages and the standard error of the mean. Three experiments were performed with 6 replicates (wells) per treatment in each experiment.

**Part 2B: Cell viability by trypan blue exclusion**

**Part 2B-I: Effects of fenretinide, genistin and genistein on A2780cp cells**

Pilot experiments were carried out to determine whether the test compounds exerted effects on ovarian cancer cell viability as assessed by direct viable cell counting using the VI-cell coulter counter. All three individual compounds (fenretinide, genistin and genistein) were found to inhibit A2780cp cell number after 48 hours without an increase in the number of dead cells, which suggests that all three compounds inhibited proliferation of A2780cp cells (Figure 13A-C). This latter effect included a 75% decrease in viable A2780cp cells in response to the maximum concentration of fenretinide (n=1), a 40% decrease in response to genistin (n=1) and 49% decrease in viable cells in response to genistein (n=1). As shown in Figures 7 to 12 the MTS assays were unable to confirm the cell viability observations, which might be mediated by interference in the MTS assay by phytochemical compounds as noted in previous studies (Wang et al, 2010).

**Part 2B-II: Effects of genistein on A2780s cells**

As shown in Figure 14, genistein had a cytotoxic effect on A2780s cells after 48 hours. There was a 65% decrease in viable A2780s cells after 48 hours exposure to genistein (n=1). This experiment suggested that the cytotoxic effect of genistein can be related to either inhibition of cell proliferation (decreased number of viable cells, red bars) or induced apoptosis (increased number of dead cells, green bar) or both.

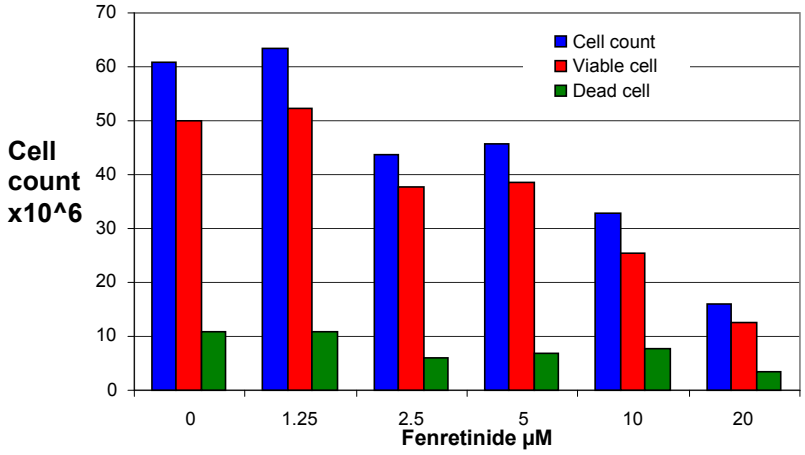
**Part 2B-III: Effects of fenretinide on ES2 cells**

In a preliminary experiment to test the effects of fenretinide on ES2 ovarian

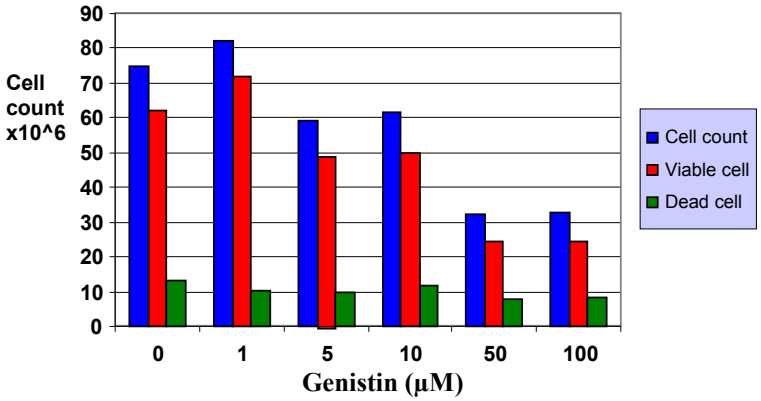
cancer cells, fenretinide had a cytotoxic effect at increasing concentrations on ES2 cell line after 48 and 72 hours (Figure 15). There was a 37% and 63% decrease in viable ES2 cells after 48 and 72 hour exposure, respectively, to fenretinide at the highest dose of 20 μM. In Figures 15A and B, the control cells were provided with the same amount of serum and DMSO as used in the treatment groups over the same period of incubation. Since no adverse effect on cell growth or viability was noted in the control group, the observed effects from the test compounds are not likely mediated by either serum starvation or DMSO cytotoxicity.

The three experiments in Part 2B determined that all three compounds were effective at suppressing proliferation of at least one of the ovarian cancer cell lines. Subsequent experiments were therefore designed to investigate those effects in greater detail.

A




B




C

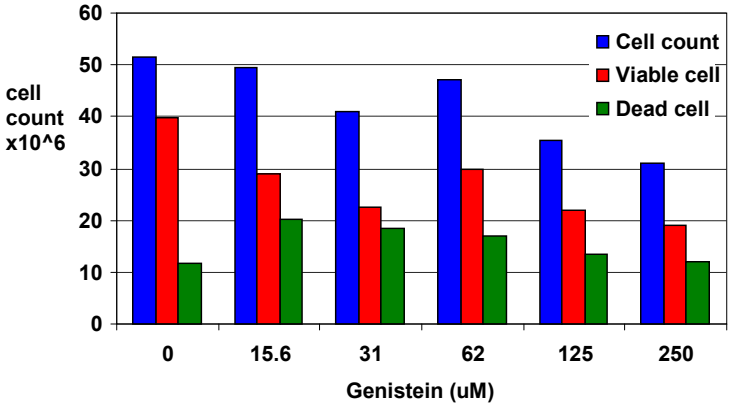



**Figure 13:** Number of total, viable and dead cell counts of A2780cp cells after 48 hours of treatment with A) fenretinide 0, 1.25, 2.5, 5, 10, 20 µM, B) genistin 0, 1, 5, 10, 50, 100 µM, and C) genistein 0, 15.6, 31, 62, 125, 250 µM.

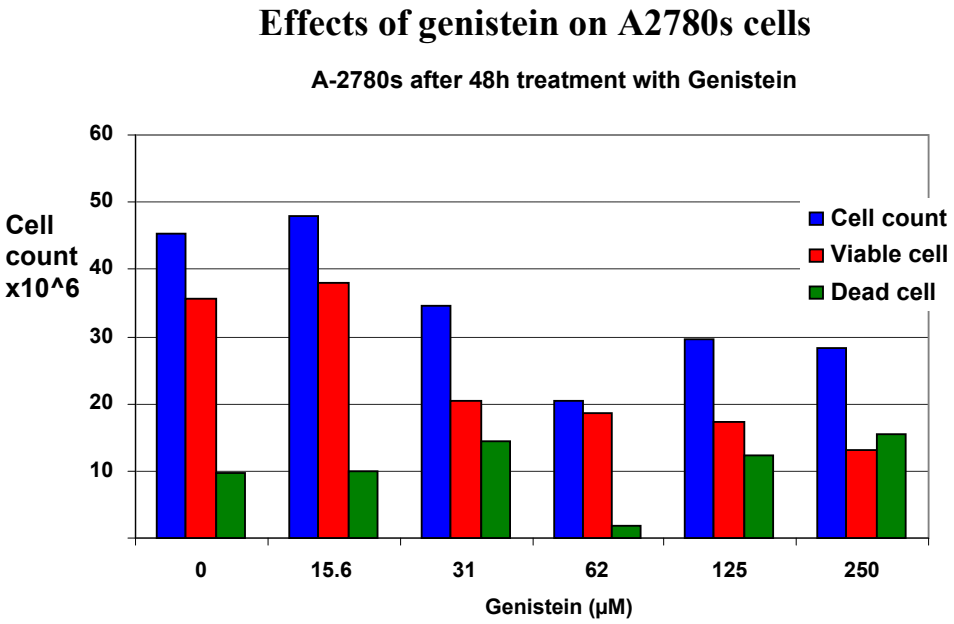
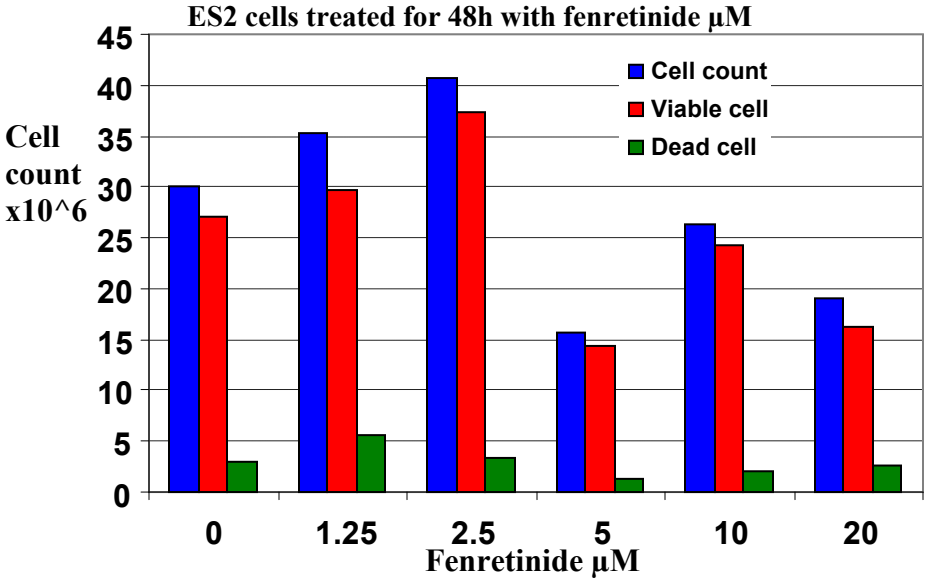



**Figure 14: Total and viable cell count of A2780s cells after 48 hrs treatment with genistein.** There is a 65% decrease in viable A2780s cells after 48 hours exposure to genistein (n=1). Cytotoxic effects of genistein may be due to inhibition of proliferation (decrease in the number of viable cells red bars) or induced apoptosis or both. One experiment was performed with 6 replicates (wells) per treatment.

**Effects of fenretinide on ES2 cells**

**A**




**B**

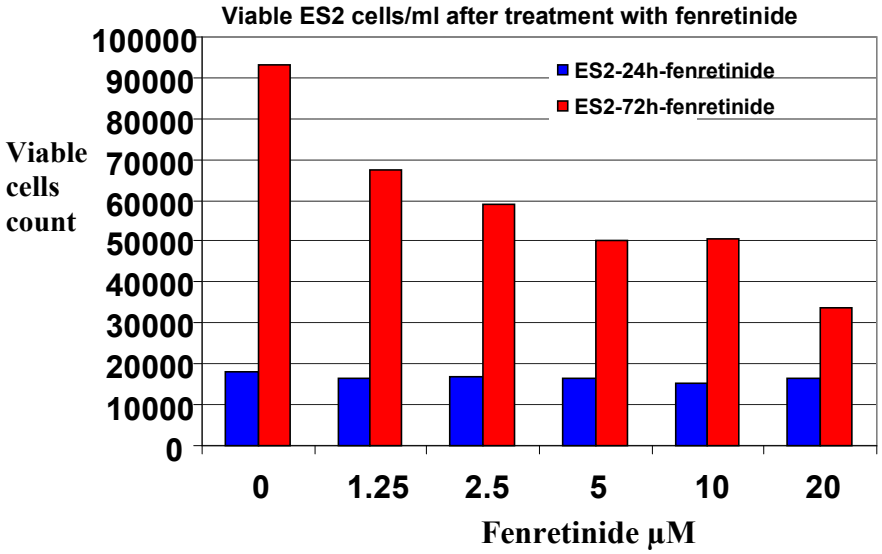



**Figure 15:** Total and viable cell counts of ES2 cell lines after treatment with fenretinide (4-HPR) for 48 hours (A) and for 24 and 72 hours (B). Fenretinide has no effect after 24 hours, but has a cytotoxic effect at increasing concentrations after 48 hours, with a 37% decrease in viable ES2 cells after 48 hours exposure to the highest concentration of fenretinide. After 72 hours, there is a 55 % decrease in ES2 cell viability at this highest dose. One experiment was performed with 6 replicates (wells) per treatment.

## PART 2C: Anti-proliferative and cytotoxic effects of Isoflavones and Fenretinide Tested at Low Concentrations

Current available evidence indicates that one of the most bio-available isoflavones from soy products is genistein, which is an aglycone form of isoflavones. In the present study we examined possible additive or synergistic effects of naturally occurring isoflavones derived from soy milk (genistein, genistin) and a synthetic retinoid drug (fenretinide). Since the aglycone form of isoflavones (genistein) from soy milk has higher bioavailability than the glucosidic form (genistin), our focus in this study was to examine the effects of genistein.

The following experiments were designed to examine the possible additive or synergistic effects of the lowest and highest concentrations of genistein, genistin and fenretinide to inhibit proliferation of ovarian cancer cells through testing cell viability and cell cycle of the A2780cp and A2780s cell lines. We used three methods to determine cell viability in the remaining experiments: 1) trypan blue viable cell counting; 2) MTS assay; and 3) cell cycle analysis by flow cytometry.

### Part 2C-Ia: Effect of fenretinide and genistein on cell viability of A2780cp cells

The viable cell count of A2780cp cells treated for 72 hours with increasing concentrations of genistein decreased (30%) with respect to the non-treated controls (Figure 16). On the other hand, fenretinide treatment decreased the number of viable cells (by ~20% at all concentrations) compared with untreated cells. When genistein and fenretinide were used in combination, the treatment decreased the number of viable cells

with increasing concentrations of genistein. Comparison of the groups indicated that the lowest concentration of fenretinide used was the most effective concentration for this drug for decreasing the number of viable cells. The results indicate that the reduction in the viable cell number was associated with both fenretinide and genistein treatments, but that genistein had more pronounced effects, both with or without fenretinide.

To determine the statistical significance of these findings, two-way ANOVA was performed along with Bonferroni post-hoc tests to allow for multiple comparisons of concentrations of fenretinide and concentrations of genistein as factors in the viability assay for the A2780cp cell line. The main effect of concentration of genistein was significant ($F(6,105)= 29.07$, $p< 0.05$). The main effect of concentration of fenretinide was also significant ($F(4,105)= 8.42$, $p < 0.05$). All pair-wise comparisons were not significantly different ($p > 0.05$) except for the combinations marked with (**) in Tables 2-7 that indicate the t values. These analyses indicate that only the lowest concentration of fenretinide (0.62 μM) decreased the number of viable A2780cp cells, which was not significantly different. There was no dose response relationship between increasing concentrations of fenretinide higher than 1.25 μM and loss of cell number and viability. In contrast, there was a dose response between increasing concentrations of genistein and a decrease in the number of viable A2780cp cells. There were no additive or synergistic anti-proliferative effects of any concentration of fenretinide and genistein when used in combination as assessed by the trypan blue assay. At all concentrations, adding fenretinide appears to be protecting the cells from the effects of genistein alone. In consideration of the findings that: a) the percentage of viable cells (data are not shown) was constant at the time of seeding to the end of the experiment (about 85% cell

viability); and b) the previous MTS assays indicated that cells were not killed by low concentrations of these drugs, the above data suggest that the compounds used at low concentrations have more pronounced effects on cell proliferation (e.g. cell cycle) rather than on cell survival or metabolic activity.

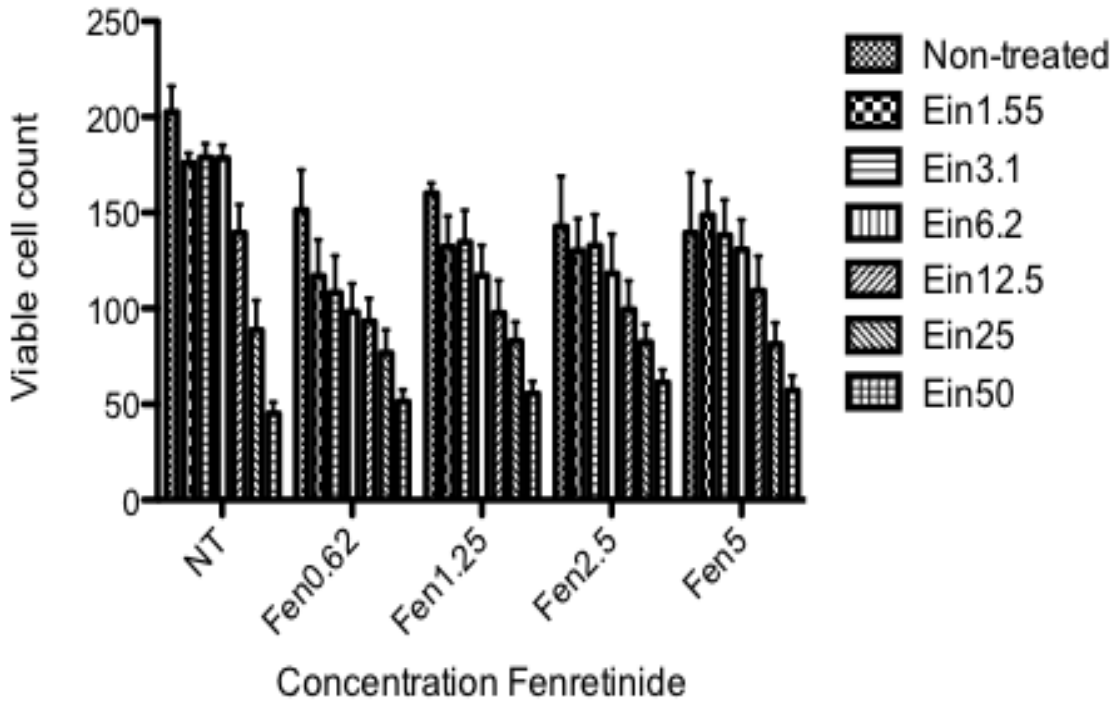



**Figure 16: Number of viable A2780cp cells after treatment with increasing doses of genistein (Ein) and fenretinide (Fen) for 72 hours.** A2780cp cells (10,000) were seeded in 1,000 µL of MEM/FBS in a 24 well plate. Media was replaced with fresh media containing serial dilutions of genistein (0, 1.55, 3.1, 6.2, 12.5, 25 and 50 µM), fenretinide (0, 0.62, 1.25, 2.5 and 5 µM) and combinations of fenretinide + genistein in MEM/FBS with 0.1% DMSO. Total numbers of cells, viable cells, and dead cells were counted with a VI-cell counter after 72 hours. Mean number of viable cell counts ± standard error of the mean from 4 independent experiments are shown here. Four experiments were performed with 6 replicates (wells) per treatment in each experiment. NT = cells treated with vehicle + 0.1% DMSO (Cell counts are *10^6).

**Table 2:** Pair-wise comparisons between non-treated (NT) cells, cells treated with Fenretinide (Fen) only and cells treated with combinations of Fen and genistein (Fen/Ein)

| | Concentration of Genistein | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Concen-tration of Fen-retinide | | NT | Ein1.55 | Ein3.1 | Ein6.2 | Ein12.5 | Ein25 | Ein 50 |
| | NT | NT | 1.230 | 1.091 | 1.111 | (**)2.913 | (**)5.257 | (**)7.272 |
| | Fen0.62 | FEN | 1.588 | 1.989 | 2.460 | (**)2.685 | (**)3.444 | (**)4.619 |
| | Fen1.25 | | 1.294 | 1.179 | 1.981 | (**)2.888 | (**)3.564 | (**)4.837 |
| | Fen2.5 | | 0.5861 | 0.4590 | 1.137 | 1.995 | (**)2.789 | (**)3.756 |
| | Fen5 | | 0.4302 | 0.06063 | 0.4100 | 1.397 | (**)2.685 | (**)3.802 |

**Table 3:** Pair-wise comparisons between cells treated with Ein 1.55 µM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Ein1.55 | Ein3.1 | Ein6.2 | Ein12.5 | Ein25 | Ein 50 |
| Concentration of Fenretinide | NT | | 0.1386 | 0.1184 | 1.683 | (**)4.027 | (**)6.042 |
| | Fen0.62 | | 0.4013 | 0.8719 | 1.097 | 1.856 | (**)3.031 |
| | Fen1.25 | | 0.1155 | 0.6871 | 1.594 | 2.269 | (**)3.542 |
| | Fen2.5 | | 0.1270 | 0.5514 | 1.409 | 2.203 | (**)3.170 |
| | Fen5 | | 0.4908 | 0.8401 | 1.827 | (**)3.115 | (**)4.232 |

**Table 4:** Pair-wise comparisons between cells treated with Ein 3.1 µM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | | | | |
|---|---|---|---|---|---|---|
| | | Ein3.1 | Ein6.2 | Ein12.5 | Ein25 | Ein 50 |
| Concentration of Fenretinide | NT | | 0.02021 | 1.822 | (**)4.166 | (**)6.181 |
| | Fen0.62 | | 0.4706 | 0.6958 | 1.455 | (**)2.630 |
| | Fen1.25 | | 0.8026 | 1.709 | 2.385 | (**)3.658 |
| | Fen2.5 | | 0.6784 | 1.536 | 2.330 | (**)3.297 |
| | Fen5 | | 0.3493 | 1.337 | (**)2.624 | (**)3.742 |

**Table 5:** Pair-wise comparisons between cells treated with Ein 6.2 µM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | | | |
|---|---|---|---|---|---|
| | | Ein6.2 | Ein12.5 | Ein25 | Ein 50 |

| | | | | | |
|---|---|---|---|---|---|
| Concentration of Fenretinide | NT | | 1.801 | (**)4.146 | (**)6.161 |
| | Fen0.62 | | 0.2252 | 0.9845 | 2.159 |
| | Fen1.25 | | 0.9065 | 1.582 | (**)2.855 |
| | Fen2.5 | | 0.8574 | 1.651 | 2.618 |
| | Fen5 | | 0.9873 | (**)2.896 | (**)3.392 |

**Table 6:** Pair-wise comparisons between cells treated with Ein 12.5 μM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | | |
|---|---|---|---|---|
| Concentration of Fenretinide | | Ein12.5 | Ein25 | Ein 50 |
| | NT | | 2.344 | (**)4.359 |
| | Fen0.62 | | 0.7593 | 1.934 |
| | Fen1.25 | | 0.6756 | 1.949 |
| | Fen2.5 | | 0.7939 | 1.761 |
| | Fen5 | | 1.288 | 2.405 |

**Table 7:** Pair-wise comparisons between cells treated with Ein 25 μM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | |
|---|---|---|---|
| Concentration of Fenretinide | | Ein25 | Ein 50 |
| | NT | | 2.015 |
| | Fen0.62 | | 1.175 |
| | Fen1.25 | | 1.273 |
| | Fen2.5 | | 0.9671 |
| | Fen5 | | 1.117 |

**Part 2C-Ib: Effect of fenretinide and genistein on cell viability of A2780s cells**

The viable cell count of A2780s cells treated for 72 hours with increasing concentrations of genistein decreased (50-80%) with respect to the non-treated condition with increasing concentrations of genistein (Figure 17). On the other hand, fenretinide treatment decreased (by ~30% only at the highest concentration of 5 μM) the number of viable cells compared with untreated cells. When genistein and fenretinide were used in combination, the treatment decreased the number of viable cells with increasing concentrations. Comparison of the groups indicated that the highest concentration of fenretinide (5 μM) used in this experiment was the most effective concentration for decreasing the number of viable cells. The results indicate that the reduction in the viable cell number was due to both fenretinide and genistein treatments, but that genistein had more pronounced effects, with or without fenretinide.

To determine the statistical significance of these findings, two-way ANOVA was performed with Bonferroni tests to allow for multiple comparisons of concentrations of fenretinide and concentrations of genistein as factors in the viability assay for the A2780s cell line. The main effect of genistein concentration was significant ($F(6,70) = 10.24$, $p < 0.05$). The effect of fenretinide concentration was not significant [$F(4,70) = 0.80$, $p > 0.05$]. All pair-wise comparisons were not significantly different ($p > 0.05$) except for the combinations marked with (**) in Tables 8-13 that indicate the t values.

Statistical analysis of dose response cell viability assay on A2780s cell lines (Tables 8 to 13) suggests that the two lowest concentrations of fenretinide (0.62 μM and 1.25 μM) do not have any effect on cell viability. There is a slight decrease (9%) of viable cells with fenretinide concentrations higher than 2.5 μM against A2780s cell lines.

There was no dose response between increasing concentrations of fenretinide and loss of cell viability but there was a dose response between increasing concentrations of genistein and loss of viable A2780s cells. Similar to the above experiments carried out with the low dose treatments, there were no additive or synergistic anti-proliferative effects between any concentrations of fenretinide and genistein in combination on A2780s cells. In all concentrations, adding fenretinide appears to be protecting cells from effects of genistein alone. In consideration of the findings that: a) percentage of viable cells (data are not shown) was steady at the time of seeding to the end of the experiment (about 85-90% cell viability); b) previous MTS assays also indicated that cells are not killed by effect of drugs at low concentrations, it appears that the test compounds used at low concentrations had only a slight effect on the cell cycle and were unable to exert cytotoxic effects on the A2780s cells. Taken together, the above findings signify that the cells appear to be alive but their cell cycle is disrupted in response to the lowest concentration of individual and combination compounds.

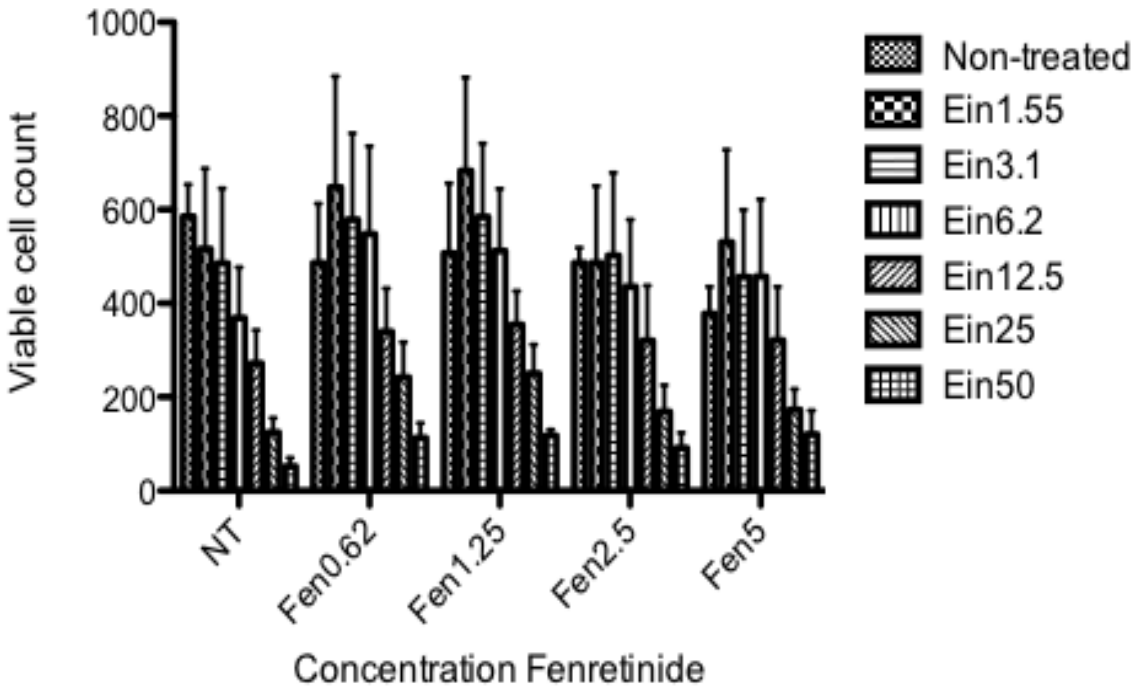



**Figure 17: Dose response of viable cell (A2780s) count after treatment with genistein and fenretinide in 72 hours.** A2780s cells (10,000) were seeded in 1,000 µL of MEM/FBS in a 24 well plate. Media was replaced with fresh media containing serial dilutions of genistein (0, 1.55, 3.1, 6.2, 12.5, 25 and 50 µM), fenretinide (0, 0.62, 1.25, 2.5 and 5 µM) and combinations of fenretinide + genistein in MEM/FBS with 0.1% DMSO. Total numbers of cells, viable cells, and dead cells were counted with a VI-cell counter after 72 hours. Mean number of viable cell counts ± standard error of the mean from 4 independent experiments are shown here. Four experiments ere performed with 6 replicates (wells) per treatment in each experiment. NT = cells treated with vehicle + 0.1% DMSO. (Cell counts are *10^6).

**Table 8:** Pair-wise comparisons between non-treated (NT) cells, cells treated with only Fenretinide (Fen) and cells treated with combinations of Fen and genistein (Fen/Ein)

| | Concentration of Genistein | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Concentration of Fenretinide | | NT | Ein1.55 | Ein3.1 | Ein6.2 | Ein12.5 | Ein25 | Ein 50 |
| | NT | | 0.3876 | 0.5595 | 1.219 | 1.761 | 2.592 | (**)2.999 |
| | Fen0.62 | | 0.9117 | 0.5243 | 0.3508 | 0.8223 | 1.373 | 2.101 |
| | Fen1.25 | | 0.9894 | 0.4350 | 0.02704 | 0.8612 | 1.448 | 2.194 |
| | Fen2.5 | | 0.001329 | 0.08973 | 0.2857 | 0.9291 | 1.786 | 2.220 |
| | Fen5 | | 0.8566 | 0.4414 | 0.4433 | 0.3200 | 1.155 | 1.447 |

**Table 9:** Pair-wise comparisons between cells treated with Ein 1.55 µM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | | | | | |
|---|---|---|---|---|---|---|---|
| Concentration of Fenretinide | | Ein1.55 | Ein3.1 | Ein6.2 | Ein12.5 | Ein25 | Ein 50 |
| | NT | | 0.1719 | 0.8317 | 1.373 | 2.205 | 2.612 |
| | Fen0.62 | | 0.3874 | 0.5609 | 1.734 | 2.285 | (**)3.012 |
| | Fen1.25 | | 0.5543 | 0.9623 | 1.851 | 2.437 | (**)3.183 |
| | Fen2.5 | | 0.08840 | 0.2870 | 0.9304 | 1.788 | 2.222 |
| | Fen5 | | 0.4153 | 0.4134 | 1.177 | 2.011 | 2.304 |

**Table 10:** Pair-wise comparisons between cells treated with Ein 3.1 µM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | | | | |
|---|---|---|---|---|---|---|
| Concentration of Fenretinide | | Ein3.1 | Ein6.2 | Ein12.5 | Ein25 | Ein 50 |
| | NT | | 0.6598 | 1.201 | 2.033 | 2.440 |
| | Fen0.62 | | 0.1735 | 1.347 | 1.897 | 2.625 |
| | Fen1.25 | | 0.4080 | 1.296 | 1.883 | 2.629 |
| | Fen2.5 | | 0.3754 | 1.019 | 1.876 | 2.310 |
| | Fen5 | | 0.001876 | 0.7614 | 1.596 | 1.889 |

:

**Table 11:** Pair-wise comparisons between cells treated with Ein 6.2 µM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | | | |
|---|---|---|---|---|---|
| Concentration of Fenretinide | | Ein6.2 | Ein12.5 | Ein25 | Ein 50 |
| | NT | | 0.5414 | 1.373 | 1.780 |
| | Fen0.62 | | 1.173 | 1.724 | 2.452 |
| | Fen1.25 | | 0.8882 | 1.475 | 2.221 |
| | Fen2.5 | | 0.6434 | 1.501 | 1.935 |
| | Fen5 | | 0.7632 | 1.598 | 1.891 |

**Table 12:** Pair-wise comparisons between cells treated with Ein 12.5 μM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | | |
|---|---|---|---|---|
| Concentration of Fenretinide | | Ein12.5 | Ein25 | Ein 50 |
| | NT | | 0.5506 | 1.278 |
| | Fen0.62 | | 0.5867 | 1.332 |
| | Fen1.25 | | 0.8573 | 1.291 |
| | Fen2.5 | | 0.8348 | 1.127 |
| | Fen5 | | 0.8315 | 1.239 |

**Table 13:** Pair-wise comparisons between cells treated with Ein 25 μM and cells treated with combinations of fenretinide and genistein (Fen/Ein)

| | Concentration of Genistein | | |
|---|---|---|---|
| Concentration of Fenretinide | | Ein25 | Ein 50 |
| | NT | | 0.7278 |
| | Fen0.62 | | 0.7457 |
| | Fen1.25 | | 0.4340 |
| | Fen2.5 | | 0.2926 |
| | Fen5 | | 0.4071 |

**Part 2C-IIa: Flow cytometric analysis of A2780s cells after treatment with genistein**

In order to further explore the anti-proliferative effects of the drug treatment and to confirm the results obtained from viable cell count assessment, cell cycle analysis of A2780s cells was conducted after 24 hours of treatment with 0 and 50 μM of genistein. Figure 18 shows a loss of G2/M phase and an increase of 34% in G1 phase indicating a cell cycle arrest in G1 phase (n=1). Further studies to confirm the effects on the cell cycle should be done with additional repetitions of these experiments.

**Part 2C-IIb: Flow cytometric analysis of A2780s cell lines after treatment with genistein and fenretinide**

Cell cycle analysis of A2780s and A2780cp cells was assessed after 24 hours of treatment with 0, 3.1, 12.5 and 50 μM of genistein and 1.25 μM of fenretinide. Comparison of Figures 18B and 19D indicates that chemotherapy sensitive and resistant cells reacted differently to the effects of 50 μM genistein. In the sensitive cell line (Figure 18B) genistein caused G1 arrest, whereas the same concentration caused G2/M arrest in the resistant cell line (Figure 19D). These differences in the stage of cell cycle arrest following exposure to genistein may be related to inherent differences in cell line characteristics that have not yet been determined. Relative to untreated cells shown in Figure 19A, Figure 19B shows a 3.15% increase of sub-G1 on A2780cp after 24 hour treatment with 3.1 μM of genistein (n=1). A low dose of 12.5 μM of genistein had no apparent effect on the cell cycle (Figure 19C; n=1); however, the addition of 1.25 μM of fenretinide to 12.5 μM genistein (Figure 19E) showed an 8% increase in sub-G1 cells after 24 hours of treatment of A2780cp cells, suggesting a combined effect of the two

compounds at those concentrations or a cytotoxic effect of fenretinide. When 50 μM genistein (Figure 19D) was tested for 24 hours, there was a 20% increase of G2/M phase and a decrease in S-phase A2780cp cells (n=1), suggesting a G2/M phase arrest. On the other hand, adding 1.25 μM fenretinide to 50 μM genistein (Figure 19F) showed a decrease in the proportion of cells in G2/M cell cycle arrest compared to genistein 50 μM alone, again suggesting that adding fenretinide may be protecting the cells from the effects of genistein. Finally, there was a 7% and 4.5% increase of G1-phase and sub-G1 phase after 24 hour administration of 50 μM genistein and 1.25 μM fenretinide (Figure 19F).

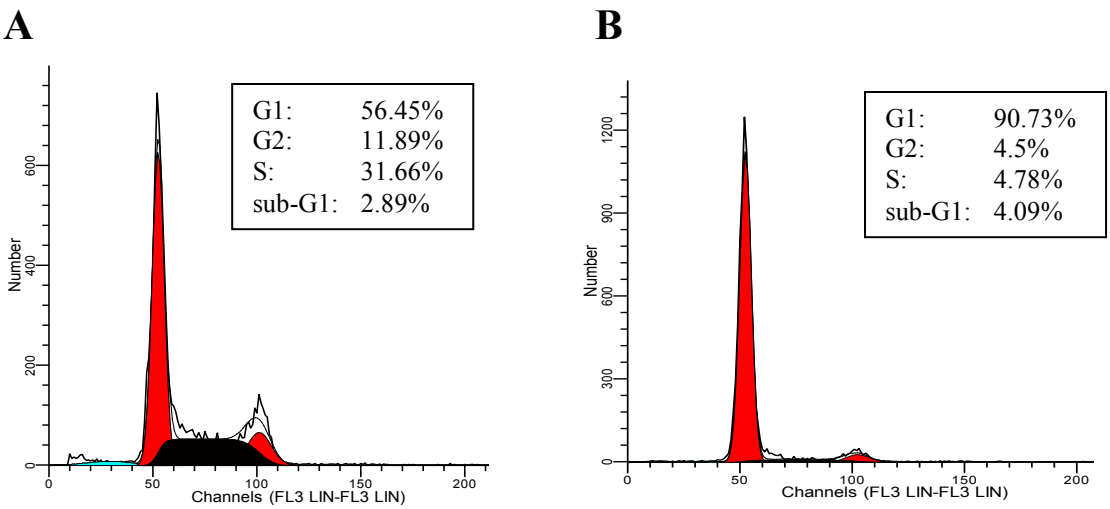



**Figure 18: Flow cytometric analyses of A2780s cells after treatment for 24 hours with genistein.** A) Control, B) genistein at 50 µM. The data show a loss of cells in G2/M and S phase after treatment with genistein, which may indicate that they arrested in G1 phase. The percentage of cells in each phase was calculated using Modfit software. One experiment was performed with 3 replicates (wells) per treatment (Number of cell are *10^5 on Y axis)

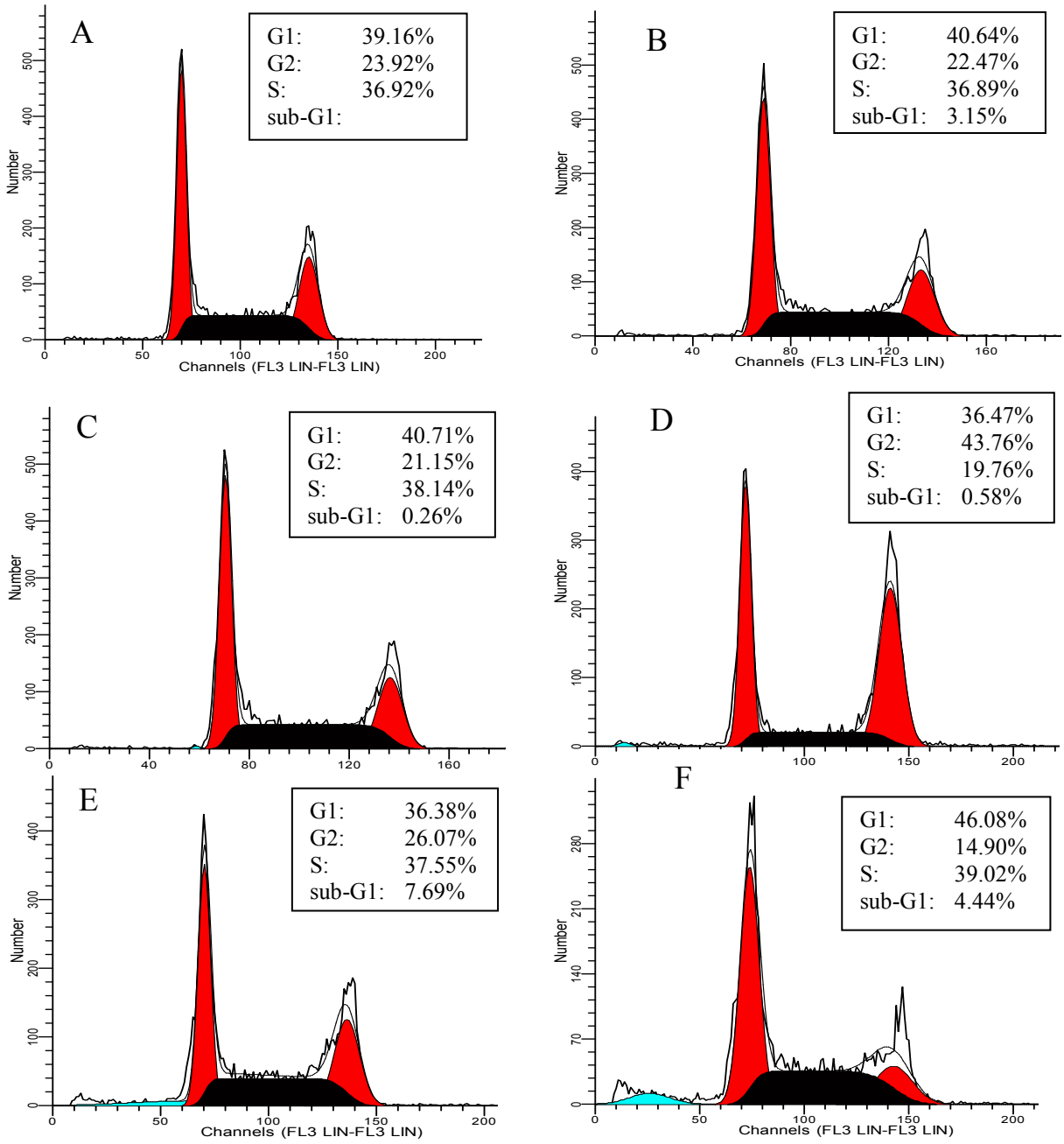



**Figure 19: Flow cytometric analyses of A2780cp cells after treatment for 24 hours with genistein alone or in combination with fenretinide.** A) control, B) genistein at 3.1 µM, C) genistein at 12.5 µM, D) genistein at 50 µM, E) 12.5 µM genistein and 1.25 µM fenretinide, and F) 50 µM genistein and 1.25 µM fenretinide. One experiment was performed with 3 replicates (wells) per treatment. (Number of cell are *10^5 on Y axis). The percentage of cells in each phase was calculated using Modfit LT software (version 3.2.1, July 08, 2008, Topsham Maryland, USA).

**Part 2C-IIIa: Effect of genistein vs. fenretinide on A2780s cells by MTS assay**

Can 3.1 μM genistein and 1.25 μM fenretinide (the lowest effective concentration of drugs against A2780s) effectively reduce the number of viable A2780s cells? According to our data from cell viability counts (A2780s cell line), we used the two lowest concentrations of fenretinide (1.25 μM) and genistein (3.1 μM) in order to minimize the effective concentration of two drugs and determine if there might be a synergistic effect of the combined compounds. To test this objective we carried out the standard cell viability assay (MTS) on A2780s cell line treated with genistein 3.1 μM and fenretinide 1.25 μM, alone or in combination for 72 hours (Figure 20).

Statistical analysis of the MTS assay data shows a similar magnitude of ODs (nM) for non-treated and treated A2780s cells with Ein 3.1 μM, Fen 1.25 μM and Fen 1.25 μM + Ein 3.1 μM for 72 hours A one-way ANOVA with Bonferroni post-hoc test was performed with Treatment (non-treated vs. Fen 1.25 μM vs. Ein 3.1 μM vs. Combo Ein 3.1 μM + Fen 1.25 μM) as a factor on the viability assay. The main effect of Treatment was not significant ($F(3,20) = 0.0445$, $p > 0.05$). All pair-wise comparisons were not significantly different ($p > 0.05$). Statistical analysis of MTS assay from this experiment also suggests that the lowest concentrations of both drugs (genistein 3.1 μM or/and fenretinide 1.25 μM) either alone or in combination does not decrease the number of viable A2780s cells.

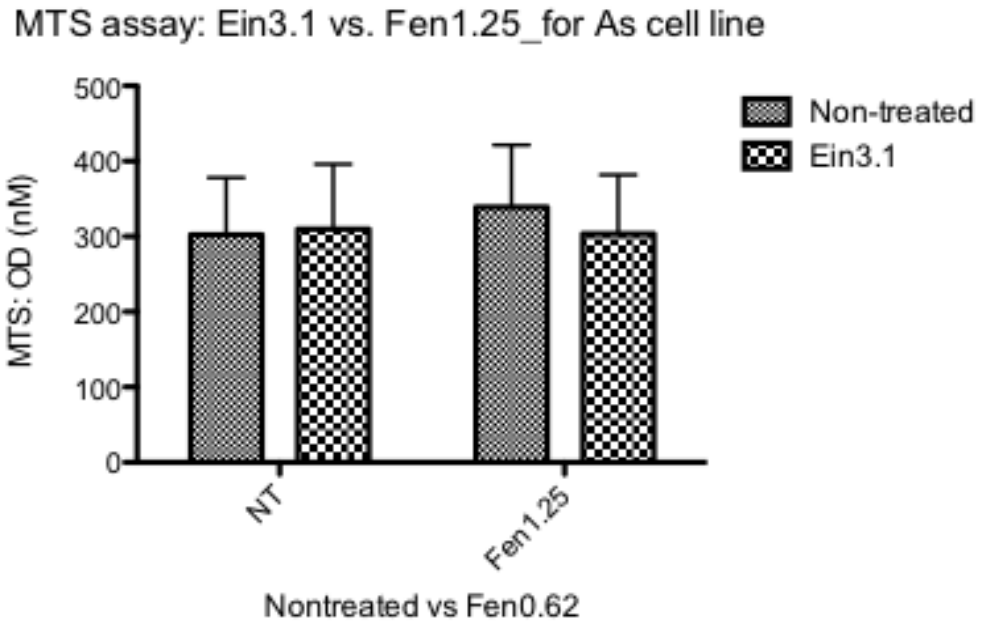



**Figure 20: Cell viability by MTS assay: effect of genistein 3.1 µM and fenretinide 1.25 µM and their combined treatment on A2780s cells after 72 hours.** As shown in this figure, there is no effect of genistein at 3.1 µM and/or fenretinide at 1.25 µM on A2780s cell line. Eight experiments were performed with 6 replicates (wells) per treatment in each experiment.

**Part 2C-IIIb: Effect of genistein vs. fenretinide on A2780cp cells by MTS assay**

We then tested if the lowest effective concentrations of drugs against A2780cp could effectively reduce the number of viable A2780s cells. Based on our data from cell viability counts for the A2780cp cell line, we used low concentrations of fenretinide (0.62 μM) and genistein (6.2 μM) to minimize the effective concentration of these two drugs and determine if there is a synergistic effect of the combined compounds. To test this objective we carried out the standard cell viability assay (MTS) on A2780cp cell line treated with 6.2 μM genistein and 0.62 μM fenretinide for 72 hours (Figure 21).

Statistical analysis of the MTS assay data indicated comparable size of ODs (nM) for non-treated and treated with Ein 6.2 μM, Fen 0.62 μM and combination Fen 0.62 μM + Ein 6.2 μM conditions for the A2780cp cell line. A one-way ANOVA with Bonferroni post-hoc test was performed with treatment (non-treated vs. Fen 0.62 μM vs. Ein 6.2 μM vs. Combo Ein 6.2 μM + Fen 0.62 μM) as a factor on the viability assay for the A2780cp cell line. The main effect of treatment was not significant ($F(3,28) = 1.057$, $p > 0.05$). All pair-wise comparisons were not significantly different ($p > 0.05$). Statistical analysis of MTS assay on A2780cp cell lines suggests that the lowest concentrations of both drugs (genistein 6.2 μM or/and fenretinide 0.62 μM) either alone or in combination does not decrease the viability of A2780cp cells.

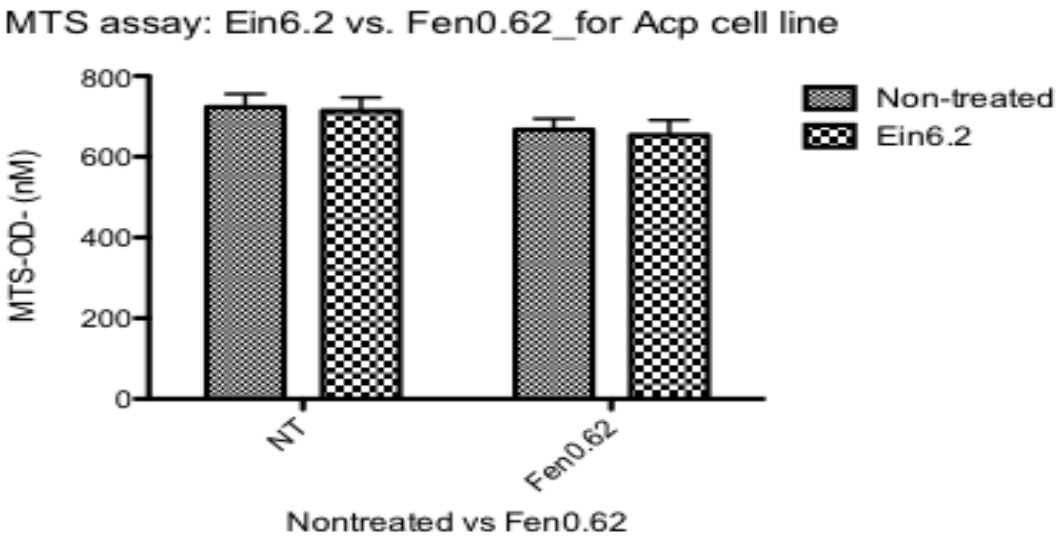



**Figure 21: Cell viability MTS assay: effect of genistein (Ein) 6.2 µM and fenretinide (Fen) 0.62 µM and their combined treatment on A2780s cells after 72 hours.** As shown in this figure, there is no effect of genistein at 3.1 µM and/or fenretinide at 1.25 µM on A2780cp cell line. Six experiments were performed with 6 replicates (wells) per treatment in each experiment.

**Part 2C-IVa: Effect of genistein and fenretinide on cell cycle of A2780s cell line**

There were contradictory results from the cell viability and MTS assays since cell viability showed a reduction in viable cell numbers and MTS did not. To verify the effect of the compounds on the A2780s cell line, cell cycle analysis was performed. A2780s cells were treated with genistein (3.1 μM) and fenretinide (1.25 μM), alone or in combination. Cells were harvested 24, 48 and 72 hours after treatment and the cell cycle was evaluated with a flow cytometer.

The results are shown in Figure 22. A two-way repeated-measured ANOVA with the Bonferroni post-hoc test was performed to allow for multiple comparisons, with Treatment (non-treated vs. Ein 3.1 μM vs. Fen.1.25 μM vs. Combo (Ein 3.1 μM + Fen 1.25 μM)) and sub-genome groups (G1 vs. G2 vs. S-phase) as factors on the 72h. Flow data for the A2780s cell line. The main effect of Treatment was not significant ($F(3,48) = 0.02$, $p > 0.05$). The effect of treatment on each analyzed sub-genome group was significant ($F(2,48) = 90.67$, $p < 0.05$). All pair-wise comparisons were not significantly different ($p > 0.05$). Similar results were obtained when the cells were analyzed after 24 and 48 hours of treatment.

The flow cytometry data indicate similar percentages of cells at each stage of the cell cycle for non-treated A2780s cells and cells treated with Ein 3.1 μM, Fen 1.25 μM and the combination, suggesting that there is no interruption in the cell cycle of A2780s cell lines after being exposed to the lowest concentrations of drugs.

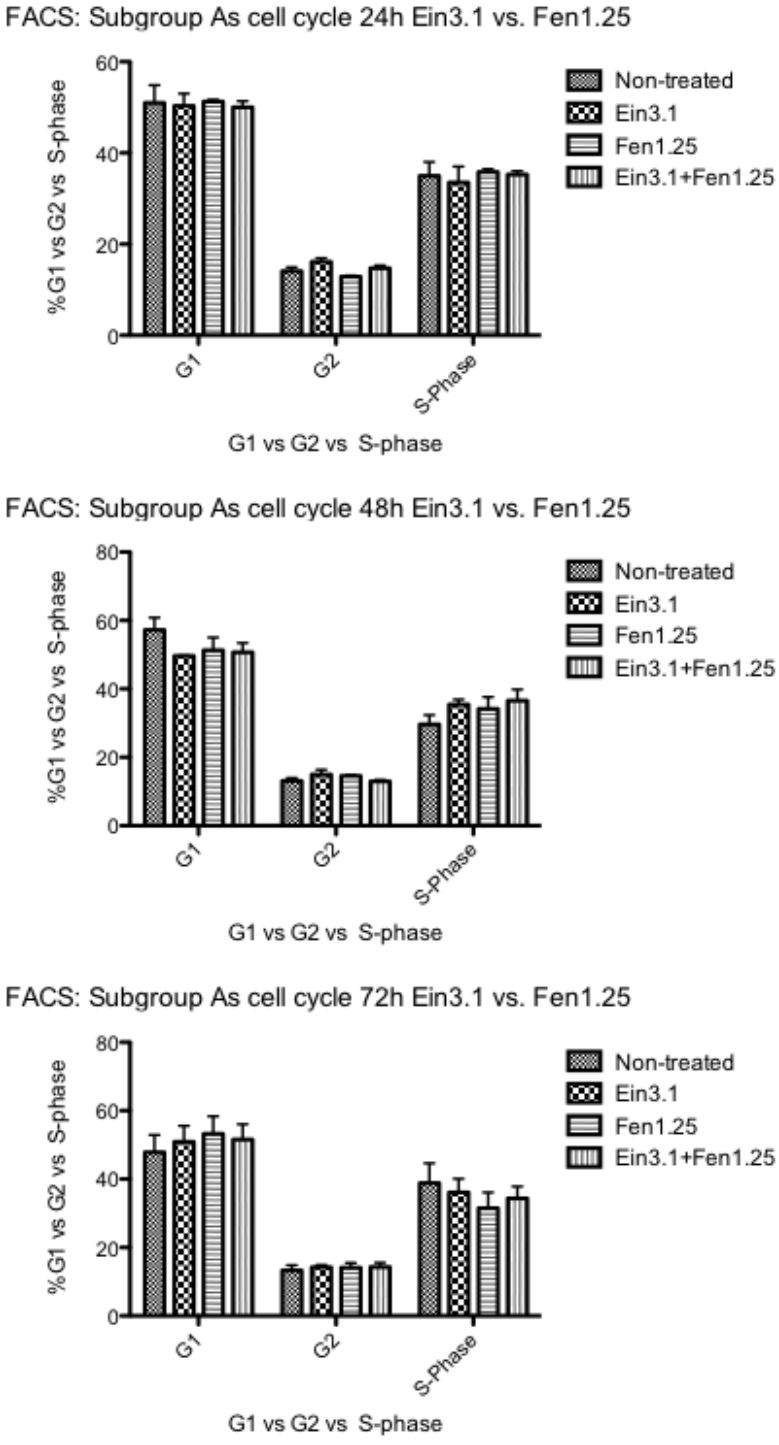



**Figure 22:** Flow cytometric analyses of A2780s cells after treatment for 24, 48 and 72 hours with genistein (Ein) 3.1 μM, fenretinide (Fen) 1.25 μM alone or in combination. Six experiments were performed with 3 replicates (wells) per treatment in each experiment.

**Part 2C-IVb: Effect genistein and fenretinide on cell cycle of A2780cp cell line**

Flow cytometric analysis was also performed on the A2780cp cells treated with genistein and fenretinide, alone and in combination (Figure 23). Statistical analysis was carried out in a manner similar to the analyses described above for the A2780s cell line. We performed a two-way repeated-measure ANOVA, Bonferroni corrected to allow for multiple comparisons, with Treatment (non-treated vs. Ein 6.2 vs. Fen 0.62 vs. Combo Ein 6.2 and Fen 0.62) and sub-genome groups (G1 vs. G2 vs. S-phase) as factors. After 72 hours, the main effect of Treatment was not significant ($F(3,60) = 0.000089$, $p > 0.05$). The effect of sub-genome groups factor was significant ($F(2,60) = 122.5$, $p < 0.05$). All pair-wise comparisons were not significantly different ($p>0.05$). Similar results were obtained when the cells were analyzed after 24 and 48 hours of treatment.

The flow cytometry data indicate similar magnitude of percentage of subgroups for non-treated and treated with Ein 6.2, Fen 0.62 and combination Fen 0.62 + Ein 6.2 conditions for the A2780cp cell line. Therefore, there is no interruption in cell cycle of A2780cp cell lines after being exposed to the lowest concentration of drugs.

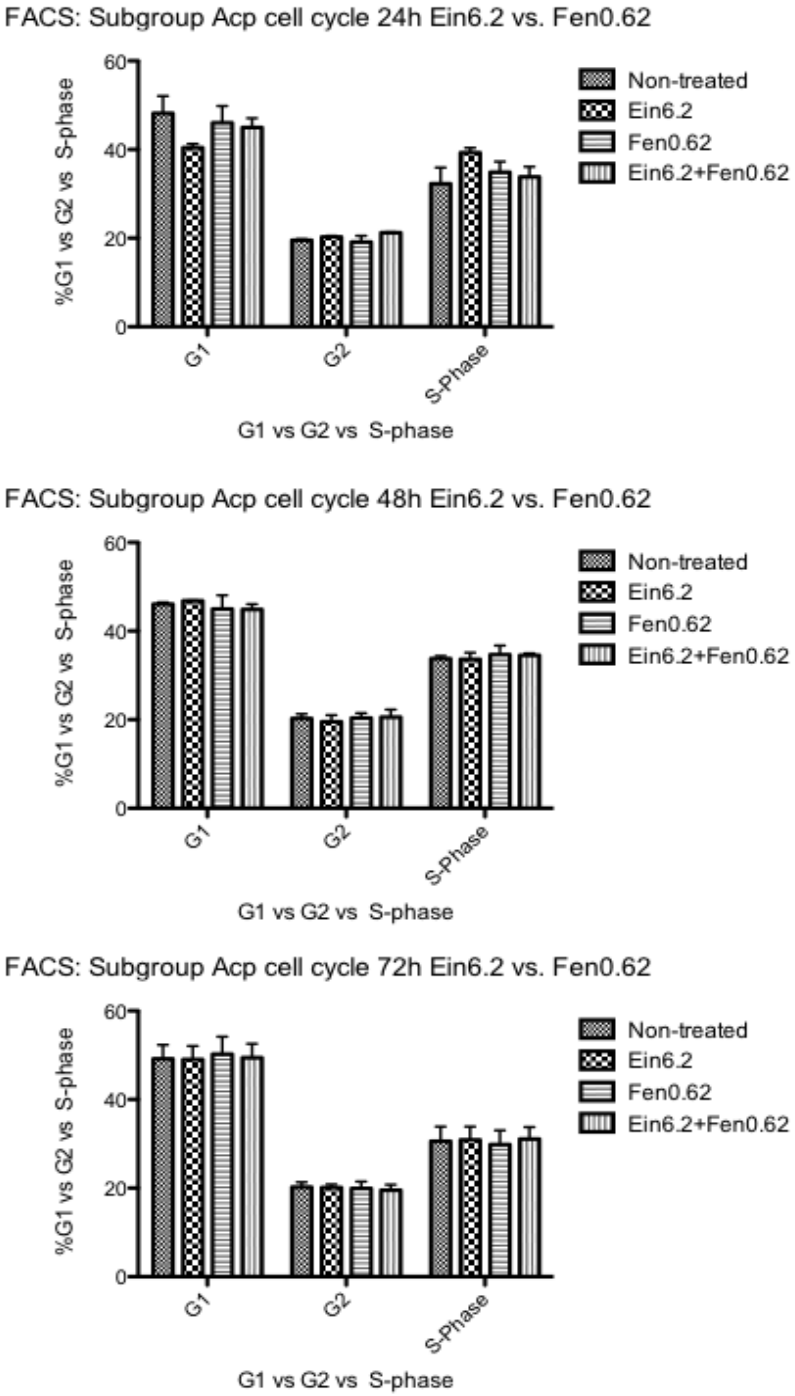



**Figure 23:** Flow cytometric analyses of A2780cp cells after treatment for 24, 48 and 72 hours with genistein (Ein) 3.1 μM, fenretinide (Fen) 1.25 μM alone or in combination. Five experiments were performed with 3 replicates (wells) per treatment in each experiment.

## Part 3D: Effect of Drugs Used at High Concentrations

Results from the MTS assay and cell cycle analyses did not confirm the slight effects on decreasing viable A2780s and A2780cp cells with low concentrations of genistein and fenretinide that was seen in the cell viability assays. We concluded that low concentrations of our drugs, alone or in combination, were not strong enough to either trigger apoptosis or inhibit cell proliferation and reduce the number of viable A2780s and A2780cp cells. Data from cell viability assays showed a slight effect of both drugs and combination drugs on mostly the A2780cp cell line and less on the A2780s cells to reduce the number of viable cells *in vitro*. We concluded that this slight effect was not due to either apoptosis or cell cycle disruption, meaning that cells were still alive but their growth rate was reduced by the lower concentrations of drugs.

In this part of the study we therefore tested the anti-proliferative effects of high concentrations of drugs in order to:

1. Investigate possible induced apoptosis by high concentrations of drugs.
2. To confirm whether or not these drugs have any effect on A2780s and A2780cp cell cycle.

### Part 3D-Ia: Effect of high concentration drugs on A2780s cell viability

A2780s cells were seeded in 1000 µL of 10% FBS/MEM-α and, after 24 hours, were treated with genistein (100 µM) and fenretinide (10 µM) for 24 hours. Total numbers of cells, viable cells, and dead cells were counted with a VI-cell counter (automated viable cell count using trypan blue dye). The results are shown in Figure 24. There was a 67% and 0% decrease in the number of viable A2780s cells when treated for

24 hours with these higher doses of genistein and fenretinide, respectively (n=2). This finding suggests a strong apoptotic effect of high dose of genistein on A2780s cells after 24 hours.

**Part 3D-Ib: Effect of genistein and fenretinide on cell cycle of A2780s cell line after 24 hours**

To assess the effect of high dose drugs on A2780s cell cycle, these cells were treated with fenretinide (Fen 10 μM) or genistein (Ein 100 μM) for 24 hours and then analyzed by flow cytometry. The results (Figure 25) indicate an increase of 27.6% and 3.1% of A2780s cells in the sub-G1 region when the cells were treated with genistein and fenretinide, respectively.

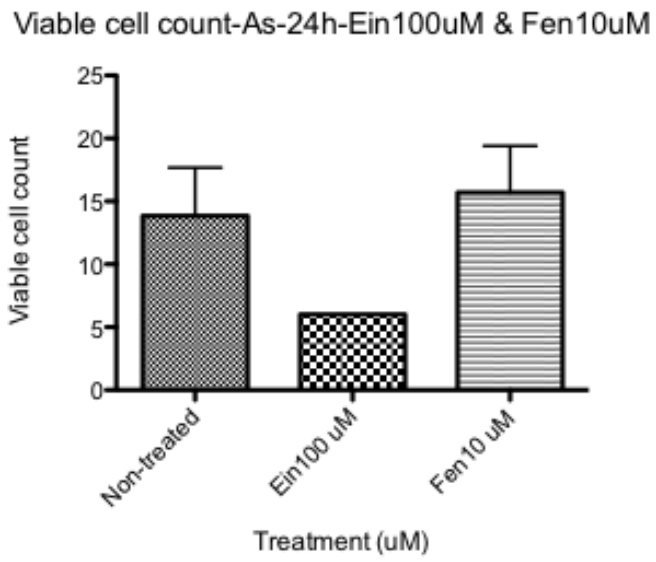
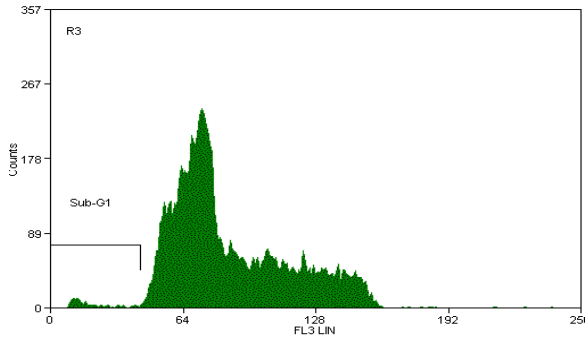
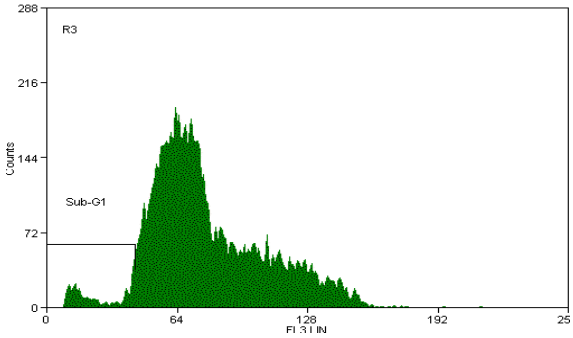



**Figure 24: A2780s viable cell count to investigate the effects of 100 µM genistein and 10µM fenretinide after 24 hours.** The numbers of viable cells were counted with a VI-cell counter (automated viable cell count using trypan blue dye) when all wells became 50% confluent (24 hours after treatment). Two experiments were performed with 3 replicates (wells) per treatment in each experiment.

## A) DMSO-Sub-G1: 1.4%




## B) Fen-10- Sub-G1: 4.5%




## C) Ein-100-Sub-G1: 29%

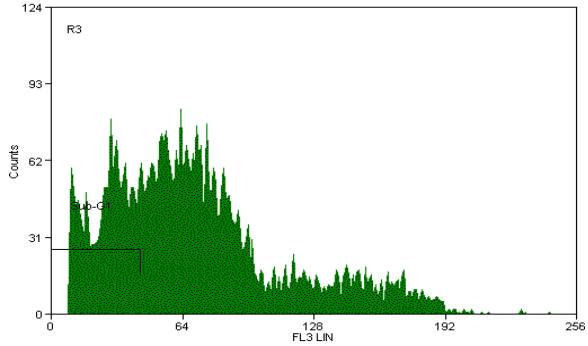



**Figure 25: Flow cytometric analyses of A2780s cells after treatment for 24 hours with genistein 100 µM or fenretinide 10 µM.** Percentages indicate the percentage of cells determined to be in the sub-G1 area. These data indicate an increase of 27.6% of A2780s cells in sub-G1 area when the cells were treated with genistein. The percentage of cells in each phase was calculated using Modfit LT software (version 3.2.1, July 08, 2008, Topsham Maryland, USA). Two experiments were performed with 3 replicates (wells) per treatment in each experiment. (Number of cell are *10^5 on Y axis).

## Part 3D-II: Effect of high concentration drugs on A2780cp cells

### Part 3D-IIa: Effect of high dose genistein nd fenretinide on viability of A2780cp cells

To determine the effects of high dose drugs on the chemo-resistant A2780cp cells, the cells were seeded in 1000 μL of 10% FBS/MEM-α and, after 24 hours, the cells were treated with genistein (100 μM), fenretinide (10 μM), alone and in combination. Total numbers of cells, viable cells, and dead cells were counted with a VI-cell counter in 24, 48 and 72 hours. The results show that (i) the viable cell count for the A2780cp cell line treated with a high concentration of fenretinide is reduced with respect to the non-treated cells for only the 72 h time intervals (Figure 26). (ii) A high concentration of genistein causes an 8- and 4-fold reduction in number of viable cells compared to the non-treated cells or cells treated with a high concentration of fenretinide only for both 48 h and 72 h time intervals, respectively. The results also indicate that for the 24 h time interval, the viable cell counts for the A2780cp cell line treated with high concentrations of either genistein or fenretinide were similar to the non-treated cells.

We ran a two-way ANOVA, Bonferroni corrected to allow for multiple comparisons, with Treatment (non-treated vs. Ein 100 μM vs. Fen 10 μM) and Time (24 h vs. 48 h vs. 72 h) as factors on the viability assay for the A2780cp cell line. The main effect of Treatment was significant ($F(2,9) = 49.43$, $p < 0.05$). The effect of Time was also significant ($F(2,9) = 52.72$, $p < 0.05$). All pair-wise comparisons marked with (**) in Table 14 were significantly different ($p < 0.05$).

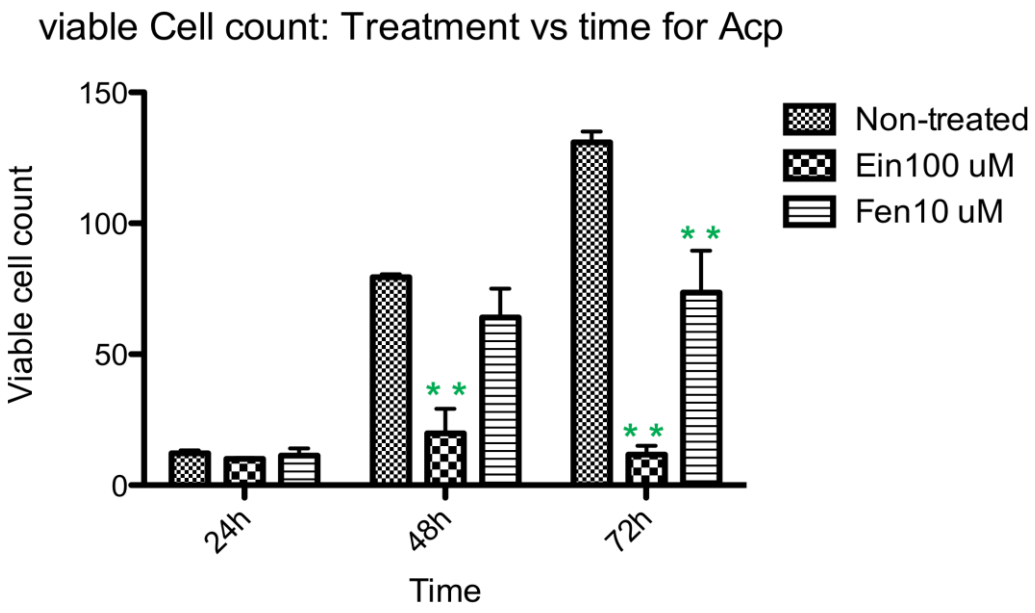



**Figure 26: A2780cp viable cell count to investigate effect of 100 µM genistein and 10 µM fenretinide after 24, 48 and 72 hours.** Three experiments were performed with 6 replicates (wells) per treatment in each experiment.

**Table 14:** Statistical analysis: Effect of genistein 100 μM and fenretinide 10μM on the viability of A2780cp cells treated for 24h, 48 and 72 hours

| | Treatment | Non-treated vs. Ein100 uM | Non-treated vs. Fen10 uM | Ein100 uM vs. Fen10 uM |
|---|---|---|---|---|
| Time | 24h | 0.1941 | 0.08678 | 0.1073 |
| | 48h | (**)5.641 | 1.460 | (**)4.181 |
| | 72h | (**)11.29 | (**)5.436 | (**)5.854 |

## Part 3D-IIb: Effect of genistein 100 μM and fenretinide 10 μM on viability of A2780cp cells treated for 24h, 48 and 72 hours, MTS cell viability assay

To confirm the effects of high dose drugs on A2780cp cell viability using an alternative method, A2780cp were treated with genistein 100 μM and fenretinide 10 μM, alone and in combination. At 24, 48 and 72 hours, the tetrazolium compound (MTS reagent) was added to assess viable cell counts. The comparison of results from MTS assay on the A2780cp cells shows that fewer viable cells are detected after treatment of A2780 cp cells with genistein than fenretinide after 72 hours (Figure 27). These results also indicate that for the 24 and 48 hour time interval, the viable cell counts for the A2780cp cells treated with high concentrations of either genistein or fenretinide were similar with the non-treated cells condition. The sensitivity of A2780cp cells to the anti-proliferative effects of genistein after 48 hours exposure as opposed to fenretinide could be related to several factors, which might include a higher cellular sensitivity to the anti-proliferative mechanisms of genistein such as diminished glucose uptake that is not associated with fenretinide treatment. Additionally, differences in the doses used might partially account the differential sensitivity seen between the two compounds.

We performed a two-way ANOVA, Bonferroni corrected to allow for multiple comparisons, with Treatment (non-treated vs. genistein 100 μM vs. Fen 10 μM) and Time (24h vs. 48h vs. 72h) as factors on the viability assay for the A2780cp cell line. The main effect of Treatment was significant ($F(2,15) = 29.04$, $p < 0.05$). The effect of Time was also significant ($F(2,15) = 166.30$, $p < 0.05$). All pair-wise comparisons marked with (**) in Table 15 were significantly different ($p < 0.05$).

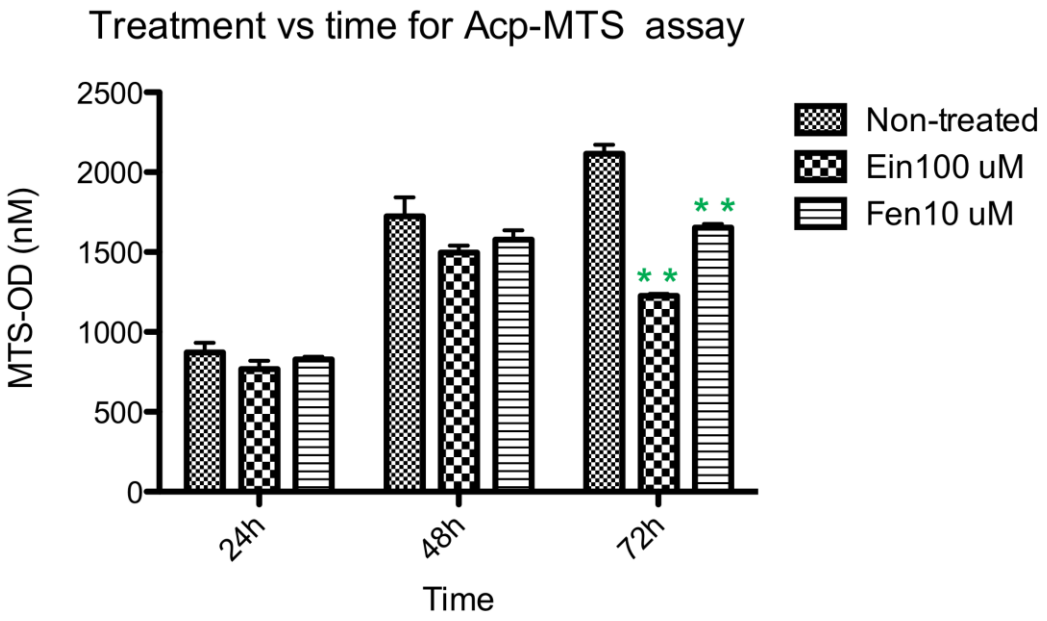



**Figure 27: A2780cp cell viability MTS assay to investigate the effects of 100 µM genistein and 10 µM fenretinide after 24, 48 and 72 hours.** Three experiments were performed with 6 replicates (wells) per treatment in each experiment.

**Table 15**: Statistical analysis: Effects of genistein 100 µM and fenretinide 10µM on the viability of A2780cp cells treated for 24h, 48 and 72 hours, MTS assay

| | Treatment | Non-treated vs. Ein100 uM | Non-treated vs. Fen10 uM | Ein100 uM vs. Fen10 uM |
|---|---|---|---|---|
| Time | 24h | 1.203 | 0.4972 | 0.7059 |
| | 48h | 2.643 | 1.686 | 0.9569 |
| | 72h | (**)8.493 | (**)4.415 | (**)4.078 |

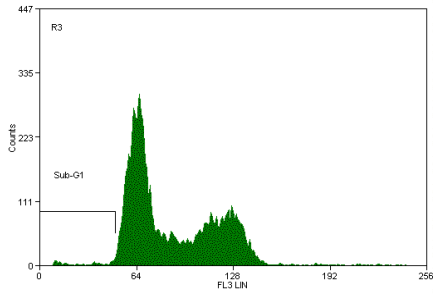
## Part 3D-IIc: Effect of high dose genistein and fenretinide on cell cycle of A2780cp cells treated for 24h, 48 and 72 hours

To determine whether the effects of high concentrations of the drugs on the A2780cp cells were due to inhibition of cell proliferation or induction of apoptosis, we carried out flow cytometry analysis with the highest concentrations of Ein (100 μM) and Fen (10 μM).

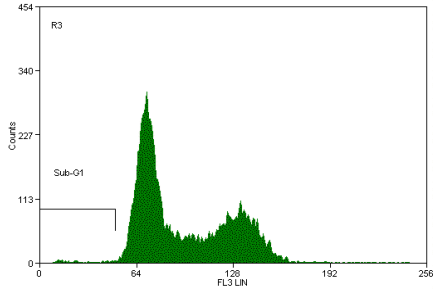
To assess the effects of high dose drugs on A2780cp cell cycle, these cells were treated with fenretinide (Fen 10 μM) or genistein (Ein 100 μM), alone or in combination for 24, 48 and 72 hours and then analyzed by flow cytometry. The results are shown in Figures 28-32. These data indicate no notable effect of fenretinide alone at any time point, but there is an increase of 14% of A2780cp cells in sub-G1 area with the combination treatment after 24 hours. As similar proportion (16-18%) are present in the sub-G1 fraction after 72 hours. The results indicate that fenretinide has no effect on the cell cycle of A2780cp cells, but genistein increases the apoptotic fraction of these cells.

# A2780cp-24 hour

**A) DMSO- Sub-G1: 1.8%**




**B) Fen-10- Sub-G1: 1.2%**




**C) Ein+Fen- Sub-G1: 15.5%**

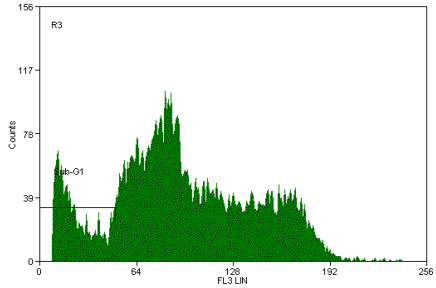
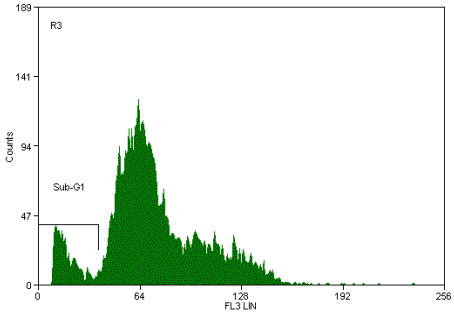


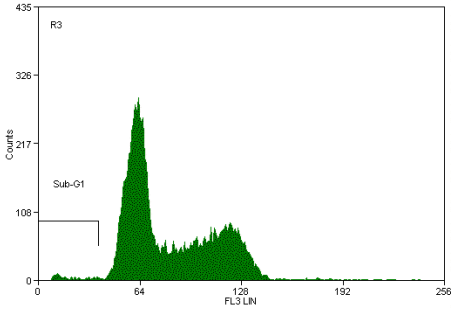

**Figure 28: Flow cytometric analyses of A2780cp cells after treatment for 24 hours: A) control B) fenretinide 10 µM and C) combination of genistein 100 µM with fenretinide 10 µM.** The three images above show the effect of combination of high concentration of genistein (100 µM) and fenretinide (10 µM) on A2780cp cell lines after 24 hours. These data indicate no effect of fenretinide alone, but there is an increase of 14% of A2780cp cells in sub-G1 area with the combination treatment. The percentage of cells in each phase was calculated using Modfit LT software (version 3.2.1, July 08, 2008, Topsham Maryland, USA). One experiment was performed with 3 replicates (wells) per treatment. (Number of cell are *10^5 on Y axis).

# A2780cp- 48 hours

**A) DMSO- Sub-G1: 9.7%**




**B) Fen-10- Sub-G1: 1.5%**




**C) Ein+Fen- Sub-G1: 11.2%**

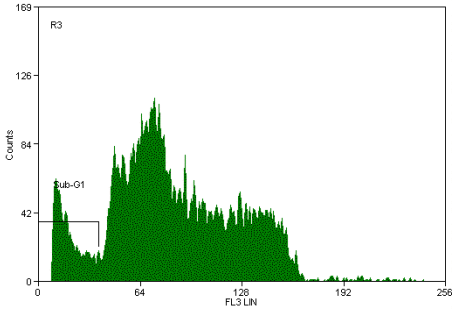
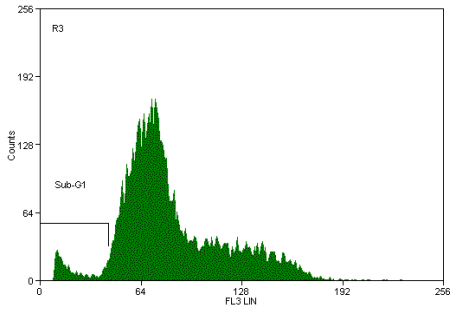


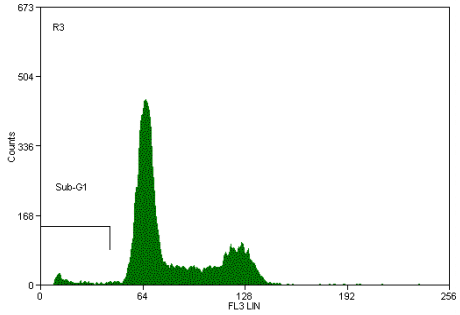

**Figure 29: Flow cytometric analyses of A2780cp cells after treatment for 48 hours: A) control B) fenretinide 10 µM and C) combination of genistein 100 µM with fenretinide 10 µM.** Above pictures show the effect of combination of genistein (100 µM) and fenretinide (10 µM) on A2780cp cell lines after 48hours. Data indicating an increase of 1.5% of A2780cp cells in sub-G1 area after 48 hours combination treatment (compare the first and third pictures above). The percentage of cells in each phase was calculated using Modfit LT software (version 3.2.1, July 08, 2008, Topsham Maryland, USA). One experiment was performed with 3 replicates (wells) per treatment. (Number of cell are *10^5 on Y axis).

# A2780cp- 72 hours- Experiment 1

**A) DMSO- Sub-G1: 4.6%**




**B) Fen-10- Sub-G1: 2.9%**




**C) Ein+Fen- Sub-G1: 17.6%**

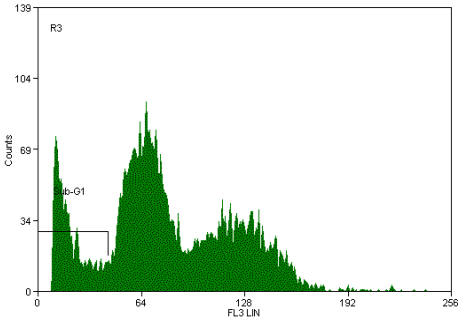
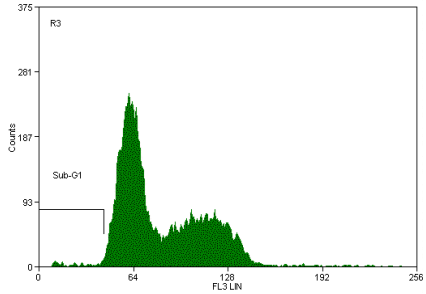


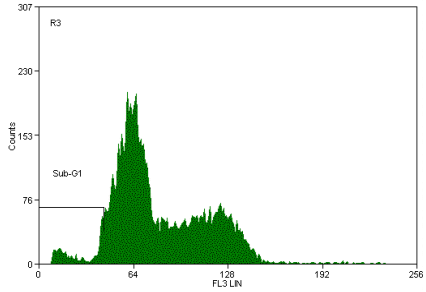

**Figure 30: Flow cytometric analyses of A2780cp cells after treatment for 72 hours: A) control B) fenretinide 10 µM and C) combination of genistein 100 µM with fenretinide 10 µM.** Above pictures show the effect of combination of genistein (100µM) and fenretinide (10 µM) on A2780cp cell lines after 72hours. Data indicating an increase of 13% of A2780cp cells in sub-G1 area (compare the first and third pictures above). The percentage of cells in each phase was calculated using Modfit LT software (version 3.2.1, July 08, 2008, Topsham Maryland, USA). One experiment was performed with 3 replicates (wells) per treatment. (Number of cell are *10^5 on Y axis).

# A2780cp- 72 hours- Experiment 2

## A) DMSO- Sub-G1: 1.4%




## B) Fen-10- Sub-G1: 5.8%




## C) Ein+Fen- Sub-G1: 16.3%

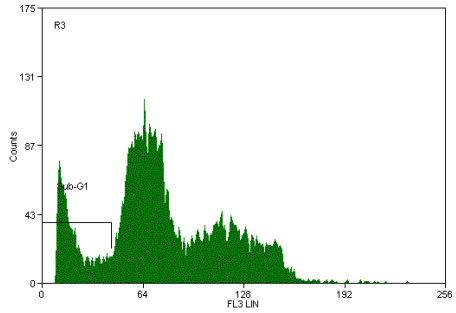



**Figure 31: Flow cytometric analyses of A2780cp cells after treatment for 72 hours: A) control B) fenretinide 10 µM and C) combination of genistein 100 µM with fenretinide 10 µM (Experiment 2).** The above images show the effect of the combination of high concentrations of genistein (100 µM) and fenretinide (10 µM) on A2780cp cells after 72 hours. These data indicate an increase of 4.4 % and 14.9% of A2780cp cells in sub-G1 area in cells treated with 10 µM fenretinide and combination of fenretinide and genistein. The percentage of cells in each phase was calculated using Modfit LT software (version 3.2.1, July 08, 2008, Topsham Maryland, USA). (Number of cell are *10^5 on Y axis) One experiment was performed with 3 replicates (wells) per treatment.

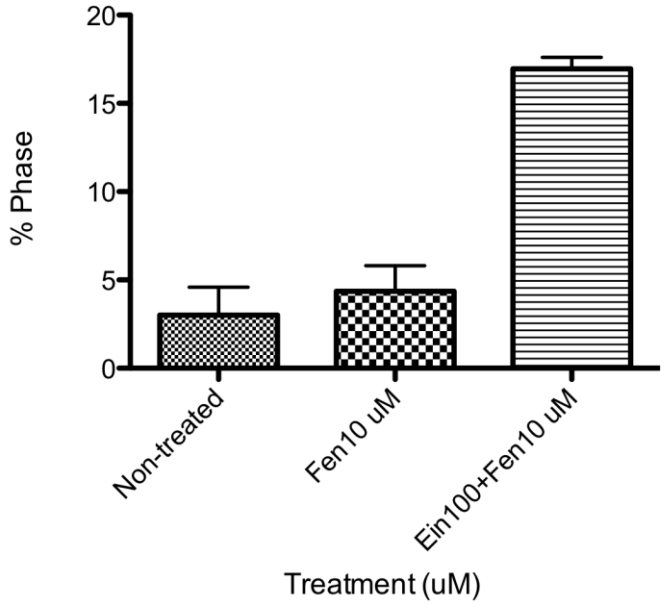



**Figure 32: Statistical analysis of (n=2) effect of fenretinide (10 µM) with or without the addition of genistein (100 µM) on A2780cp cell line.** The results indicate a significant increase ($p<0.05$) in the sub-G1 fraction when the A2780cp cells were treated with the combination of Ein 100 + Fen 10 µM for 72 hours. There was no effect of fenretinde alone. Data were analyzed by a one-way ANOVA, Bonferroni corrected to allow for multiple comparisons. The percentage of cells in each phase was calculated using Modfit software. One experiment was performed with 3 replicates (wells) per treatment. (Number of cell are *10^5 on Y axis)

# CHAPTER 4: DISCUSSION

In pilot experiments, potent anti-proliferative effects of fresh soy milk and soy kefir on A2780cp and ES2 ovarian cancer cells were observed after 1-5 day treatments with 5-10% (v/v) concentrations of fresh and lyophilized soy milk and soy kefir. Similarly, dramatic decreases in A2780cp cell viability after 5 day treatment with lyophilized soy milk (0-2% w/v) and soy kefir (1% w/v) were observed in a dose response manner. The comparison of the $IC_{50}$ of soy milk and soy kefir showed a slightly lower potency for soy kefir as compared to soy milk at the same doses, which suggests that some of the bioactive compounds from soy milk were degraded during the fermentation process. Although previous studies have not tested the anti-proliferative effects of soy milk or soy kefir fractions in tumor cell cultures, the above findings are in agreement with human population studies showing that soy milk appears to have chemopreventative properties against various types of cancers. For example, lower cancer rates are associated with higher consumption of soy products in women in Asian populations (Korde et al, 2009, Butler et al, 2010, Wu et al, 2008, 2009, Sakaucki et al, 2007 and Klein et al, 2006). Likewise, Hwang et al (2009) reported that the bioactive isoflavone genistein found in relatively high concentrations in soy products is associated with lowering the risk of prostate cancer in patients who consumed soy products in their daily diet.

Soy milk contains several bioactive compounds that can either induce apoptosis or decrease the proliferation rate of ovarian cancer cells *in vitro*; however, most human tumor cell culture studies have implicated genistein as the most potent anti-cancer soy constituent. Also, the combination of genistein and the anti-cancer agent fenretinide at

physiologically relevant doses has been shown to induce cell cycle arrest associated with apoptosis in neuroblastoma cells (Janardhanan et al (2009). There have been only a limited number of studies, however, regarding the anti-proliferative effects of either genistein or fenretinide in ovarian cancer cell lines. Hence, another set of preliminary experiments were carried out to clarify whether fenretinide, genistein and the less bioavailable isoflavones form, genistin, can inhibit proliferation of A2780cp cells using a range of low to high concentrations of the test compounds. One of the pilot experiments showed 75%, 40% and 49% decreases in viable A2780cp cells in response to high concentrations of fenretinide (20 μM), genistin (250 μM) and genistein (250 μM), respectively. The latter results could be related to either inhibition of cell proliferation or apoptosis induced by the test compounds, which was explored in a subsequent series of dose ranging studies. In one set of studies relatively low concentrations of genistein or genistin ranging from 3.1 to 50 μM were examined either singly or in combination with relatively low concentrations of 0.62 to 5 μM fenretinide. The lowest concentration of fenretinide of 0.62 μM tested is physiologically reachable after consuming 200 mg/day of fenretinide (Villani et al, 2004). In another set of experiments supra-physiological doses of both genistein (100 μM) and fenretinide (10 μM) were tested both singly and in combination. Only a few cancer cell culture studies have tested for anti-proliferative effects of genistein in cancer cells at doses that are closer to physiological concentrations (<10 μmol) as most studies have used genistein concentrations of 50 or 100 μM. This might be relevant since lower concentrations of genistein (<10 μM) appear to act primarily act as an estrogen agonists to exert anti-proliferative effects whereas higher

genistein concentrations (>10 μM) appear to be associated with multiple biological actions to exert cellular growth suppression (Lim et al., 2006).

Since to our knowledge, no studies to date have tested ovarian cancer cells for anti-proliferative effects of genistein or genistin at doses less than 10 μM, the identification of the lowest effective anti-proliferative and cytotoxic concentrations of genistein and genistin was studied in ovarian cancer cell cultures. The latter isoflavones were examined either alone or in combination with fenretinide to test for possible combinational anti-proliferative and cytotoxic effects. Since preliminary results showed that the more bioavailable isoflavone genistein exerted significantly more potent anti-proliferative effects than genistin in cisplatin-resistant (A2780cp) and sensitive A2780 (A2780s) cell lines, subsequent studies focused on the anti-proliferative effects of genistein alone or in combination with fenretinide using more physiologically relevant concentrations. A dose response relationship was observed in terms of loss of viable A2780cp and A2780s cells with increasing concentrations of genistein ranging from 3.1 to 50 μM. Interestingly, the A2780cp cells showed a greater sensitivity to genistein showing anti-proliferative effects at 3.1 μM in comparison to A2780s cells that only showed an anti-proliferative effect with genistein co-treatment at the 12.5 μM dose.

Cell cycle analysis was carried out to determine possible effects on apoptosis induced after 24 hours by 50 μM genistein in A2780s cells. The cell cycle distribution in A2780s cells showed a 34% increase in G1 phase after 24 hour treatment with 50 μM of genistein. Although further confirmatory studies are needed, the result suggests a cell cycle arrest in the G1 phase, suggesting that this is the mechanism by which genistein at 50 μM can impair proliferation of ovarian cancer cells. In agreement with the above

finding, Ouyang et al. (2009) reported that human ovarian cancer HO-8910 cells exposed to 50 μM genistein exhibited an activation of the DNA damage checkpoint pathway, which induces extensive DNA damage, and triggers G2/M phase arrest and apoptosis. Similarly, Janardhanan et al (2009) showed an increase in G0/G1 in neuroblastoma cell lines exposed to either 25 or 50 μM genistein. Using microarray analysis, Konstantakopoulos et al. (2006) examined the human endometrial cancer cell line, Ishikawa, at both physiological (0.5, 5 and 50 μM) and supra-physiological concentrations (500 μM) of genistein. They reported that physiologically relevant treatment with genistein at 5 μM induced multiple changes in the expression of certain genes that were mostly involved in a variety of pathways leading to the reduction of tumorigenesis.

Fenretinide treatment in the present thesis study showed no effect on cell cycles in A2780cp and A2780s cells when these cell lines were exposed to 0.62 μM and 1.25 μM fenretinide, respectively. This latter result agrees with previous studies showing no effect of 0.5 μM fenretinide treatment on cell cycles in neuroblastoma cell lines (Janardhanan et al., 2009). On the other hand, Janardhanan et al (2009) have reported additive effects on G0/G1 arrest by co-treatment with 0.5 μM fenretinide and either 25 or 50 μM genistein. Combination treatment of fenretinide (0.62 and 1.25 μM) with lower genistein doses of 3.1 and 6.2 μM used in the A2780cp and A2780s cell lines showed no additive effects in the present thesis work. The varying results between the above two studies could be related to differences in genistein doses or the responsiveness of the cell lines tested.

Using concentrations of fenretinide ranging from 1.25 to 5 μM in A2780s cell lines, viable cell counts measured by trypan blue exclusion indicated that only the 5 μM

fenretinide dose showed anti-proliferative effects, exhibiting a 35% decrease in viability after 72 hours of treatment. Although previous studies have not tested fenretinide treatments at less than 10 μM on ovarian cancer cells, other studies involving neuroblastoma cell lines have shown that fenretinide alone at the 0.5 μM dose did not significantly decrease cell viability (Janardhanan et al., 2009). In contrast to the above genistein findings, the chemo-resistant A2780cp cells were less sensitive to fenretinide treatment with only a 25% decrease in cell viability observed at the 5 μM fenretinide dose. This might suggest that certain mechanisms of action of fenretinide markedly differ from genistein, which may have allowed cross-resistance of cisplatin-resistant cells to the anti-proliferative effects of fenretinide but not genistein. Gossner et al., 2007 reported that genistein at concentrations of 50 and 100 μM down-regulate AKT, which can inhibit glucose uptake to trigger phagocytosis and cell death since AKT is an important glucose metabolism regulator. This latter cytotoxic mechanism has not been reported for either cisplatin or fenretinide.

Fenretinide at concentration of 10 μM has been shown to induce mitochondrial depolarization and activate caspase-9 and caspase-3, but not caspase-8, and so induce apoptosis in ovarian cancer CA-OV-3 and SKOV-3 cells after 48 hours of treatment (Holmes et al., 2003). Similarly, Suzuki et al. (1999) reported apoptosis in human cervical carcinoma cells after exposing them to 10 μM of fenretinide for 3 days, which resulted in the release of cytochrome c, generation ROS, activation of caspase-3 leading to DNA fragmentation and apoptosis. Cisplatin causes DNA cross-linking that interferes with mitosis cell division. As the DNA damage is too extensive to be repaired by DNA repair mechanisms, this triggers apoptosis (Fuertes et al., 2003). Interestingly, Solomon et

al. (2008) and Lim et al. (2006) have reported that pre-treatment of 25 and 50 μM genistein for 24 hours sensitizes the drug resistant ovarian and mammary cell cancer lines to chemotherapeutics, respectively. This latter result could be related to higher drug sensitivity resulting from effects of genistein on anti-proliferative pathways other than those affected by chemotherapeutic drugs such as down-regulation of the AKT pathway. In the present study, however, no additive or synergistic anti-proliferative effects were observed in either the A2780cp or A2780s cells from the combination of fenretinide and genistein as assessed by flow cytometry, cell viability or MTS assay. The data showed similar magnitudes of percentage of subgroups for non-treated cells and cells treated with the combination of 3.1 μM genistein and 1.25 μM fenretinide.

In contradiction to the present findings, Janardhanan et al (2009) reported that co-treatment of genistein (25 or 50 μM) with 0.5 μM fenretinide caused an additive reduction of viable neuroblastoma cells in the two neuroblastoma cell lines, SH-SY5Y and SK-N-BE2. They showed that combination treatment of genistein and fenretinide induced differentiation, over-expression of E-cadherin, reactivation of multiple tumor suppressors and an induction of G1/S phase arrest associated with early cell cycle exit. The contrasting findings with the thesis results might be due to inherent differences in the tested cell lines or differences in the combinational doses used between the two studies. It is possible that future studies using higher doses of genistein or pre-treatment with genistein prior to fenretinide exposure may result in either additive or synergistic actions. On the other hand, the addition of fenretinide at concentrations ranging from 2.5-5 μM concomitant with genistein (12.5-50 μM) in A2780cp or A2780s cells diminished the impact of genistein to decrease cell viability as intermediate cell viability was observed in

relation to the two treatments. This latter finding may suggest that fenretinide co-treatment with genistein in the tested ovarian cancer cell lines partially inhibits the anti-proliferative effects of genistein by undetermined mechanisms. One possible mechanism of action could be related to the induction by fenretinide of higher intracellular concentrations of ceramide (Tani et al., 2007). Ceramide can be hydrolyzed to sphingosine that, in turn, undergoes phosphorylation to form sphingosine 1-phosphate via sphingosine kinase (Tani et al., 2007). Sphingosine 1-phosphate has been demonstrated to stimulate AKT activation in human hepatocytes via the sphingosine 1-phosphate receptor (Osawa et al., 2001). The latter effect of fenretinide could thus counteract the genistein-mediated down-regulation of AKT indicated to be a significant cytotoxic mechanism of action of genistein on tumor cells (Gossner et al., 2007).

In contrast to the trypan blue analyses that demonstrated anti-proliferative effects in A2780cp or sensitive A2780s cells from the exposure of genistein, experiments using MTS to examine for anti-proliferative effects showed no anti-proliferative or cytotoxic effects of genistein at any of the tested concentrations in both ovarian cancer cell lines. The contrasting results might be explained by previous studies that have suggested a limitation of the utilization of MTS-based viability assays with respect to the testing of anti-proliferative effects of plant polyphenols such as (-)-epigallocatechin-3-gallate (EGCG) found in high concentrations in green tea. An underestimation of the cytotoxic effects of EGCG in human cell lines was recently reported using the MTS-based viability assay (Wang et al., 2010). The MTS assay is based on the formation of purple formazan crystals from the reduction of the MTS reagent by metabolically active cells (Mosmann et al., 1983). Although the formazan dye color formation is directly correlated to viable

cell numbers, some phytochemicals can interact with the MTS reagent or affect succinate dehydrogenase activity (Hsu et al., 2003 and Devika et al., 2008). Hence, the anti-proliferative and cytotoxic effects of genistein are thus more accurately investigated using a direct staining assay such as trypan blue, or calcein AM and live/dead assay and more than one cytotoxicity assay should be used.

The experiments described herein have suggested that low genistein doses could trigger apoptosis and inhibit cell proliferation. Higher fenretinide doses were needed to inhibit cell proliferation although fenretinide exhibited less anti-proliferative effects relative to genistein. No combinational anti-proliferative effects between genistein and fenretinide were observed, however, which could have been due to the relatively low doses used of the test compounds. Previous tumor cell studies have typically tested higher concentrations of both genistein (70-100 μM) and fenretinide (10 μM). Hence, additional experiments were carried out using A2780s and A2780cp cells to evaluate whether higher doses of the test compounds could exert combinational effects on cell proliferation, apoptosis or cell cycle arrest on the ovarian cancer cells. To test this latter objective, higher concentrations of both compounds were used, i.e., 100 μM genistein and 10 μM fenretinide. To the best of our knowledge, this is the first examination of cytotoxic effects of 100 μM genistein either alone or in combination with 10 μM fenretinide on cisplatin resistant and sensitive ovarian cancer cells.

There was a 67% decrease in viable cell count of A2780s cells when treated for 24 hours with a high dose of 100 μM genistein. In A2780cp cells, the same treatment did not lead to a significant decrease in cell viability after 24 hours. This latter result appears to contradict the present thesis findings showing a greater sensitivity of A2780cp cells to

the anti-proliferative effects of genistein at the lower doses of 3.1-50 μM. The latter apparent contradiction might be related to other multiple biological effects suggested to occur with genistein when used at high concentrations (Lim et al., 2006). Further studies are needed to examine the mechanisms for the differential sensitivities of the two cell lines.

After 48- and 72-hour exposure to genistein at 100 μM, however, a 70% and 80% decrease in cell viability was observed in A2780cp cells indicating that a strong cytotoxic effect of genistein at 100 μM occurs in both cell lines. Several other studies have reported potent cytotoxic effects from exposure to > 70-100 μM genistein as opposed to cytostatic effects at lower genistein doses in various tumor cell lines including stomach cancer cell lines (Yanagihara et al., 1993), neuroblastoma cells (Arabinda et al., 2006) and ovarian cancer cells (Choi et al., 2007). Genistein treatment at a concentration of 100 μM has previously been reported to inhibit cell proliferation of SKOV-3 cancer cells in a dose dependent manner at 24 and 48 hours (Choi et al., 2007). Arabinda et al., (2006) have indicated 24 hour treatment of neuroblastoma cells with genistein at concentrations of 50 and 100 μM resulted in increased free intracellular $Ca^{++}$ release leading to induction of MPT, generation of ROS and apoptosis.

Treatment of cells for 24 hours with a high dose of fenretinide (10 μM) did not affect cell viability in either cell line. Prolonged exposure with fenretinide, however, was associated with a 25% and 48% decrease in the viability of A2780cp cells after 48 and 72 hour treatment, respectively. Appierto et al, (2004) has indicated that c-Fos as a member of the AP-1 transcription factor plays a role in mediating fenretinide-induced growth inhibition in ovarian cancer cells.

Flow cytometric analysis of A2780 cp cells treated with a combination of 100 μM genistein and 10 μM fenretinide for 72 hours revealed a three-fold increase in the proportion of cells in the sub-G1 phase. The above data thus indicate that the combination of high concentrations of genistein (100 μM) and fenretinide (10 μM) can induce apoptosis in A2780cp cell lines. It is not clear regarding whether or not genistein or fenretinide exerted additive effects on apoptosis as cell cycle analysis data was not obtained regarding treatment with either genistein or fenretinide alone. It is possible that both compounds exerted pro-apoptotic effects at the tested concentrations.

A previous report has indicated 100 μM genistein treatment causes cell cycle arrest at G2/M phase of SKOV-3 cancer cells within 24 hours (Choi et al, (2007). Also, Appierto et al, (2004, 2007) have shown *PLAB* genes and c-Fos contribute to fenretinide-induced apoptotic activity in A2780 ovarian cancer cells when exposed to 10 μM fenretinide. They also showed that ceramide, which is involved in fenretinide-induced apoptosis, was also involved in c-Fos induction. Morales et al, (2007) reported that fenretinide increases ceramide levels in mitochondria that lead to ROS generation causing inactivation of *Bak* and *Bax*, loss of mitochondrial membrane permeabilization, and consequently cell death.

The work herein presents with a few limitations. *In vitro* cell culture models may not be representative of *in vivo* phenomena including the effects of gut metabolism that could alter isoflavone structures via microbial or intestinal metabolism. Hence, results from such studies are difficult to extrapolate, although they are well-suited for the exploration of mechanistic cellular and molecular interactions. Future experiments can be designed to investigate the possible anti-proliferative impact of isoflavone metabolites

produced via the action of human digestive enzymes and gut microbes using the automated, computerized human dynamic gut model (Prakash et al. 2011). Thus, the effects of isoflavones metabolites isolated from the gut model could be tested using human enterocytes Caco2 bilayers to identify the post-absorptive effects of isoflavones metabolites on ovarian cancer cells. In addition, the use of ovarian non-cancer cell lines can provide further information as to the possible selectivity of the effects of the drugs tested. Time constraints have prevented the collection of some data, which may have provided further information as to the behavior of ovarian cancer cell lines in response to the compounds tested.

Future experiments could involve: i) prior exposure of ovarian cancer cell lines to genistein prior to any treatment with anti-proliferative agents in order to explore sensitizing effects to chemotherapeutic drugs, especially in resistant cell lines; and ii) evaluation of various metabolic and mitochondrial functions (ROS, MPT, eNOS, expression of CYP24 enzyme, vitamin D receptor genes) to explore anti-proliferative, apoptotic and cytotoxic mechanism(s) of action of genistein either alone or in combination with chemotherapeutic agents.

In summary, the present thesis findings indicate that genistein can exert anti-proliferative and pro-apoptotic effects in cisplatin-resistant and sensitive A2780 ovarian cancer cells at a wide range of doses including doses closer to physiological concentrations. Significantly, the sensitivity of the two cell lines to anti-proliferative effects of genistein differed according to dose used as physiological concentrations were paradoxically associated with more potent effects in cisplatin-resistant ovarian cancer cells. Fenretinide co-treatment with low concentrations of genistein, however, showed an

antagonistic effect of fenretinide on the anti-proliferative effects of genistein, which demonstrates that certain drug-genistein combinations, may not necessarily be advantageous. Further mechanistic evaluations regarding the efficacy of the more physiologically relevant low dose genistein treatment either singly or in combination with other chemotherapeutic agents bears further study.

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