REVERSAL OF BREAST CANCER RESISTANCE PROTEIN MEDIATED MULTIDRUG RESISTANCE IN MCF-7 BREAST ADENOCARCINOMA CELL LINE

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ABSTRACT

REVERSAL OF BREAST CANCER RESISTANCE PROTEIN MEDIATED MULTIDRUG RESISTANCE IN MCF7 BREAST ADENOCARCINOMA CELL LINE

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Resistance to various chemotherapeutic agents is a major problem in success of cancer chemotherapy. One of the primary reasons of development of multidrug resistance (MDR) is the overexpression of ATP binding cassette (ABC) transporter proteins. Breast cancer resistance protein (BCRP) belongs to ABC transporter family and encoded by *ABCG2* gene. BCRP is mainly expressed in *MDR1* (P-glycoprotein) lacking breast cancer cells. Overexpression of BCRP leads to efflux of chemotherapeutic agents at higher rates, therefore, decreased levels of intracellular drug accumulation. Despite the fact that several chemical modulators claim to restore BCRP-mediated increased drug efflux, these modulators were shown to display various side effects, precluding their clinical use. Therefore, to reverse BCRP-mediated MDR phenotype by a modulator with minimum cytotoxicity may increase clinical benefits and minimize side effects.

Objective of current study was to reverse zoledronic acid resistance in MCF7 breast cancer cell line by impairing the function of BCRP. The effect of treatment with BCRP modulator Biochanin A on the expression levels of *BCRP*, *MRP1* and *MDR1* genes was analyzed by qPCR. The cytotoxicity of Biochanin A in sensitive and zoledronic acid resistant MCF7 cells and the reversing effect of Biochanin A on zoledronic acid resistance were determined.

The results showed that BCRP was significantly overexpressed in zoledronic acid resistant MCF7 cell line while the expressions of *MRP1* and *MDR1* genes were not changed relative to sensitive cells. The expression of BCRP was not affected by Biochanin A treatment in resistant cells. Cytotoxicity analysis demonstrated that Biochanin A did not display toxic effects on both cell lines. Treatment with Biochanin A resulted in sensitization of resistant cells to zoledronic acid in a dose dependent manner, however, it showed no effects in parental sensitive MCF7 cell line. Approximately 55% reversal of BCRP-mediated MDR phenotype was achieved by Biochanin A treatment in zoledronic acid resistant MCF7 cells.

It was shown that Biochanin A effectively restored zoledronic acid cytotoxicity in resistant cells, therefore it could be a proper candidate for further *in vivo* applications and therapeutic use.

Key words: BCRP, zoledronic acid, MDR reversal, Biochanin A

ÖZ

MCF7 MEME KANSERİ HÜCRE HATTINDA MEME KANSERİ DİRENÇLİLİK PROTEİNİNDEN KAYNAKLANAN ÇOKLU İLAÇ DİRENÇLİLİĞİNİN GERİ ÇEVRİLMESİ

Urfalı, Çağrı

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Kemoterapi ilaçlarına karşı geliştirilen dirençlilik kanser kemoterapisinin başarısını engelleyen en önemli etkendir. Çoklu ilaç dirençliliğinin ortaya çıkmasındaki en önemli nedenlerden biri ATP bağlanma kaseti (ABC) taşıyıcı proteinlerinin fazla ifade edilmesidir. ABCG2 gen ürünü olan meme kanseri dirençlilik proteini (BCRP) bu taşıyıcı protein ailesinin bir üyesidir. BCRP genellikle MDR1 (çoklu ilaç dirençliliği ile ilgili protein) ifadesi olmayan meme kanseri hücrelerinde ifade edilmektedir. BCRP'nin fazla ifade edilmesi kemoterapi ilaçlarının hücre dışına atılmasına ve hücre içinde düşük ilaç düzeylerine neden olmaktadır. Bazı kimyasallar BCRP'den kaynaklanan ilaç atımını engelleseler de, bu kimyasalların farklı toksik yan etkileri belirlenmiştir. Bu nedenle, BCRP'den kaynaklanan çoklu ilaç dirençliliğini toksik olmayan bir kimyasalla engellemek klinik faydaları artırıp yan etkileri azaltacaktır.

Bu çalışmanın amacı MCF7 meme kanseri hücre hattında BCRP'den kaynaklanan zoledronik asit dirençliliğini geri çevirmektir. BCRP inhibitörü olan Biochanin A muamelesinin *BCRP*, *MRP1* ve *MDR1* genlerinin ifade düzeyine olan etkisi kantitatif eş-zamanlı PZR yöntemiyle incelenmiştir. Biochanin A'nın ilaca duyarlı ve dirençli MCF7 hücrelerindeki etkisi sitotoksisite analizleriyle belirlenmiştir. Ayrıca Biochanin A'nın zoledronik asit dirençliliğini geri çevirmedeki etkisi incelenmiştir.

Sonuçlara göre, zoledronik aside dirençli olan MCF7 hücre hattında *BCRP* ifadesi anlamlı bir şekilde artmıştır. Aynı zamanda *MRP1* ve *MDR1* ifadesi gen düzeylerinde bir değişim olmadığı gösterilmiştir. *BCRP* gen ifadesinin Biochanin A muamelesine bağlı olarak değişmediği görülmüştür. Sitotoksisite analizleri Biochanin A'nın ilaca duyarlı ve dirençli hücrelerde toksik etkileri olmadığını göstermiştir. Biochanin A ile muamele edilmiş dirençli hücrelerin, doza bağımlı bir şekilde zoledronik aside duyarlı hale geldiği belirlenmiştir. Buna karşın ilaca duyarlı MCF7 hücreleri Biochanin A muamelesinden etkilenmemiştir. Zoledronik aside dirençli MCF7 hücrelerinde ilaç dirençliliği yaklaşık %55 oranında geri çevrilmiştir.

Biochanin A, kendisi toksik etki göstermemesine karşın, hücrelerde zoledronik asit toksisitesini önemli bir şekilde artırmıştır. Bu sonuçlara göre, Biochanin A ilaç dirençliliğinin geri çevrilmesi amaçlı uygulamalar ve *in vivo* çalışmalar için uygun bir ajan olabilir.

Anahtar kelimeler: BCRP, zoledronik asit, dirençliliğin geri çevrilmesi, Biochanin A

To my precious family

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ABC	ATP-Binding Cassette
BCRP	Breast Cancer Resistance Protein
BP	Bisphosphonate
bp	Base Pair
cDNA	Complementary Deoxyribonucleic Acid
DEPC	Diethylpyrocarbonate
dH_2O	Distilled Water
DMSO	Dimethyl Sulfoxide
dNTP	Deoxy Nucleotide Triphosphate
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
FC	Fold Change
FR	Fold Reversal
IC50	Inhibitory Concentration 50
LRP	Lung Resistance Protein
MCF-7/S	Sensitive MCF-7
MCF-7/Zol	Zoledronic Acid Resistant MCF-7
MDR	Multidrug Resistance
MRP1	Multidrug Resistance Protein 1
NBD	Nucleotide Binding Domain
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
P-gp/MDR1	P-glycoprotein/Multidrug Resistance Protein 1
qPCR	Quantitative Real-Time Polymerase Chain Reaction
R	Relative Resistance Index

RNA	Ribonucleic Acid
rpm	Revolution per minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standart Error of the Means
TAE	Tris-acetate-EDTA
TMD	Transmembrane Domain
V	Volt
w/v	Weight per volume
ZOL	Zoledronic Acid

CHAPTER 1

INTRODUCTION

1.1 Biology of Cancer

Cancer is defined as a group of diseases which could be characterized by uncontrolled cell proliferation, even invasion of neighbouring sites and metastasis of abnormal cells to distant tissues. American Cancer Society had predicted approximately 1.5 million new cancer cases to emerge and half-million deaths due to multiorgan failure associated with different types of cancer only in the US (American Cancer Society, 2010). World Health Organization declared that cancer was the primary cause of death worldwide and had accounted 7.6 million deaths (13% of all deaths) in 2008 (World Health Organization, 2008). Turkish Ministry of Health declared that approximately 200,000 cancer cases occured in 2006. Additionally, cancer-related deaths have the second rank after heart diseases-related deaths, covering approximately 15% of all deaths (Turkish Ministry of Health, 2006).

Cancer occurs through a multistep process which converts normal cell into malignant ones (Boland and Goel, 2005). The generation of malignant cells require massive genetic alterations including the activation or amplification of oncogenes which lead to overproliferation and the inactivation of tumor suppressor genes which are normally responsible for limiting cell growth. The genetic alterations that result in development of tumor cells can be caused by both internal and external factors. Internal factors include point and frameshift mutations which can lead to partial or complete loss in protein function, chromosomal breakages or rearrangements,

intrinsic chemical instability of certain DNA bases, epigenetic alterations, hormone levels, free radicals generated by metabolic processes and immune system alterations (Bertram, 2001; American Cancer Society, 2010). On the other hand, viral or bacterial infecitons, ionizing or UV radiation, exposure to chemical carcinogens and smoking can be listed as external factors (Bertram, 2001; Rieger, 2004). Both internal and external factors change genetic and cellular systems to initiate or promote cancer growth (American Cancer Society, 2010).

Bertram (Bertram, 2001) suggested that it is necessary to activate/inactivate five major pathways in the genesis of cancer; *(i)* development of independence in growth simulatory signals, *(ii)* development of a refractory state to growth inhibitory signals, *(iii)* development to resistance to programmed cell death, *(iv)* development of an infinite proliferative capacity and *(v)* development of angiogenic potential. Hanahan and Weinberg (Hanahan and Weinberg, 2000) proposed six essential properties of cancer cells as sustained proliferative signaling, insensitivity to growth suppressors, activation of invasion and metastasis, induction of replicative immortality, sustained angiogenesis and evasion of apoptosis. Recent studies demonsrated that, in addition to six hallmarks of cancer, malignant cells also have the capability of deregulation of cellular energetics and evasion of immune destruction (Hanahan and Weinberg, 2011). Emergence of additional hallmarks confirmed that cancer cells continue to gain new properties and become even more dangerous (Karp, 2002).

1.2 Breast Cancer

Breast cancer is the most common cancer among women worldwide. American Cancer Society (American Cancer Society, 2011) predicted 280,130 new brest cancer cases (230,480 invasive and 57,650 *in situ*) to occur in the United States in 2011; of which, 2% comprised cancer cases in men. Additionaly, breast cancer has the second rank in cancer-related deaths in women. In 2011, estimated breast cancer associated deaths were determined as approximately 40,000. Besides, Turkish Ministry of

Health declared that breast cancer was the most common cancer in women, comprising 25% of all cancer cases (Turkish Ministry of Health, 2006).

Breast cancer is generally initiated in the lobules (milk-producing glands) or in the ducts (the channels which drain milk to nipple from the lobules). Rarely, it could originate in stromal tissue which includes fatty and fibrous connective tissue of the breast (http://www.breastcancer.org/symptoms/understand_bc/what_is_bc.jsp). Malignant breast tumor cells can invade neighboruing healthy cells and nearby lymph nodes, before they enter blood circulation. Lung and bones are favorable metastatic sites for breast cancer (Keen and Lennan, 2011).

Breast cancer could be developed due to a number of factors including age, weight (being overweight or obese), smoking, use of alcohol, long menstruation history, use of oral contraceptives, combined use of hormones after menapause and birth at old ages (American Cancer Society, 2011; Tyczynski *et. al*, 2002). Although 95% of breast cancer cases occur spontaneously, familial history and genetic tendency could also affect the rate of occurence of breast cancer. Several studies demonsrated that the estimated lifetime risk of breast cancer is found to be 80% in women with inherited *BRCA1* and *BRCA2* mutations (King *et. al*, 2003; Foulkes *et. al*, 2003; Serova *et. al*, 1997). Risk control is essential for these individuals to avoid breast cancer development or recurrence.

1.3 Treatment Strategies of Breast Cancer

Breast cancer treatmet generally involves surgery, radiation therapy, hormonal therapy, targeted therapy and chemotherapy. The type of the treatment is determined according to the size and location of tumor, stage of cancer, cell turnover rate, status of hormones and their receptors, status of axillary lymph nodes and menapausal status as well as age of the patient, general health conditions and patient preference

(American Cancer Society, 2010; NIH Consensus Development Conference Statement, 2000).

1.3.1 Surgery

Surgery is generally the first choice of breast cancer treatment (NIH Consensus Development Conference Statement, 2000) . According to the tumor size and stage, the type of surgery is determined. Lumpectomy, also known as breast conserving surgery, is the surgical removal of tumor with clear margins (American Cancer Society, 2010). Lumpectomy is often preferred when tumor size is less than 5 cm and there is *in situ* carcinogenesis (Veronesi *et. al*, 2002). Lumpectomy is usually applied in combination of radiation. On the other hand, mastectomy is the surgical removal of entire breast tissue and frequently used to treat larger tumors with invasive characteristics (American Cancer Society, 2010. Dissection of axillary lymph nodes also takes place in advanced breast cancers (http://www.cancer.gov/cancertopics/pdq/treatment/breast/).

1.3.2 Radiation Therapy

Radiation therapy (radiotherapy) is based on the use of high-energy beams to distrupt tumor cells. Radiotherapy could be applied before surgery to shrink tumor size (if tumor size is larger than 5 cm) or after surgery to kill any remaining cancer cells (http://www.cancer.gov/cancertopics/factsheet/Therapy/radiation). Moreover, it has been used to destroy ovaries to prevent estrogen stimulation in ER-positive breast cancers (NIH Consensus Development Conference Statement, 2000).

Radiotherapy kills cancer cells by directly damaging DNA structure or generating free radicals within the cells which can further create DNA damages (http://www.cancer.gov/cancertopics/factsheet/Therapy/radiation).

Radiation therapy is a highly effective and targeted approach. Studies showed that radiation therapy could reduce risk of recurrence by 70%. However, some side-effects could be seen in different tissues, mostly in skin (http://www.breastcancer.org/treatment/radiation).

1.3.3 Hormonal Therapy

Approximately 75% of breast cancers express increased levels of estrogen receptor α (ERα) (Dowsett, 2001). Hormonal therapy is required in such cancer cases to prevent cancer cells from being stimulated from estrogen. Estrogen deprivation is achieved by either blocking estrogen receptor through drugs including tamoxifen and raloxifene or suppression of estrogen synthesis through aromatase inhibitors such as anastrazole (NIH Consensus Development Conference Statement, 2000). Tamoxifen, an antagonist of ER, was shown to reduce breast cancer risk and increase overall survival (Vogel *et. al*, 2005). Randomized trials and meta-analyses demonstrated that tamoxifen combined with chemotherapy decreased the risk of recurrence in post-menopausal women. Selective estrogen receptor modulator (SERM) raloxifene also was shown to reduce the risk of invasive breast cancer by 76% (Cummings *et. al*, 1999). However, recent studies revealed that HER2 overexpression leads to resistance to hormonal therapy (Dowsett, 2001).

1.3.4 Targeted Therapy

Targeted therapy involves the use of monoclonal antibodies and tyrosine kinase inhibitors to minimize the side effects of other types of therapies in breast cancer treatment. Trastuzumab (Herceptin®) is a monoclonal antibody aganist human epidermal growth factor 2 (HER2) which was found to be overexpressed in 25% of invasive breast cancers and associated with poor disease-free survival (Nahta *et. al*, 2006). Similar to Herceptin®, lapatinib (Tykerb®) and bevacizumab (Avastin®) have

been approved to be used in advanced breast cancer treatment (American Cancer Society, 2010). Although targeted therapy is well tolerated compared to chemotherapy, high response rates could not be achieved (Livingston and Esteva, 2001).

1.3.5 Chemotherapy

Chemotherapy is the use of anticancer drugs to kill cancer cells. The main goal of chemotherapy is to weaken or destroy the cancer cells and to reduce risk of recurrence (http://www.breastcancer.org/treatment/chemotherapy/medicines.jsp).

There are various chemotherapeutics available for different types of cancer. Anthracyclines (doxorubicin, epirubicin), taxanes (docetaxel, paclitaxel), mitoxantrone and vinorelbine are frequently used in breast cancer chemotherapy (O'Shaughnessy, 2005; Glück, 2005). In advanced stages of breast cancers, chemotherapeutics are not sufficient in terms of effectiveness. Additional drugs such as bisphosphonates (BPs) are required to increase the rate of cell death and to prevent metastasis (Maricic, 2006).

Chemotherapeutics could be introduced to patients orally or intravenously. The application of chemotherapeutics is done in regular cycles, however, the duration between two cycles are determined with respect to the stage of cancer and location of the tumor (http://www.fccc.edu/cancer/types/breast/treatment/chemotherapy.html). These anticancer drugs could be given as a single agent (monotherapy) or in combination with other agents (chemotherapy regimens). Use of chemotherapy regimens increases effectiveness of chemotherapy and cause shrinkage or disappreance of tumor in 60% of all breast cancer patients (http://www.breastcancer.org/treatment/chemotherapy/medicines.jsp).

Beside traditional chemotherapy, adjuvant and neoadjuvant chemotherapies are used to increase the benefits of cancer treatment. Adjuvant chemotherapy is given after surgery to get rid of any remaining cancer cells. Adjuvant chemotherapy increases the chance of long-term disease-free survival in breast cancer patients. On the other hand, neoadjuvant chemotherapy is applied before surgery to shrink tumor size of large tumors which can not be removed surgically. (http://www.cancer.gov/cancertopics/factsheet/Therapy/adjuvant-breast).

1.3.5.1 Zoledronic Acid (Zometa®, Reclast®)

In most cases surgery, chemotherapy and hormonal therapy could effectively treat the primary tumor. However, it is obvious that breast cancer cells could escape the primary site and tend to develop secondary tumors in bone where they could be protected from adjuvant therapy (Lipton, 2011). Moreover, bone is known as a beneficial metastatic site because of its high blood flow due to large amount of red blood marrow and abundance of growth factors and chemokines (Wong and Pavlakis, 2011). Approximately 75% of women with breast cancer experience bone metasteses (Coleman, 1997).

Bone metastasis occurs as an organized and multistep process involving tumor intravasation, survival of tumor cells in circulation, extravasation into secondary site, initiation of tumorigenesis and angiogenesis (Wong and Pavlakis, 2011). Zoledronic acid (Zometa®, Reclast®) belongs to bisphosphonate (BP) class agents which are important and well-established drugs in treatment of wide range of cancers, including breast cancer, prostate cancer and multiple myeloma.

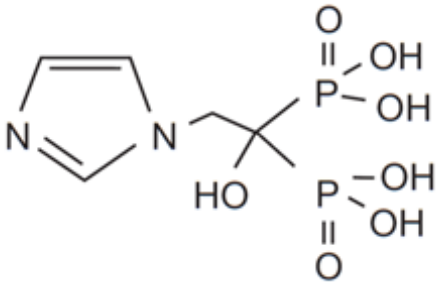


Figure 1.1 Structure of zolenoid acid (Wong and Pavlakis, 2011)

Zoledronic acid is known and frequently used to interfere with cell-to-bone matrix attachment to prevent metastasis to bone (Maricic, 2007). Zoledronic acid inhibits osteoclast mediated bone resorption to block the release of growth factors such as TGF-β and IGF-1 as well as interleukins and chemokines which are essential for tumor initiation and growth in bone. Moreover, studies showed that N-telopeptide of type I collagen (NTX) levels were elevated during osteoclast mediated bone resorption, however, zoledronic acid modulates microenvironment of bone by normalizing the levels of NTX in order to block the growth of secondary tumors in bone (Coleman *et. al*, 2011). The re-modification of bone microenvironment by zoledronic acid also affects the survival of disseminated tumor cells and prevents tumor recurrence (Gimsing *et. al*, 2010). The anti-metastatic activities of zoledronic acid have powerful effects on prevention of skeletal related events (SREs) such as pathological fractures, hypercalcemia and spinal cord compression which are associated with increased morbidity and mortality in women with breast cancer (Saad *et. al*, 2007). Another important activity of zoledronic acid is that zoledronic acid exerts its effects on farnesyl diphosphate synthase which is a key enzyme in formation of isoprenoid lipids such as ubiquinone and dolichol. These lipids are further required to post-transcriptionally modify and activate small GTPases. Preventing the activation of these GTPases which include Ras, Rho and Rab families, zoledronic acid indirectly regulate the important signaling pathways and cellular processes for osteoclast function (Roelofs *et. al*, 2006). Beside the anti-metastatic and regulatory activities, preclinical studies showed that zoledronic acid exhibits direct and indirect anticancer activities including the direct inhibiton of

cancer cell proliferation, induction of apoptosis, inhibition of vascularization (Traina, 2009), activation of antitumor T cell immunity and synergy with other cytotoxic drugs (Jagdev, 2001).

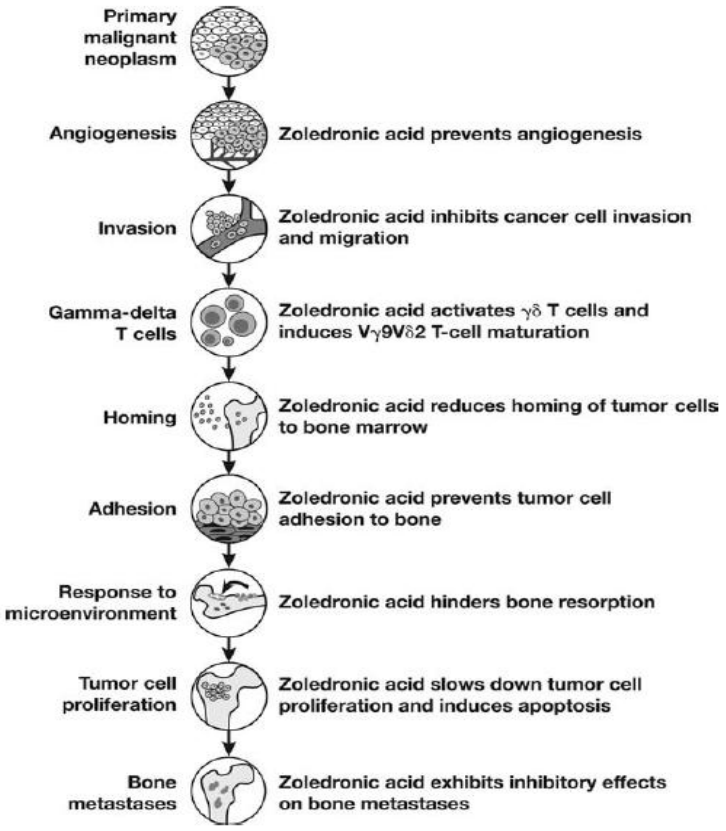


Figure 1.2 Action mechanisms of zoledronic acid (Coleman *et. al*, 2001)

1.4 Multidrug Resistance (MDR)

Multidrug resistance (MDR) is a term which is used to describe different mechanims of tumor cells to avoid cytotoxic effects of anticancer drugs (Simon and Schindler, 1994). Drug resistant tumor cells can develop simultaneous resistance to various functionally and structurally different chemotherapeutics (Krishna and Mayer, 2000). Multidrug resistance is the major obstacle in the success of chemotherapy, causing failure in cancer treatment in approximately 90% of cancer patients (Longley and Johnston, 2005).

MDR could be intrinsicly present in cancer cells prior to any treatment with chemotherapeutics and inherited to the daughter cells from the drug-resistant parent, as well as it could be acquired during the course of chemotherapy (Ejendal and Hrycyna, 2002) (Figure 1.3).

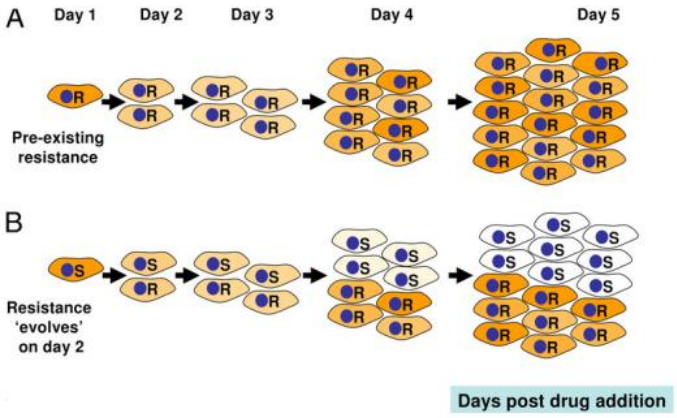


Figure 1.3 Evolution of drug resistance during drug addition (Robinson *et. al*, 2011)

Tumor cells are heterogenous in terms of cellular composition. Some cells could be drug-sensitive, whereas the rest could be drug-resistant. Chemotherapeutics kills drug-sensitive cells, remaining drug-resistant cells alive at a higher population, therefore, as drug-resistant cells proliferate, treatment will fail due to resistant tumor cell population.

1.4.1 MDR Mechanisms

Multidrug resistance could be acquired through various mechanims. Multidrug resistance mechanisms could be classified into two major categories as non-cellular and cellular based resistance mechanims (Krishna and Mayer, 2000). Non-cellular resistance mechanims are generally associated with solid tumors including poor vascularization of solid tumors (Jain, 1987) and acidic environment in hypoxic tumors leading resistance to weak bases (Demant *et. al*, 1990). Cellular-based resistance mechanisms could further be examined in two different groups; non-classical MDR phenotypes and transport-based MDR mechanisms. Non-classical

MDR phenotypes include alterations in drug metabolism, altered drug targets, changes in the activity of certain detoxification pathways such as Cytochrome P450 (CYP) and Glutathione-S-transferase (GST) enzymes (Gottesman *et. al*, 2002), decreased permeability of cellular and nuclear membranes for decreased drug accumulation, alterations in various enzymes such as topoisomerases, changes in DNA binding and alterations in regulation of apoptotic pathways and DNA repair mechanisms (Simon and Schindler, 1994).

1.4.1.1 Transport-based MDR Mechanisms

One of the most common mechanisms to develop multidrug resistance is increased drug efflux (Morrow *et. al*, 2009). The drug efflux is achieved by certain transmembrane proteins which belong to ATP-binding cassette (ABC) transporter superfamily. ABC transporter protein family is one of the most ancient and largest protein families among different species. So far, 28 ABC transporters in *Saccharomyces*, 51 in *Drosophila*, 58 in *Caenorhabditis*, 129 in *Arabadopsis* and 69 in *E.coli*, which accounts for 5% of its genome, have been identified (Linton , 2007). Similarly, 48 ABC transporter proteins have been discovered in humans (Gottesman *et. al*, 2002).

Even though they have been discovered in drug resistant cell lines, ABC transporters are expressed in normal tissues including kidney, intestine, liver, placenta, blood-brain barrier, pancreas and testes. The function of ABC transporters is varying due to the localization of the proteins, however, they mostly involve in the uptake, transport and distribution of endogenous substrates as well as these of exogenously administered drugs (Table 1.1).

Table 1.1 Localization, function and possible substrates of common ABC transporters (Gottesman *et. al*, 2002).

Common Name	Systematic name	Tissue	Non-chemotherapy substrates	Chemotherapy substrates (known and suspected)
PGP/MDR1	ABCB1	Intestine, liver, kidney, placenta, blood–brain barrier	Neutral and cationic organic compounds, many commonly used drugs	Doxorubicin, daunorubicin, vincristine, vinblastine, actinomycin-D, paclitaxel, docetaxel, etoposide, teniposide, bisantrene, homoharringtonine (STI-571)
MDR2	ABCB4	Liver	Phosphatidylcholine, some hydrophobic drugs	Paclitaxel, vinblastine
MRP1	ABCC1	All tissues	Glutathione and other conjugates, organic anions, leukotriene C4	Doxorubicin, epirubicin, etoposide, vincristine, methotrexate
MRP2, cMOAT	ABCC2	Liver, kidney, intestine	Similar to MRP1, non-bile salt organic anions	Methotrexate, etoposide, doxorubicin, cisplatin, vincristine, mitoxantrone
MRP3	ABCC3	Pancreas, kidney, intestine, liver, adrenal glands	Glucuronate and glutathione conjugates, bile acids	Etoposide, teniposide, methotrexate, cisplatin, vincristine, doxorubicin
MRP4	ABCC4	Prostate, testis, ovary, intestine, pancreas, lung	Nucleotide analogues, organic anions	Methotrexate, thiopurines
MRP5	ABCC5	Most tissues	Nucleotide analogues, cyclic nucleotides, organic anions	6-Mercaptopurine 6-Thioguanine
MRP6	ABCC6	Liver, kidney	Anionic cyclic pentapeptide	Unknown
MXR, BCRP, ABC-P	ABCG2	Placenta, intestine, breast, liver	Prazosin	Doxorubicin, daunorubicin, mitoxantrone, topotecan, SN-38
BSEP, SPGP	ABCB11	Liver	Bile salts	Paclitaxel
ABCA2	ABCA2	Brain, monocytes	Steroid derivatives, lipids	Estramustine

Most mammalian ABC transporters consist of 2 major structural domains: the transmembrane domain (TMD) which contains hydrophobic transmembrane α-helices and the hydrophilic intracellular nucleotide binding domain (NBD) (Figure 1.4). TMD is not conserved in terms of amino acid composition. The unconserved amino acid structure suggested that TMD determines substrate specificity for a particular ABC transporter. On the other hand, since NBDs are binding sites for ATP molecules, hence they are evolutionarily conserved among all organisms (Ejendal and Hrycyna, 2002).

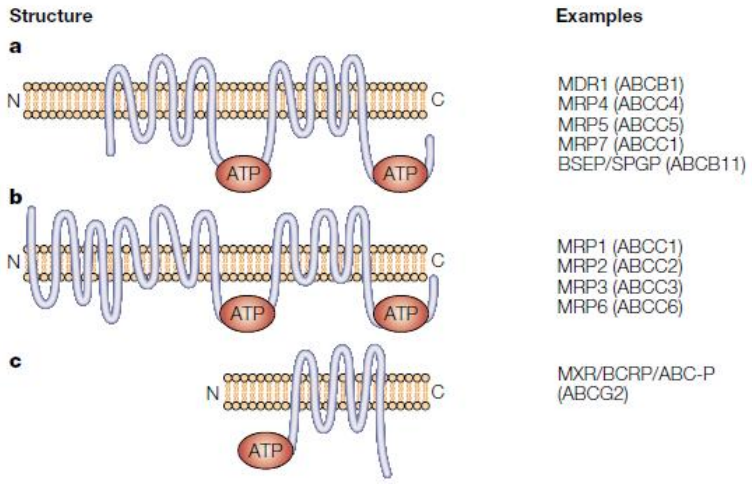


Figure 1.4 Structures of ABC transporters that involve in drug resistance (Gottesman *et. al*, 2002)

Functional studies showed that reduced drug accumulation was reversed in ATP-depleting conditions (Bates *et. al*, 2001). It confirmed that ABC transporters achieve transport of various cytotoxic agents aganist concentration gradient through hydrolysis of ATP molecules (Mao and Unadkat, 2001).

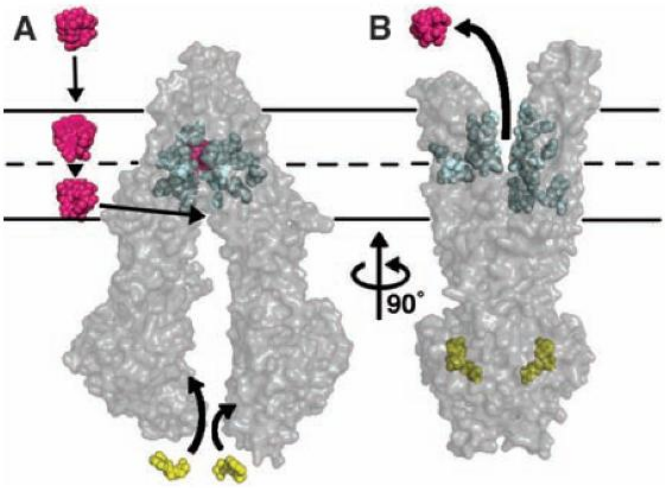


Figure 1.5 Substrate transport by P-gp. Substrate (magenta) binds to drug-binding pocket (cyan). Through ATP (yellow) hydrolysis, P-gp undergoes conformational change and substrate is transported to outside of the cell (Aller *et. al*, 2009).

Although most of ABC transporters are embedded in plasma membrane, some of transporters could be found on intracellular organelles and in cytoplasm. For instance, a 110 kDa ABC protein was discovered predominantly in lysosomes (Simon and Schindler, 1994). Moreover, lung resistance protein (LRP) have been firstly identified in P-gp lacked non-small cell lung cancer cell lines. LRP is found in cytoplasm as a major component of ribonucleoprotein complex called as vault. The vault complexes are responsible for nucleocytoplasmic and vesicular transport of drugs (Scheffer *et. al*, 1995).

1.4.1.1.1 P-glycoprotein (P-gp/MDR1)

P-glycoprotein (P-gp) is the first human ABC transporter to be identified. P-gp is the product of *MDR1* gene which is localized on human chromosome 7q21 (Ambudkar *et. al*, 2003). With a 170 kDa molecular weight, P-gp consists of 2 transmembrane domains, each with 6 transmembrane segments and 2 nucleotide binding domains (Figure 1.6), therefore, it is called as a full transporter (Linton, 2007).

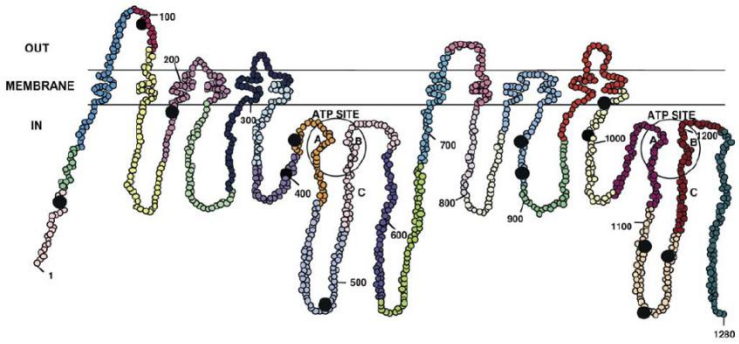


Figure 1.6 Schematic representation of P-gp (Ambudkar *et. al*, 2003)

P-gp is normally expressed in epithelia of intestines, kidney, liver, testes and ovaries (Ambudkar *et. al*, 2003) and protects these tissues from external and internal toxins (Gottesman *et. al*, 2002). However, overexpression of P-gp is commonly observed in malignancies. Approximately 40% of breast cancers are known to overexpress P-gp.

P-glycoprotein could recognize numerous compounds which range between 330 to 4000 Daltons (Aller *et. al*, 2009) including anthracyclins (doxorubicin, epirubicin), taxenes (docetaxel, paclitaxel) and *Vinca* alkaloids (vincristine) (Kim, 2002).

Although P-gp modulators such as verapamil and promethazine are effective in terms of inhibiton of P-gp mediated drug efflux (Dönmez *et. al*, 2011), they are limited in use due to their cytotoxic nature (Modok *et. al*, 2006).

1.4.1.1.2 Multidrug Resistance Protein 1 (MRP1)

Multidrug resistance protein 1 (MRP1) is a large protein with a molecular weight of 190 kDa. MRP1 is also a full transporter similar to P-gp, however, it has an asymmetrical structure due to having five additional transmembrane segments (Linton, 2007). The first transmembrane domain is called TMD_0 and thought to provide higher affinity for organic anions (Leonard *et. al*, 2003).

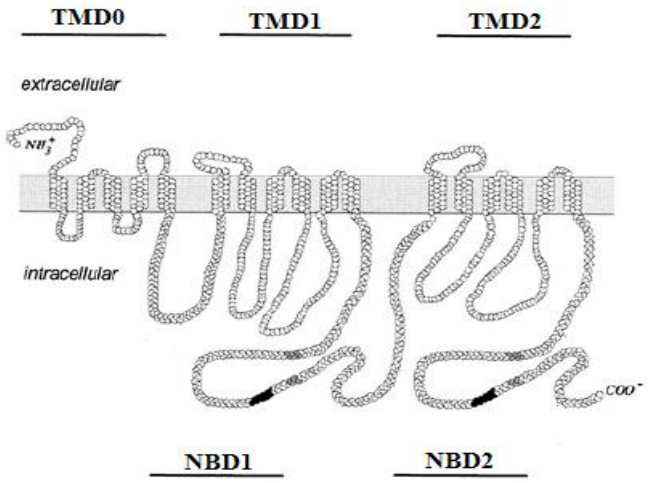


Figure 1.7 Schematic representation of membrane topology of MRP1 (adapted from Kern *et. al*, 2004)

By decreasing intracellular drug accumulation, MRP1 provides resistance to various chemically unrelated cytotoxic agents such as epipodophyllotoxins (etoposide, teniposide), anthracyclines (daunorubicin, doxorubicin) and methotrexate (Leonessa and Clarke, 2003). Recent studies showed that MRP1 is able to transport mitoxantrone in glutathione conjugated form (Morrow *et. al*, 2006). Because of the capability of transporting glutathione conjugates, MRP1 is called as GS-X pump and acts as detoxification factor inside the cell.

1.4.1.1.3 Breast Cancer Resistance Protein (BCRP)

Breast cancer resistance protein (BCRP/ABCG2/MXR) is a member of subfamily G of ABC transporter family (Doyle and Ross, 2003). BCRP was identified in doxorubicin resistant-verapamil selected MCF7 cells (Doyle *et. al*, 1998). Later, it was mapped to human chromosome 4q22, downstream of a TATAless promoter (Knutsen *et. al*, 2000).

BCRP is a small protein consisting of 655 amino acid with a molecular weight of 72 kDa (Doyle and Ross, 2003). In contrast to other ABC transporters, BCRP consists of one pair of transmembrane domain with 6 membrane spaning regions and nucleotide binding domain (Figure X), therefore, it is named as a half transporter (Bates *et. al*, 2001). Recent studies showed that BCRP could function as a homodimer or homotetramer (Mao and Unadkat, 2005).

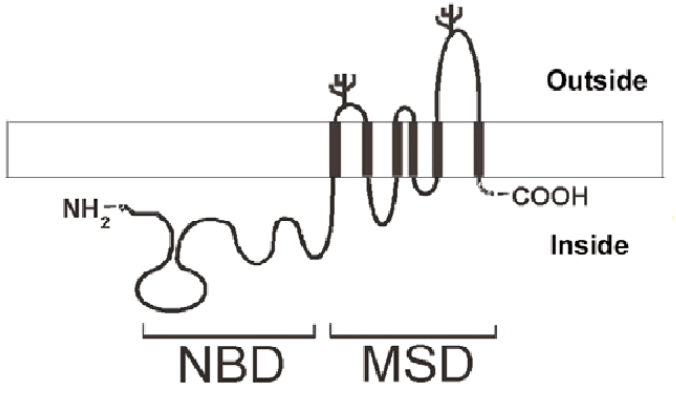


Figure 1.8 Proposed membrane topology model of BCRP (Mao and Unadkat, 2005)

BCRP is constitutively expressed in blood-brain barrier, small intestine and liver, all of which have important roles in drug metabolism and disposition. Moreover, expression of BCRP at a higher rate in placenta suggested that BCRP may involve in the elimination of fetal waste products and protection of fetus from potential toxins (Ewart and Howells, 1998). Different studies showed that BCRP is predominantly localized in cellular membrane (Maliepaard *et. al*, 2001). On the other hand, BCRP was found on lysosomal membranes and doxorubicin-positive intracellular vesicles in BCRP-transfected HeLa cells (Rajagopal and Simon, 2003). The location of BCRP is correlated with its function to reduce drug accumulation inside the cell and keep drugs away from the cellular targets.

BCRP has a wide range of substrates including both cytotoxic agents and organic molecules. The substrates of BCRP includes a potent anthracenedione called mitoxantrone, nucleoside analogs used in treatment of HIV infections and anthracyclines (Table 1.2). Structure-function studies demonstrated that substrate specificity of BCRP is closely related with amino acid at position 482. Wild type BCRP containing an arginine (Arg) at position 482 does not recognize and transport anthracyclines such as daunorubicin and doxorubicin, however, the mutants having a threonine (Thr) or glycine (Gly) do. On the other hand, both wild type and mutants recognize mitoxantrone and Hoechst 33342 (Robey *et. al*, 2003).

Table 1.2 Substrates of BCRP (Mao and Unadkat, 2005)

Drug	Organic Molecule
Anthracyclines*	Fluorophores
Daunorubicin	Rhodamine 123*
Doxorubicin	Lysotracker Green*
Epirubicin	Prazosin-BODIPY
	Hoechst 33342
Anthracenes	
Mitoxantrone	Conjugates
Bisantrene	Estrone-3-sulfate (E_1S)
Aza-anthrapyrazole (BBR 3390)	4-MUS
	E3040S
Camptothecin Derivates	TLC-S
Topotecan	4-MUG
SN-38	E3040G
9-amino-camptothecin	$E_2$17βG
Irinotecan	DNP-SG
Diflomotecan	
Polyglutamates*	Other Molecules
Methotrexate	Phosphatidylserine
Methotrexate-Glu_2	Pheophorbide α
Methotrexate-Glu_3	Protoporphyrin IX
	PhIP
Nucleoside Analogs	GV196771
AZT	Genistein
AZT 5′-monophosphate	
Lamivudine (3TC)	
Other Drugs	
Prazosin	
Indolocarbazole	
Topoisomerase I inhibitors (NB-506; J-107088)	
Flavopiridol	
ErbB1 tyrosine kinase inhibitor (CI1033)	
Imatinib mesylate (STI571)	
Pantoprazole	

* Whether these compounds are substrates of BCRP depends on the amino acid at position 482.

In vitro resistance to zoledronic acid was firstly reported by Kars *et. al*, and it was found that the resistance to zoledronic acid in MCF7 human breast cancer cell line could be associated with overexpression of a resistance related transporter protein called breast cancer resistance protein (BCRP) (Kars *et. al*, 2007).

1.5 Reversal Strategies for MDR in Cancer Cells

Since the development of multidrug resistant is the primary cause of failure in chemotherapy, it is essential to overcome MDR in order to maximize clinical outcome. So far, several MDR-reversal strategies have been proposed to obtain higher concentrations of cytotoxic agents (Tiwari *et. al*, 2009). As mentioned before, overexpression of ABC transporter proteins is one of the most important reasons in the development of MDR phenotype, therefore, ABC transporters are main targets to reverse multidrug resistance in different types of cancers. Borowski *et. al* (Borowski *et. al*, 2005) classified the MDR-reversal strategies, which target ABC transporters, into two main categories; (*i*) the control of expression of ABC transporters and (*ii*) the control of function of ABC transporters.

Expression of ABC transporters can be controlled at transcriptional level by different approaches such as modification of promoter, RNA-interference technology and use of ribozymes (Borowski *et. al*, 2005). The first means to control the expression of transporters is to modulate gene promoters. BCRP contains an estrogen response element (ERE) in the promoter region, hence the expression of BCRP can be affected by estrogen levels. Toremifene (a selective estrogen receptor modulator) caused reduced BCRP expression upon binding to ERE and interfering with transcription machinery (Zhang *et. al*, 2010). RNA-interference (RNAi) technology involves the use of small non-coding RNA molecules to control the transcription of the target molecules. It was demonstrated that siRNA-mediated destruction of BCRP mRNA leads to significant decrease in growth of cancer cells (Ee *et. al*, 2005). Use of ribozymes (small RNA molecules which catalyze site-specific cleavage of the target) is another way to affect the target mRNA (Phylactou, 2001). Transfection of human gastric carcinoma cell line with anti-BCRP ribozyme was found to increase intracellular drug accumulation by 80% (Kowalski *et. al*, 2002). Although *in vitro* studies yielded remarkable results, the effectiveness of RNAi and ribozyme strategies has not been confirmed by animal experiments (Borowski *et. al*, 2005).

1.5.1 Modulation of BCRP Function

The function of BCRP is modulated by combination therapy which includes the simultaneous use of cytotoxic drugs and augmenting agents. The augmenting agents are named as modulators, inhibitors, chemosensitizers or reversing agents regardless of their structure and function (Borowski *et. al*, 2005).

Table 1.3 BCRP modulators (Mao and Unadkat, 2005)

Inhibitor	IC_{50} (nM)
GF120918	50
Fumitremorgin C (FTC)	1,000
Ko132	190–270
Ko134	85–110
Iressa (Gefitinib or ZD1839)	300
Imatinib mesylate (STI571 or Gleevec)	170
EKI-785	100
CI1033	3700
Novobiocin	50–100
Estrone	ND
Diethylstilbestrol	ND
Tamoxifen, TAG-11 and TAG0139	ND
Reserpine	ND
VX-710 (Biricodar or Incel)	ND
Tryprostatin A	ND
Flavonoids (chrysin and biochanin A)	low μM ranges
Ritonavir	19 500
Saquinavir	19 500
Nelfinavir	12 500
Omeprazole	10 000–50 000

* Several of the inhibitors such as CI1033 are BCRP substrates.
ND indicates not determined.

One strategy to modulate BCRP function is the use of non-substrate agents. Poor substrates of BCRP including camptothecin derivatives (Perego *et. al*, 2001; Zhang *et. al*, 2008) and antimetabolites such as cytarabine (Stam *et. al*, 2004) are advantageous in reversal of BCRP-mediated MDR because they do not disturb the normal biological function of BCRP in constitutively expressed tissues. However, it is difficult to identify non-substrate agents in random testing since BCRP has a broad range of substrates. Beside these, GF120918 and Fumitremorgin C (FTC) are

important modulators of BCRP. GF120918 (a second-generation P-gp inhibitor) was found to increase drug accumulation in BCRP expressing cells with low inhibitory concentrations (Doyle and Ross, 2003). Fumitremorgin C is derived from *Aspergillus fumigatus* was demonstrated to completely reverse mitoxantrone resistance in colon carcinoma cell lines at 1-5 μM concentrations (Mao and Unadkat, 2005). Although effective in inhibiton of BCRP-mediated drug transport, these modulators could exert side effects and their use is precluded due to their neurotoxicity (Borowski *et. al*, 2005).

1.5.1.1 Flavonoid Class Modulators

Flavonoid class agents are natural polyphenolic compounds which are widely found in foods and herbs including soy products and grape. Flavonoids are categorized as isoflavonoids and neoflavonoids depending on their precursor. 3-phenylchromen-4-one (3-phenyl-1,4-benzopyrone) structure is the precursor of the isoflavonoids whereas neoflavonoids are derived from 4-phenylcoumarine (4-phenyl-1,2-benzopyrone). More than 4000 types of flavonoids have been identified. All of them are phenyl-benzopyrenes which are formed by two benzene rings combined with a heterocyclic pyrone or pyrane (Gallego *et. al*, 2007).

Flavonoids are known to exhibit a number of beneficial properties for human health due to their interactions with a number of cellular targets. Both isoflavonoids and neoflavonoids were found to act as free-radical scavenger and have antioxidant activity *in vitro* (Burda and Olezsek, 2001). It was suggested that flavonoids may stabilize free oxygen species by hydrogenation or formation of complexes with these species. Flavonoids were shown to display anti-inflammatory and antiviral functions as well as they may involve in prevention of cardiac diseases due to anti-thrombotic properties (Di Pietro *et. al*, 2002; Gallego, *et. al*, 2007). Morris and Zhang (Morris and Zhang, 2006) showed that flavonoids have anti-estrogenic effects. Flavonoids may interfere with estrogen stimulation in estrogen receptor (ER) positive cells

through competition with estrogen for ER. Moreover, flavonoids might induce various mechanisms that affect cancer cells and inhibit tumor invasion through different pathways (Figure 1.9) (Kanadaswami *et. al*, 2005).

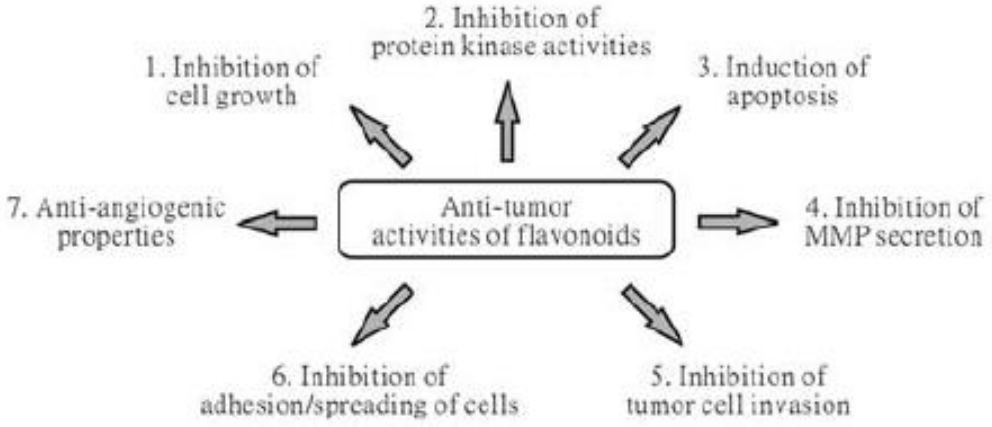
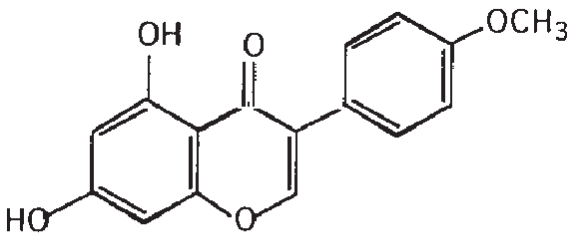


Figure 1.9 Antitumor activites of flavonoids (Kanadaswami *et. al*, 2005)

1.5.1.1.1 Biochanin A

Biochanin, a member of isoflavonoids, is one of the most potent BCRP inhibitors. Biochanin A is a natural product which is derived from red clover (*Trifolium pratense).* Red clover isoflavone extracts, containing Biochanin A predominantly, are commercially available as dietary supplements and widely used for relieveing post-menopausal symptoms in women and maintaining the prostate health in men (Zhang *et. al*, 2009).



Biochanin A

Figure 1.10 Structure of Biochanin A (Peterson *et. al*, 1998)

Although metabolism of Biochanin A and other flavonoids frequently takes place in liver, intestines and kidneys, Biochanin A could be also metabolized in breast. Biochanin A has a high clearance rate and poor bioavailability (<4%) due to this extensive metabolism. It could be a disadvantage because it is rapidly cleared from the circulation. However, Biochanin A is metabolized into genistein (another isoflavonoid class agent) and its conjugates which have also inhibitory effects on BCRP (Moon *et. al*, 2006). This may prolonge the effects of Biochanin A on BCRP-mediated multidrug resistance.

Biochanin A may have antiproliferative activities. It was revealed that Biochanin A inhibited the growth of human prostate cancer cells (Peterson and Barnes, 1993), mammary cancer cells (Peterson *et. al*, 1996) and cancer cells established from the gastrointestinal tract (Yanagihara *et. al*, 1993). Moreover, Biochanin A and its structurally related isoflavonoids were effective on induction of apoptosis by interfering cell cycle kinetics (Balabhadrapathruni *et. al*, 2000). Beside these properties, several studies demonstrated that Biochanin A could be effective to increase intracellular drug accumulation in BCRP-overexpressing cancer cell lines (Zhang *et. al*, 2004).

1.6 Objectives of the Study

The aim of this study is to reverse BCRP mediated zoledronic acid resistance with an novel natural agent. Blocking the function of BCRP can increase the sensitivity of resistant cells to zoledronic acid, therefore, the success of chemotherapy.

The objectives of this study are as below:

- Determination of expression levels of *BCRP*, *MRP1* and *MDR1* in parental and zoledronic acid resistant MCF7 cell lines
- Evaluation of antiproliferative effect of zoledronic acid on parental sensitive and zoledronic acid resistant MCF7 cell lines and determination of inhibitory concentration 50 (IC50) of zoledronic acid
- Investigation of cytotoxicity of Biochanin A in parental sensitive and zoledronic acid resistant MCF7 cell lines and determination of inhibitory concentration 50 (IC50) of Biochanin A
- Determination of changes in expression levels of *BCRP*, *MRP1* and *MDR1* after Biochanin A treatment
- Evaluation of chemo-sensitivity of zoledronic acid resistant MCF7 cells in response to Biochanin A treatment at different concentrations

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Lines

Parental sensitive MCF7 human breast carcinoma cell line was donated by Şap Institute, Ankara, Turkey. Zoledronic acid resistant (MCF7/Zol) subline was developed from parental MCF7 cell line (MCF7/S) previously in our laboratory by stepwise selection of cells in increasing drug concentrations with a final drug concentration of 8 μM zoledronic acid. Final MCF7/Zol subline was found to express high levels of BCRP (Kars *et. al*., 2007).

2.1.2 Chemicals and Reagents

Zoledronic acid was kindly provided by Novartis, Pharma AG, Switzerland. 5 mM stock solution was prepared with sterile distilled water and stored at -20^{0}C. Biochanin A was obtained from Sigma-Aldrich, USA. Biochanin A was dissolved in dimethyl sulfoxide (DMSO) with a stock concentration of 50 mM and stored at -20^{0}C.

RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Biochrom AG, Germany. Phosphate buffered saline (PBS) and DMSO were purchased from Sigma-Aldrich, USA. Trypsin-EDTA, trypan blue, gentamycin and XTT Cell Proliferation Assay Kit were obtained from Biological Industries, Israel. TRIzol® was purchased from Sigma-Aldrich, USA. Diethylpyrocarbonate (DEPC), ethanol, isopropanol and agarose were obtained from Applichem, Germany. High Range RNA ladder, 50 bp DNA ladder, dNTP set, 6X loading dye, Moloney-Murine Leukemia Virus Reverse Transcriptase and *Taq* DNA polymerase were purchased from Fermentas, Lithuania. FastStart Universal SYBR Green Master Kit (Rox) were obtained from Roche Diagnostics, Switzerland.

2.1.3 Primers

BCRP, MRP1, MDR1 and *β-actin* primers were obtained from Alpha DNA, Canada. Primer sequences, locations on exons and amplicon sizes are shown in Table 2.1.

Table 2.1 Primers used in gene expression analyses

Primer	Sequence	Location	Amplicon Size
BCRP Sense	5' AGATGGGTTTCCAAGCGTTCAT3'	Exon 15	91 bp
BCRP Antisense	5' CCAGTCCCAGTACGACTGTGACA3'	Exon 16	
MRP1 Sense	5'TGTGGGAAAACACATCTTTGA3'	Exon 18	80 bp
MRP1 Antisense	5'CTGTGCGTGACCAAGATCC3'	Exon 19	
MDR1 Sense	5'ACAGAAAGCGAAGCAGTGGT3'	Exon 15	62 bp
MDR1 Antisense	5'ATGGTGGTCCGACCTTTTC3'	Exon 16	
β-actin Sense	5'CCAACCGCGAGAAGATGA3'	Exon 3	97 bp
β-actin Antisense	5'CCAGAGGCGTACAGGGATAG3'	Exon 4	

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Line and Culture Conditions

Parental sensitive MCF7 cells (MCF7/S) and zoledronic acid resistant cells (MCF7/Zol) were maintained in 12 mL of RPMI 1640 medium (Appendix A) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) and 1% (v/v) gentamycin in T75 filter cap tissue culture flasks (Greiner Bio-one, Germany). Cells were incubated at 37^0C in a humidified atmosphere with 5% (v/v) CO_2 in a Heraeus incubator (Hanau, Germany).

2.2.1.2 Subculturing (Passaging)

Sunculturing is required when 80% confluency was reached in order to keep the cells actively growing and ready for experimentation (Freshney, 1994). Subculturing involves releasing cells from monolayer surface area and transferring them into new culture flasks. Subculturing was carried out by trypsinization. Briefly, medium was discarded and cells were washed with 3-4 mL of PBS to remove waste products as well as traces of serum which is known to inactivate trypsin. 1 mL of trypsin-EDTA was added and cells were incubated at 37^0C for 5 minutes to activate trypsin. Detached cells were taken into 15 mL Falcon tubes (Greiner) and centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded to remove traces of trypsin and pellet was resuspended and homogenized in medium containing serum. Appropriate number of homogenized cells were transferred into new culture flasks. Zoledronic acid was added with the final concentration of 8 μM in order to maintain resistance.

2.2.1.3 Freezing Cells

Cells were trypsinized to detach as described previously. Detached cells were suspendend in 5 mL of medium containing serum and centrifuged at 1000 rpm for 5 min. Supernatant was discarded and pellet was homogenized in 5 mL of PBS. Then cell suspension was centrifuged at 1000 rpm for 5 min. After discarding supernatant, cells were resuspended in 1 mL of freezing medium consisting of 10% (v/v) DMSO and 90% heat inactivated FBS. Optimum concentration of cells should be approximately $2x10^6$ cells/mL. The final cell suspension was taken into cryovials (Greiner Bio-One, Germany) and immediately immersed into ice. Then the cells were kept at -20°C for 1-2 hours and transferred to -80°C for overnight incubation. Finally, the cryovials were transferred to liquid nitrogen for long term storage.

2.2.1.4 Thawing Cells

Cryovials were taken from liquid nitrogen storage tank. Frozen cells were incubated at 37^0C until melting. Immediately after melting, the cells were taken into 15 mL Falcon tubes (Greiner Bio-One, Germany) because DMSO in freezing medium is toxic to cell at temperatures above 4^0C. The cells were centrifuged at 1000 rpm for 5 min. Supernatant was discarded to remove freezing medium and cells were resuspended in 3 mL of culture medium contaning 10% FBS. Then cells were seeded into culture flasks and the volume was completed to 7 mL.

2.2.1.5 Viable Cell Count by Trypan Blue Exclusion Method

Trypan Blue dye selectively stains cells, providing discrimination between dead and live cells. Since membrane of live cells is intact, trypan blue dye is not taken into the cell (Freshney, 1987). Therefore, viable cells remain colorless whereas dead cells with damaged membrane appear as blue.

Cells were trypsinized as described previously. After centrifugation and homogenization in medium containing serum, appropriate volume of the cell suspension was mixed with 0.5% trypan blue solution with a ratio of 9:1. 20 µL of cell suspension–trypan blue mixture was taken on Neubauer hemacytometer (Bright-line, Hausser Scientic, USA) and cells were counted under phase contrast microscopy (Olympus, USA).

The hemacytometer consists of 16 large squares, each containing 16 small squares. One small square has a volume of 0.00025 mm^3. Cell counting experiments were performed in triplicates. Cell concentration was calculated according to the formula below (Equation 2.1) :

Cell number/mL = Average cell count per square x Dilution factor x 4 x 10^6 (2.1)

2.2.2 Gene Expression Analyses

2.2.2.1 Isolation of Total RNA

All the glassware and other equipments were treated with DEPC prior to RNA isolation to inactivate RNases which cause RNA degradation.

Total RNA isolation was carried out using TRIzol® reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions. All centrifugation steps were performed at 4^0C.

Cells were treated with 1 mL of TRIzol® reagent after washing with 3-4 mL of PBS. Then, the cells were briefly detached using a cell scraper. Skipping trypsinization step would help to isolate intact RNA molecules by preventing degradation. Cell lysates were taken into 2 mL Eppendorf tubes (Greiner Bio-One, Germany) and

pipetted for several times for homogenization. The cell lysates were incubated at room temperature for 5 min. 200 µL of chloroform was added and mixed thoroughly for 15 seconds. After incubation on ice for 15 min, the cell lysates were centrifuged at 12000 g for 15 min. Three layers were formed; aqueous upper layer containing RNA, white middle layer containing precipitated DNA and pinkish bottom layer containing organic molecules. The upper phase was taken into a new tube and 500 µL of 100% ice-cold isopropanol was added. Samples were incubated at room temperature for 10 min prior to centrifugation at 120000 g for 10 min. Supernatant was discarded and RNA pellet was washed with 1 mL of 75% Ethanol. After centrifugation at 12000 g for 10 min, supernatant was removed and RNA pellet was air-dried. The RNA pellet was redissolved in appropriate volume of nuclease free dH_2O (Fermentas). Then RNA samples were incubated at 55^0C for 15 min to distrupt secondary structures and stored at -80^0C.

2.2.2.2 RNA isolation after Biochanin A treatment

MCF7/Zol cells were seeded in cell culture flasks and treated with 5, 10, 20 and 40 µM of Biochanin A for 72 hours.

Total RNA isolation was performed by TRIzol® reagent (Sigma-Aldrich, USA) as described previously.

2.2.2.3 Quantitation of the Isolated RNA

The concentration and purity of isolated RNA samples were determined by measuring optical densities at 260 nm and 280 nm using NanoDrop 2000C spectrophotometer (Thermo Fischer Scientific, USA). Absorbance at 260 nm is used to calculate RNA concentration while ratio of absorbance at 260 nm to 280 nm indicates purity of RNA.

The concentration of RNA was calculated using formula below (Equation 2.2) :

$[RNA]\ \mu g/mL = A_{260} \times 40.0$

where

A_{260} = absorbance at 260 nm

The average extinction coefficient of RNA was taken as 40.0. (2.2)

Pure RNA sample should have an A_{260}/A_{280} ratio of 2.0±0.1.

2.2.2.4 Agarose Gel Electrophoresis of RNA

The intactness of RNA samples and the presence of DNA contamination were investigated by horizontal agarose gel electrophoresis.

1 g of agarose was weighed and dissolved in 50 mL of 1X TAE buffer (Appendix B). The mixture was boiled in microwave oven until agarose completely melted. After cooling of gel solution, 3 µL of ethidium bromide solution (Appendix B) was added. The gel solution was poured into electrophoresis apparatus and comb was placed. After the gel solidified, 5 µL of RNA sample was mixed with 5 µL of 2X loading dye (Appendix B) and loaded. The samples were run on 2% (w/v) agarose gel at 80V for 60 min and visualized by UV gel acquisition system.

2.2.2.5 Reverse Transcription (cDNA synthesis)

cDNA synthesis were carried out with 5 µg of total RNA and 20 pmol either of *BCRP, MRP1, MDR1* and *β-actin* gene specific primer.

5 µg total RNA, 20 pmol of gene specific antisense primer and nuclease free dH_2O (Fermentas) were mixed into DEPC treated sterile 0.5 mL Eppendorf tube (Greiner) with a final volume of 11 µL. The sample was incubated at 70^0C for 5 min to break the secondary structures of RNA. Then, 4 µL of 5X reaction buffer, 2 µL of 10 mM dNTP mix and 2.5 µL of nuclease free dH_2O (Fermentas) were added. The reaction mixture was incubated at 37^0C for 5 min to allow primer annealing. Afterwards, 0.5 µL Moloney-Murine Leukemia Virus Reverse Transcriptase (Fermentas) was added and the mixture was incubated at 42^0C for 60 min for cDNA synthesis. Finally, incubation at 72^0C for 10 min was performed to terminate the reaction. cDNA was stored at -20^0C.

2.2.2.6 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Quantitative real-time polymerase chain reaction (qPCR) enables quantitation of PCR products subsequently with the amplification step. This method provides a basis for the determining of the precise concentration of the product as well as preventing the possible end-point detection problems caused by traditional PCR. SYBR Green is a frequently used fluorescent dye which intercalates the double strands of DNA. The accumulation of PCR products in each cycle intensifies the signal generated by the intercalation of SYBR Green, enabling to monitor the change in the quantity of products.

qPCR experiments were conducted in Rotor-Gene 6000 (Corbett Research, Australia). FastStart Universal SYBR Green Master (Rox) kit (Roche Diagnostics, Switzerland) was used to perform qPCR according to the manufacturer's instructions. The reaction mixture contained 10 µL 2X master mix, 2.8 µL template cDNA, 0.4 µL of sense and antisense primers and 6.4 µL nuclease free water. The reaction was carried out in 0.2 mL PCR tubes (Greiner Bio-one, Germany). Each sample was prepared in triplicates. No template control containing nuclease free water instead of template cDNA was used to detect background signal. qPCR conditions for *BCRP, MRP1, MDR1* and *β-actin* genes are represented in Table 2.3.

Table 2.2 qPCR conditions of *BCRP, MRP1, MDR1* and *β-actin* genes

	BCRP	**MRP1**	**MDR1**	**β-actin**
Activation	95^0C, 10 min	95^0C, 10 min	95^0C, 10 min	95^0C, 10 min
Denaturation	95^0C, 20 sec	95^0C, 20 sec	95^0C, 20 sec	95^0C, 20 sec
Annealing	58^0C, 30 sec	55^0C, 30 sec	55^0C, 30 sec	58^0C, 30 sec
Extension	72^0C, 45 sec	72^0C, 45 sec	72^0C, 30 sec	72^0C, 30 sec
Melting	50^0C - 99^0C	50^0C - 99^0C	50^0C - 99^0C	50^0C - 99^0C
Cycle number	45	45	45	45

Amplification results were plotted as fluorescence versus threshold cycle number.

After amplification, a melting analysis was performed to check whether non-specific products had been generated. Melting step was carried out by gradually increasing the temperature from 50^0C to 99^0C and subsequently monitoring the change in the fluorescent signal. Same PCR products generated by the specific primer pair have same melting temperature, giving same melting peaks in melt-curve analysis.

2.2.2.7 Quantitation of qPCR products

The method used to determine relative quantitaion of qPCR products was $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The data is represented as fold change in gene expression. The fold change in gene expression is determined by normalization with respect to an internal control gene and relative to a reference group (i.e. untreated control sample). The changes in gene expression of *BCRP*, *MRP1* and *MDR1* were determined according to the formula below (Equation 2.3):

Fold change = $2^{-\Delta\Delta Ct}$

$$\Delta\Delta Ct = (Ct_{target} - Ct_{internal\ control})_{treatment} - (Ct_{target} - Ct_{internal\ control})_{no\ treatment} \quad (2.3)$$

Fold changes of *BCRP*, *MRP1* and *MDR1* genes were normalized to the internal control gene β-actin and calculated for each sample relative to no-treatment control. The threshold cycle (C_T) values for each gene were represented in Appendix C.

2.2.2.8 Statistical Analysis

All data represent three independent experiments, each of which run in triplicates. The data were expressed as mean ± standart error of the means (SEM). The results were evaluated by one-way ANOVA test using GraphPad Prism 5.0 Software (GraphPad Software Inc, USA). Tukey's Multiple Comparison Analysis was applied to compare different groups. The mean differences were significant at the 0.05 level.

2.2.3 Determination of Antiproliferative Effect with XTT reagent

2.2.3.1 Determination of Antiproliferative Effect of Zoledronic acid

Cell proliferation or viability was measured as a function of redox potential of metabolically active cells by using XTT Cell Proliferation Assay Kit (Biological Industries, Israel). Live cells can reduce XTT reagent, which is a tetrazolium salt, to orange colored formazan crystals. The formazan crystals are water soluble and their intensities could be spectrophotometrically measured at a specific wavelength. The dye absorbance measured is proportional to the number of metabolically active cells (Biological Industries, 2002).

Briefly, MCF7/Zol cells were trypsinized and diluted in complete medium without antibiotics. Cells were seeded to 96-well plates starting from the second column (10000 cells/well). Cells were incubated at 37^0C to allow them to attach. After overnight incubation, medium was discarded to remove unattached cells. The first column was medium control column (medium without cells) and the second column was cell control column (untreated cells). Into the first and second columns, 150 µL of medium was added and to the columns 4 to 12, 50 µL of medium was added. The third column (high dose drug column) contained concentrated zoledronic acid (400 µM). The concentrated drug was serially diluted by taking 150 µL portion from the third column and passing it to the next column. Finally, all volumes were completed to 150 µL by adding 100 µL medium to the columns 4 to 12. Cells were incubated at 37^0C for 72 hours.

The antiproliferative effect of zoledronic acid was also investigated in MCF7/S cells. MCF7/S cells were seeded to 96-well plates (10000 cells/well) and subjected to the same serial dilutions to assess the cell proliferation. 200 µM zoledronic acid was used as highest drug concentration.

XTT and activator reagents were added to the plates according to the manufacturer's manual after 72 hour incubation period was completed. Cells were incubated at 37^0C for 4 hours in order that XTT reagent was reduced to formazan compund. The optical density of soluble formazan compound was measured at 492 nm with a Anthos 2010 96-well plate reader (Biochrom, Germany).

Inhibitory concentration 50 (IC50) of a specific agent is defined as the concentration of the drug which inhibits a particular biological activity by 50%. IC50 is calculated by plotting % cell proliferation versus concentration graphs. To plot % cell proliferation vs concentration graph, the intensity of the formazan crystal formed by the reduction of tetrazolium salts was converted to percent cell proliferation. The proliferation of control cells (cell control column) was accepted as 100% and the proliferation of the cells at different concentrations of zoledronic acid was calculated accordingly. Resistance index (R) is the ratio of IC50 of resistant cell line to sensitive cell line (Dalton *et. al*, 1986).

Resistant indices (R) were calculated by using the formula below (Equation 2.4):

R = IC50 of resistant cell line / IC50 of sensitive cell line (2.4)

2.2.3.2 Determination of Antiproliferative Effect of Biochanin A

Cell proliferation in concentration gradient of Biochanin A was determined as previously described. Concentrated Biochanin A (45 μM for MCF7 cells and 150 μM for MCF7/Zol cells) was serially diluted with the same dilution rate.

Biochanin A was dissolved in DMSO (Applichem) prior to cell proliferation assays. The fact that DMSO exerts toxic effects on the cells at 37^0C, the top and bottom horizontal rows were left as DMSO control columns in Biochanin A testing plates in

order to eliminate the effect of DMSO on the cells. DMSO was subjected to the same serial dilutions as Biochanin A.

IC50 values of Biochanin A in MCF7 and MCF7/Zol cells were determined as described previously.

2.2.3.3 Determination of Reversal Effect of Biochanin A

Cell proliferation in concentration gradient of zoledronic acid in the presence of Biochanin A was determined in the previously described manner. Concentrated zoledronic acid was serially diluted with the same dilution rate. The maximum concentration of zoledronic acid for MCF7/S cells was 200 μM while it was 400 μM for MCF7/Zol cells. Cells were treated with either 5,10, 20 or 40 μM Biochanin A. The top and bottom horizontal rows were left as DMSO control columns. IC50 values were calculated for each group of cells.

Fold reversal (FR) is expressed as the ratio of the IC50 of the resistant cell line without any treatment to resistant cells which are treated with a particular modulator (Wu *et. al*, 2003).

Fold reversal was determined by the following formula (Equation 2.5):

Fold reversal = IC50 of resistant cells / IC50 of Biochanin A treated cells (2.5)

2.2.3.4 Statistical Analysis

All cytotoxicity experiments were carried out in triplicates of three independent experiments. The data were expressed as mean ± standart error of the means (SEM) and evaluated by one-way ANOVA test using GraphPad Prism 5.0 Software (GraphPad Software Inc, USA). Tukey's Multiple Comparison Analysis was applied to compare different groups. The mean differences were significant at the 0.05 level.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Isolation of Total RNA

Isolated total RNAs were analyzed on 2% agarose gel prior to cDNA synthesis (Figure 3.1). Ribosomal RNAs (rRNAs) comprise approximately 80-85% of total RNA sample, therefore, they could be visualized on agarose gel. The presence of distinct and sharp bands which corresponded to 28S and 18S rRNA, respectively, without any smear appearence indicated that the isolated RNA samples were intact and suitable for further analysis. The RNA samples were further examined by NanoDrop 2000C spectrophotometer (Thermo Fischer Scientific, USA) to check the purity of sample. The RNA samples with the nucleic acid/protein (A260/A280) ratio of 1.8-2.0 were used in gene expression analyses.

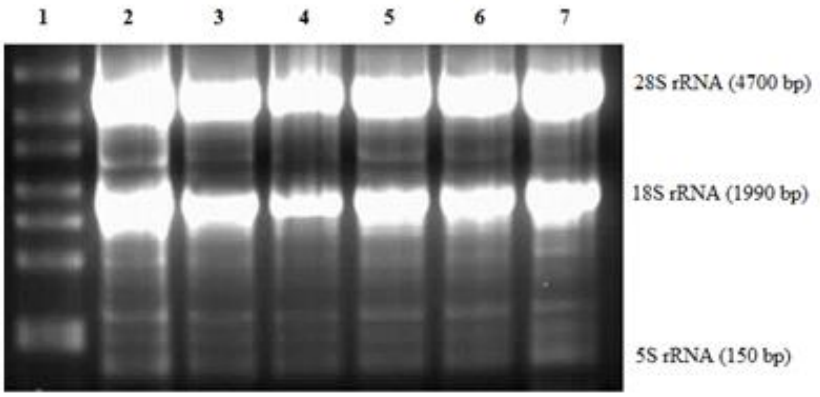
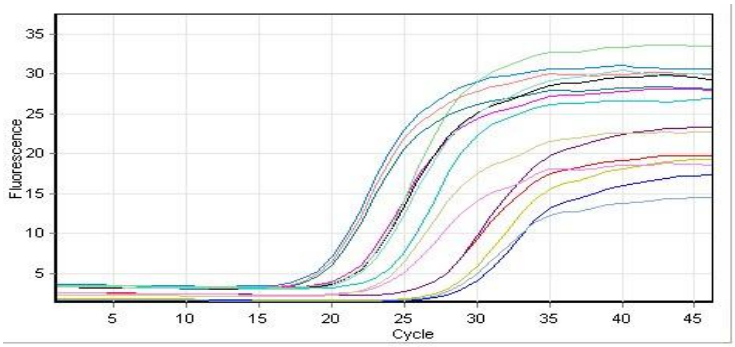


Figure 3.1 Total RNAs isolated from MCF7/S (Lane 2) and MCF7/Zol cells treated with Biochanin A at different concentrations (Lanes 3-7) on 2% agarose gel (High Range RNA ladder was Lane 1).

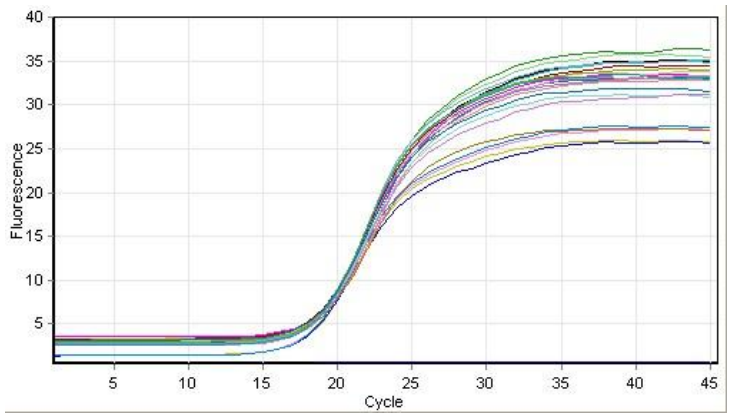
3.2 Quantitative Real-Time Polymerase Chain Reactin (qPCR) : Expression analysis of *BCRP, MRP1* and *MDR1* genes

The change in the expression of *BCRP*, *MRP1* , *MDR1* and *β-actin* genes in parental MCF7/S cell line and resistant MCF7/Zol subline was quantified by qPCR before and after cells were treated with different concentrations of Biochanin A for 72 hours. Amplification curves were displayed as fluorescence vs threshold cycle number (Figure 3.2).

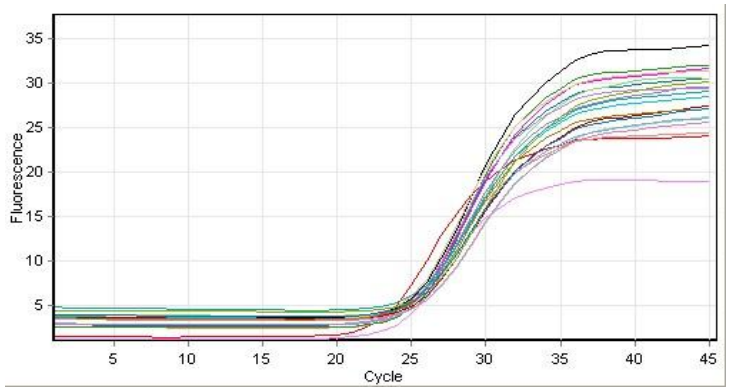
a)



b)



c)



d)

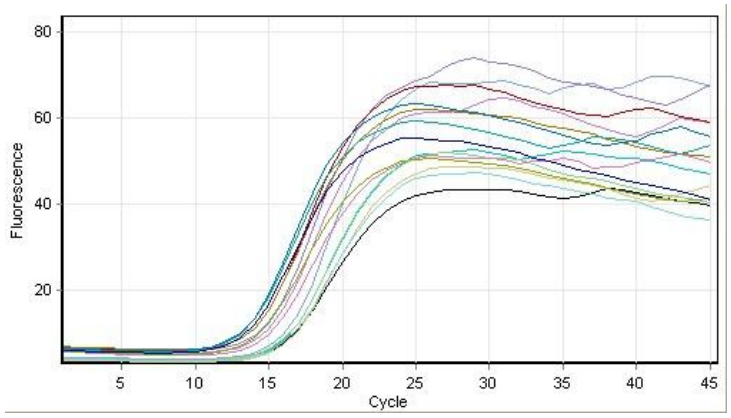


Figure 3.2 Amplification curves for a) *BCRP*, b) *MRP1*, c) *MDR1* and d) *β-actin* genes in MCF7/S and MCF7/Zol cell lines

Melting curve analysis was performed after each run by measuring the signal generated from dissociation of DNA as the temperature increased. The presence of nonspecific amplification was checked by monitoring the dissociation kinetics of the qPCR products (Figure 3.3).

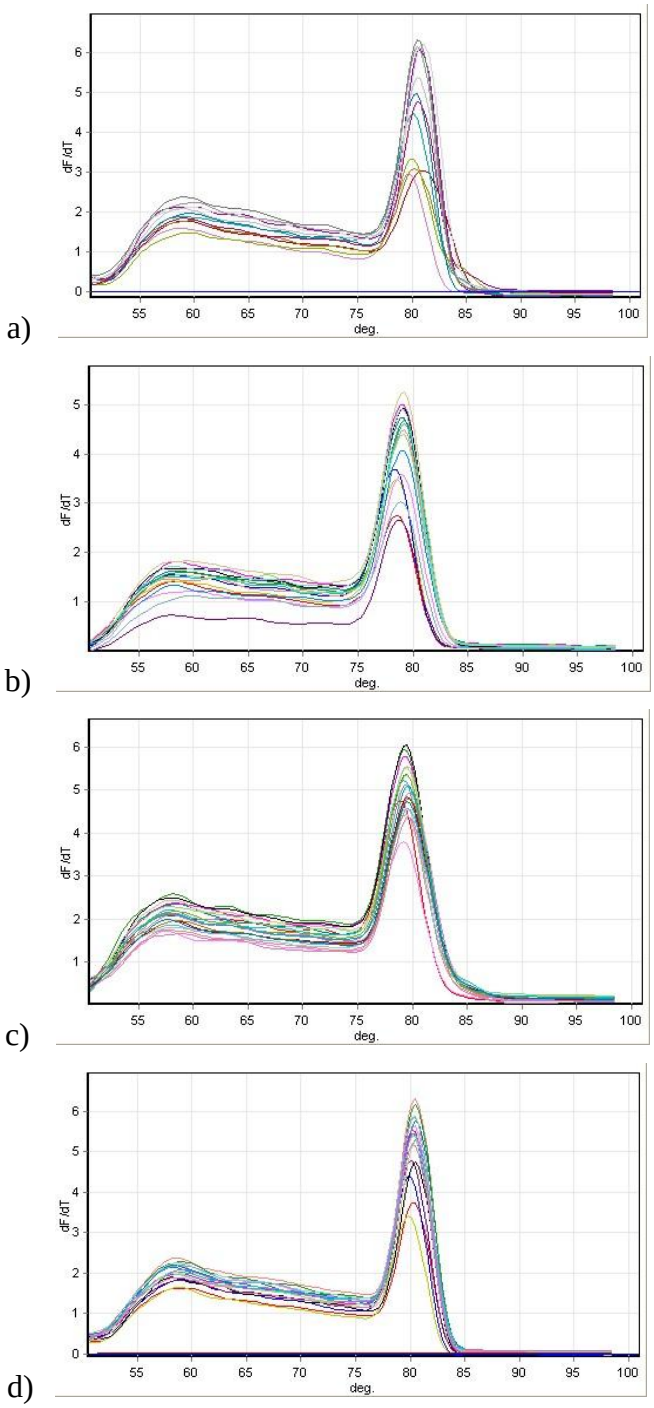


Figure 3.3 Melt-curve analysis for a) *BCRP*, b) *MRP1*, c) *MDR1* and d) *β-actin* genes in MCF7/S and MCF7/Zol cell lines.

As demonstrated in Figure 3.3, amplification products generated by a particular gene-specific primer had melting peaks at the same temperature, indicating that only the expected products were amplified. The absence of melting peaks corresponding other temperatures showed that there were no nonspecific products generated. The qPCR products of *BCRP*, *MRP1*, *MDR1* and *β-actin* were further examined by agarose gel electrophoresis and demonstrated in Appendix D.

The quantitation data of *BCRP*, *MRP1* and *MDR1* in MCF7/Zol cell line were normalized with respect to the internal control gene *β-actin*. The fold changes in the expression of *BCRP*, *MRP1* and *MDR1* were determined relative to MCF7/S parental cell line by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The relative fold changes were displayed as bar graphs in Figure 3.4.

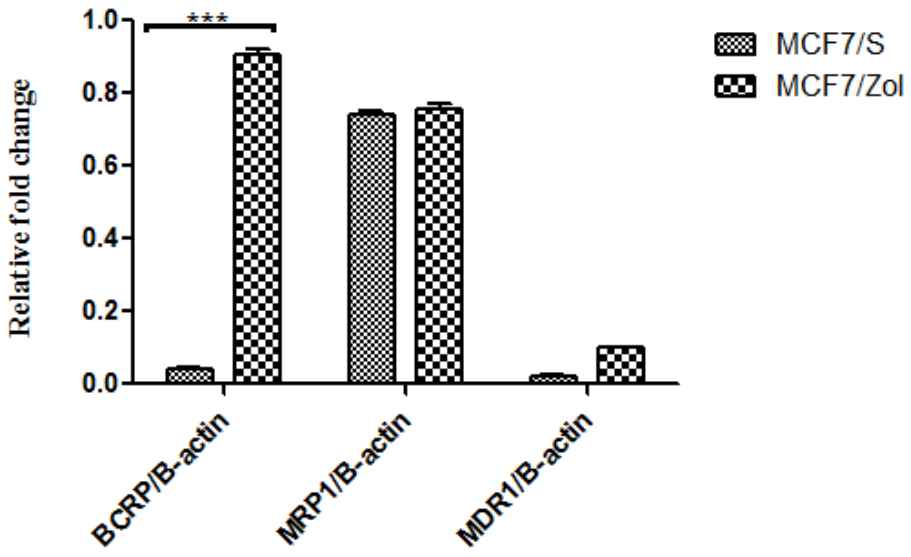


Figure 3.4 Expression of *BCRP*, *MRP1* and *MDR1* in parental sensitive (MCF7/S) and zoledronic acid resistant (MCF7/Zol) cell lines (******* Results were significant with a $p<0.001$)

According to Figure 3.4, parental MCF7/S cells did not intrinsicly express BCRP. However, BCRP expression was significantly upregulated in MCF7/Zol cell line ($p<0.05$). The development of resistance to zoledronic acid was firstly reported by

Kars *et. al* (Kars *et. al*, 2007) and high levels of BCRP expression was observed in MCF7/Zol cell line. In this study, the change in the expression level of BCRP in MCF7/Zol cell line was quantified relative to MCF7/S cell line by qPCR analysis. It was deduced from qPCR experiments that BCRP was expressed approximately 90-fold in MCF7/Zol cell line, compared to parental MCF7/S cell line. *MDR1* expression was not observed in MCF7/S cells. A slight increase in the expression of *MDR1* gene was detected in MCF7/Zol cells, however, the change in the expression of *MDR1* was not statistically significant. The intrinsic *MRP1* expression was observed in MCF7/S cell line, however the expression of *MRP1* gene did not show a noteworthy change as the cells gained resistance to zoledronic acid. The results indicated that although the development of multidrug resistance involves different cellular pathways including altered targets, altered cellular detoxification systems, reduced apoptosis and enhanced repair mechanisms, the *de novo* expression of BCRP could be the predominant transport-based resistance mechanism in MCF7/Zol cell line. Since BCRP overexpression caused to confer resistance to zoledronic acid in MCF7/Zol cells, zoledronic acid could be a substrate of BCRP. These results showed a correlation with previous findings (Kars *et. al*, 2007).

Earlier studies showed that estrogen exposure induced the expression of BCRP mRNA in ER-positive cell lines and it was found that the promoter of BCRP accomodates an estrogen responsive element (ERE) (Ee *et. al*, 2004). Biochanin A is a member of major phytoestrogens which compete with estrogen and it was found that Biochanin A displays anti-estrogenic effects in the cells (Kuiper *et. al*, 1998). Moreover, expression profiling experiments showed that treatment with several isoflavonoids including Biochanin A caused a significant downregulation in breast cancer-related genes such as *BRCA1* (Ise *et. al*, 2005) which also contain an ERE in their promoter region (Xu *et. al*, 1997), similar to BCRP. In this context, BCRP expression may be downregulated with respect to the treatment with Biochanin A in MCF7/Zol cells. To assess the effects of Biochanin on BCRP mRNA, MCF7/Zol cells were treated with different concentrations (5,10, 20 and 40 μM) of Biochanin A for 72 hours. Expressions of *BCRP*, *MRP1* and *MDR1* after Biochanin A treatment

were investigated by qPCR. The results were interpreted as relative fold changes and demonstrated as bar graphs in Figure 3.5, 3.6 and 3.7, respectively.

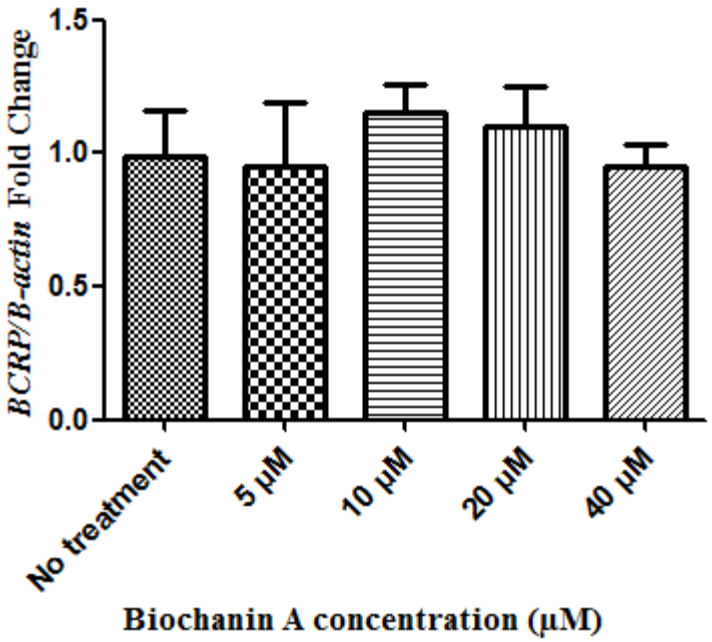


Figure 3.5 Expression of *BCRP* gene after Biochanin A treatment at different concentrations for 72 hours

Interestingly, the expression of *BCRP* gene level did not show a statistically significant change after treatment with Biochanin A for 72 hours at either concentrations (Figure 3.5). The treatment with Biochanin A did not affect the expression of internal control gene *β-actin*. The results demonstrated that Biochanin A did not have any significant effect on BCRP mRNA. Moreover, there are no studies reporting that Biochanin A or other isoflavonoids could cause a significant change in the expression of ABC transporter proteins at mRNA level. Therefore, it could be concluded that Biochanin A may exhibit its effects at translational or activity level, rather than at transcriptional level.

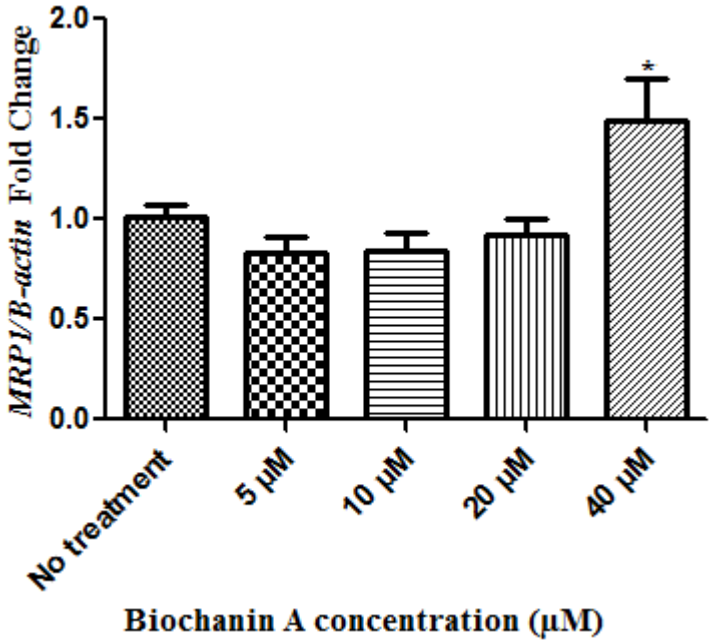


Figure 3.6 Expression of *MRP1* gene after Biochanin A treatment at different concentrations for 72 hours.

As displayed in Figure 3.6, *MRP1* expression did not show a significant change after treatment with Biochanin A at 5, 10 and 20 μM concentrations for 72 hours. However, the expression of *MRP1* was increased when treated with 40 μM of Biochanin A. The increase in the expression level suggested that the mechanism of resistance could shift to upregulation of MRP1. The shift in the resistance mechanism may be caused by that MCF7/Zol cells could try to avoid increased cytotoxic effects of zoledronic acid due to the impaired function of BCRP with respect to Biochanin A treatment.

The treatment with Biochanin A at either concentrations did not affect *MDR1* expression, unlike *MRP1*. The expression level of *MDR1* did not demonstrate a significant change after Biochanin A treatment (Figure 3.7)

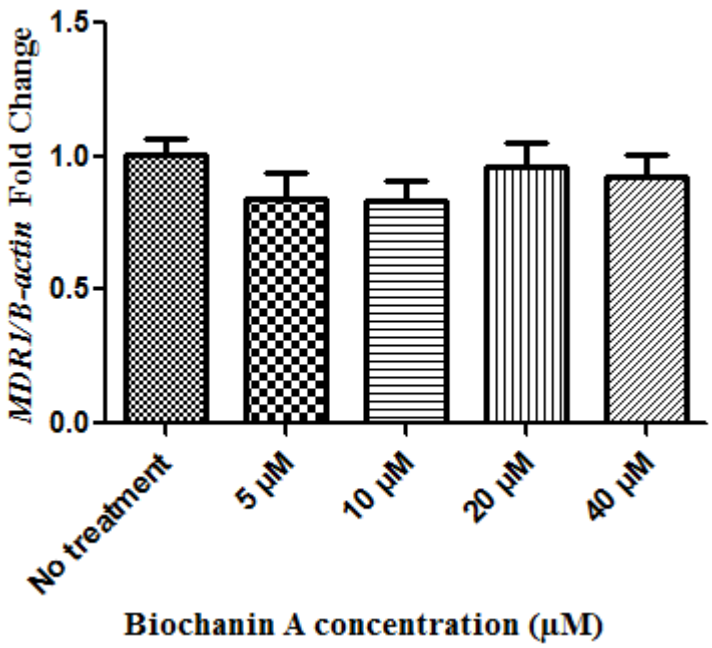


Figure 3.7 Expression of *MDR1* gene after Biochanin A treatment at different concentrations for 72 hours

Previous studies conducted by several laboratories showed that Biochanin A affected the MDR1 (P-gp)-mediated drug efflux. It was revealed that the oral bioavailability and pharmacokinetics of three well-known P-gp substrates, paclitaxel, digoxin and fexofenadine, were altered after Biochanin A treatment. The oral absorption of these drugs was enhanced when they were coadministered with Biochanin A (Peng *et. al*, 2006). Additionally, Biochanin A treatment caused incerased accumulation of daunomycin, vinblastine, paclitaxel and doxorubicin in different P-gp expressing cell lines (Zhang *et. al*, 2009). The results indicated that Biochanin A could modulate the function of MDR1 protein but not the expression of *MDR1* gene.

Table 3.1 Fold changes in expression of *BCRP*, *MRP1* and *MDR1* genes

Cell Line	Biochanin A Treatment	FC (*BCRP*)	FC (*MRP1*)	FC (*MDR1*)
MCF7/Zol	No treatment	0.98 0.43	0.99 ± 0.17	1.01 ± 0.15
	5 μM	0.95 ± 0.68	0.82 ± 0.14	0.86 ± 0.19
	10 μM	1.15 ± 0.35	0.85 ± 0.17	0,85 ± 0.20
	20 μM	1.10 ± 0.41	0.93 ± 0.28	0.96 ± 0.15
	40 μM	0.94 ± 0.32	1.49 ± 0.29	0.92 ± 0.19

Fold changes were represented as "mean ± SEM". SEM values were determined from three independent experiments, each run in triplicates.

3.3 Cell Proliferation Assay with XTT Reagent : Reversal of Zoledronic Acid Resistance

Zoledronic acid resistant (MCF7/Zol) cell line was developed previously in our laboratory by stepwise selection of MCF7 cells at increasing concentrations of zoledronic acid in two years. The final subline was found to be resistant to 8 μM zoledronic acid. It was determined by XTT cell proliferation assay that MCF7/Zol cell line had an inhibitory concentration (IC50) value of 340.36 ± 12.64 μM (Kars *et. al*, 2007). MCF7/Zol cells were stored in liquid nitrogen for a long time period until they had been thawed for experimentation. Prior to current study, MCF7/Zol cells were checked by XTT cell proliferation assay whether they have still been resistant to 8 μM zoledronic acid. MCF7/Zol cells were treated with a concentration gradient of zoledronic acid (with the highest drug dose of 400 μM) for 72 hours. % cell proliferation was determined relative to the proliferation cell control (no treatment control) group. To determine IC50 value of zoledronic acid, % cell proliferation versus concentration graphs were plotted (Appendix E) and it was found that MCF7/Zol cells had an IC50 value of 327.01 ± 11.28 μM.

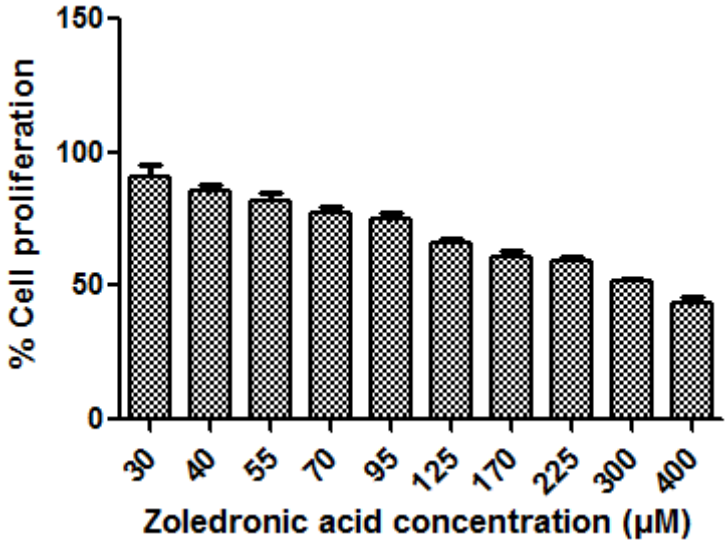


Figure 3.8 Profile of cell proliferation of MCF7/Zol cells at increasing concentrations of zoledronic acid

Consequently, parental MCF7 cells were exposed to increasing concentrations of zoledronic acid with the highest drug dose of 200 µM for 72 hours. It was found that IC50 value of zoledronic acid was 95.64 ± 2.35 µM in MCF7/S cell line.

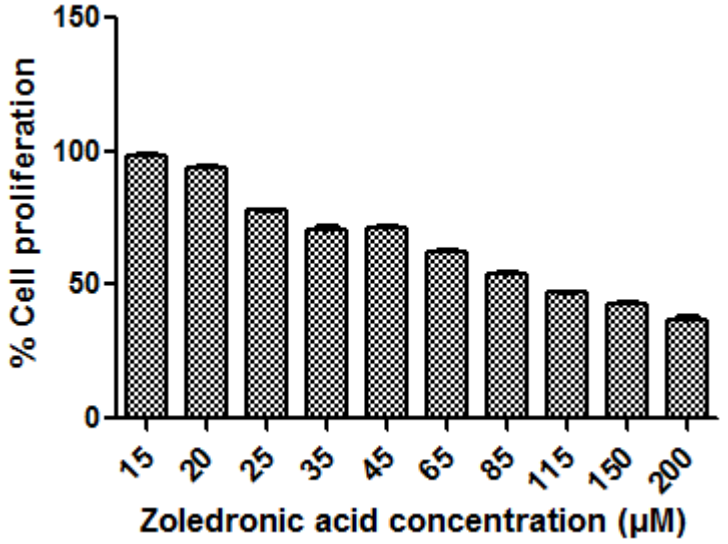


Figure 3.9 Profile of cell proliferation of MCF7/S cells at increasing concentrations of zoledronic acid

To determine the fold-resistance of MCF7/Zol cell line, resistance index (R) of zoledronic acid was calculated by the Equation 2.4. Resistance index of zoledronic acid in MCF7/Zol cell line was found as 3.42. The results of cell proliferation analysis showed that MCF7/Zol subline was approximately 3.5 fold resistant to zoledronic acid, compared to parental MCF7/S cell line. The results were consistent with the previous findings (Kars *et. al*, 2007).

Zoledronic acid is the first bisphosphonate which is approved by Food and Drug Administration (FDA) in the USA. Several studies demonstrated that zoledronic acid reduced cancer cell migration and metastasis to bone, prevented angiogenesis, decreased proliferation of cancer cells and induced apoptosis remarkably (Coleman *et. al*, 2001; Saad *et. al*, 2007; Traina, 2009). Moreover, zoledronic acid was shown to enhance cytotoxicity of other chemotherapeutic agents including taxenes and anthracyclines (Jadgev, 2001; Knight *et. al*, 2005). These properties of zoledronic acid make it the most frequently used bisphosphonate as the adjuvant therapy in breast cancer treatment. Although *in vivo* resistance to zoledronic acid had not been reported, the decrease in efficiency of zoledronic acid was known due to long-term use (Knight *et. al*, 2005). Since zoledronic acid has important direct and indirect effects on tumor growth and progression, the reduced activity of zoledronic acid may cause higher rates of cancer cell proliferation and metastasis. Hence, it is crucial to reverse zoledronic acid resistance in order to improve benefits of cancer chemotherapy.

Even though, multidrug resistance may be influenced by various factors, mechanisms and pathways, gene expression analysis by qPCR demonstrated that BCRP overexpression could be the predominant mechanism in development of zoledronic acid in MCF7 breast cancer cell line. Therefore, zoledronic acid resistance could be overcomed by modulating BCRP. There have been several BCRP modulators available. Two of the most potent BCRP inhibitors are Fumitremorgin C (FTC) and GF120918. These BCRP inhibitors have approximately IC50 values of 1000 nM and 50 nM, respectively, in BCRP-transfected breast cancer cell lines. Having such low

IC50 values, both FTC and GF120918 were shown to be effective to increase mitoxantrone (a known substrate of BCRP) accumulation in cancer cells (Allen *et. al*, 1999; Rabindran *et. al*, 2000). However, both inhibitors displayed cytotoxic effects in normal healthy cells that constitutively express BCRP. Furthermore, FTC showed neurotoxic side effects which limit its use in clinic (Borowski *et. al*, 2005). Therefore , it is crucial to use a modulator with maximum benefits and minimum side effects.

In the current study, a natural isoflavonoid class BCRP modulator, Biochanin A, was selected to reverse BCRP-mediated zoledronic acid resistance in MCF7 cell line. Previous studies showed that Biochanin A had IC50 values at low micromolar ranges in several cell lines (Mao and Unadkat, 2005). XTT cell proliferation assay was performed to assess the cytotoxicity of Biochanin A in parental sensitive and zoledronic acid resistant MCF7 cells. % cell proliferation at different concentrations of zoledronic acid was represented relative to no treatment control. IC50 values of Biochanin A in MCF7/S and MCF7/Zol cells were determined from % cell proliferation versus concentration plots in Figure 3.10 and 3.11, respectively.

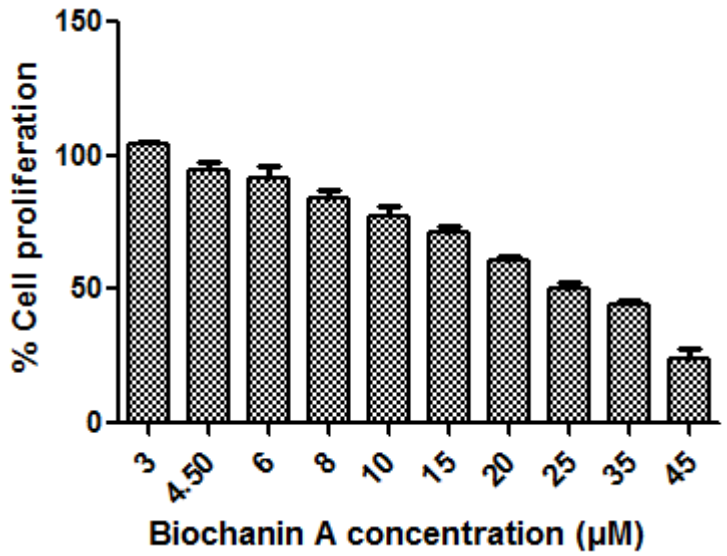


Figure 3.10 Profile of cell proliferation of MCF7/S cells at increasing concentrations of Biochanin A

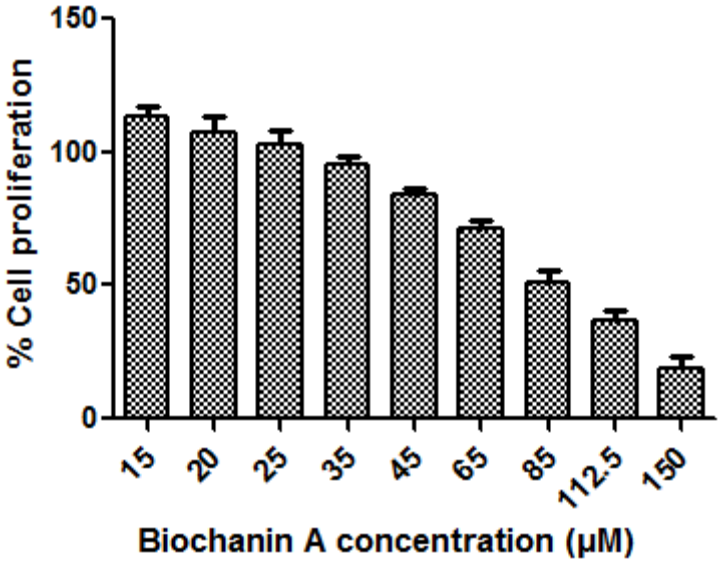


Figure 3.11 Profile of cell proliferation of MCF7/Zol cells at increasing concentrations of Biochanin A

IC50 value of Biochanin A was found as 25.47 ± 2.62 in MCF7/S cell line and as 93.17 ± 6.31 in MCF7/Zol subline. The results showed that Biochanin A has moderately high IC50 values, indicating that high concentrations of Biochanin A is necessary to display cytotoxic effects. Given the fact that Biochanin A exhibits low cytotoxicity, compared to other BCRP modulators, it could be a proper candidate to reverse BCRP-mediated zoledronic acid resistance.

Table 3.2 IC50 values of Biochanin A in MCF7/S and MCF7/Zol cell lines

Cell Line	**Drug**	**IC50 ± SEM (µM)†**
MCF7/S	Biochanin A	25.47 ± 2.62
MCF7/Zol	Biochanin A	93.17 ± 6.31

† SEM values were obtained from three independent experiments.

Interestingly, IC50 value of Biochanin A in MCF7/Zol cell line was higher than that of Biochanin A in MCF7/S cell line. It was found from XTT cell proliferation assay results that 3.66 fold more Biochanin A concentration was required to kill 50% of the

MCF7/Zol cells, when compared to parental MCF7/S cells. The need of higher concentrations of Biochanin A in BCRP-overexpressing MCF7/Zol cells may indicate that there could be a physical interaction between Biochanin A and BCRP. The physical interaction between BCRP and its modulator could be established in two ways. The first type of interaction is that Biochanin A may be a substrate of BCRP (Wang *et. al*, 2003). The stimulation of ATPase activity of BCRP when the BCRP-transfected cells were treated with different types of isoflavonoids suggested that there is an interaction between isoflavonoids and substrate recognition site of BCRP (Morris and Zhang, 2005). Moreover, it was known that estrogen and its derivatives (estrogen-sulfate conjugates) are substrates of BCRP. The structural similarity of isoflavonoids with estrogens could be a further evidence of that isoflavonoids may be the substrates of BCRP (Morris and Zhang, 2005). Additionally, isoflavonoids could be high-affinity substrates of BCRP so that the inhibitory effect is through competition with chemotherapeutic agents. The higher affinity isoflavonoids may cause the accumulation of anticancer agents inside the cells up to toxic cncentrations (Di Pietro *et. al*, 2002). On the other hand, Biochanin A could be a partial substrate of BCRP. Partial substrates were shown as better antagonists, although they bind to transporter proteins with lower affinity. The other type of interaction between BCRP and Biochanin A could be that Biochanin A could bind to ATP-binding region of BCRP to block ATP hydrolysis in order to inhibit ATP-hydrolysis driven drug efflux (Wang *et. al*, 2003). The interaction between nucleotide binding domain (NBD) of ABC transporters and isoflavonoids was firstly reported by structure-activity analysis of P-glycoprotein. It was found that isoflavonoids genistein could bind NBD2 of P-glycoprotein, interfering with ATP hydrolysis (Morris and Zhang, 2005). The important structural features for isoflavonoid-BCRP interaction are not identical with P-gp, however, the mechanism of binding is very similar (Counseil *et. al*, 1998). Even though the interaction of isoflavonoids with NBD of BCRP has not been studied in detail, it was thought that isoflavonoids could mimic adenosine ring of ATP so that it may easily bind to nucleotide binding domain of BCRP (Cooray *et. al*, 2004).

Szakacs *et. al* suggested that three major strategies to overcome transport-based multidrug resistance in cancer include the co-administration of pump-inhibitors with cytotoxic agents (engage), the use of cytotoxic agents which bypass transport-based drug efflux (evade), and the utilization of colleteral sensitivity of resistant cells (exploit) (Szakacs *et. al*, 2006). Given the results of previous studies, it may be concluded that Biochanin A could engage substrate binding domain or ATP binding site of BCRP, interfering with either zoledronic acid binding or ATP-hydrolysis driven zoledronic acid efflux, respectively.

To determine the reversing effect of Biochanin A on zoledronic acid resistance, MCF7/S and MCF7/Zol cells were treated with zoledronic acid in the presence of Biochanin A for 72 hours. Biochanin A concentrations were carefully chosen in order to eliminate the antiproliferative effect of Biochanin A. Cell proliferation analyses results showed that in MCF7/Zol cells, 100% proliferation was obtained in when they were treated with Biochanin A upto 40μM. At concentrations higher than 40 μM, Biochanin A displayed a gradual decrease in proliferation of MCF7/Zol cells. Similarly, in MCF7/S cells, 100% cell proliferation was achieved at 4 μM of Biochanin A. Biochanin A showed antiproliferative effects in MCF7/S cells at concentrations more than 4 μM. Therefore, 5, 10, 20 and 40 μM concentrations of Biochanin A were used to determine the reversing effect of Biochanin A on BCRP mediated zoledronic acid resistance and 4 μM Biochanin A was used to detect the effect of Biochanin A in the cells which did not express BCRP. DMSO was used as negative control in order to eliminate any toxic effects caused by DMSO. Proliferation of MCF7/S and MCF7/Zol cells in the concentration gradient of zoledronic acid along with Biochanin A was determined by plotting % cell proliferation versus concentration graphs (Figure 3.12 and Figure 3.13).

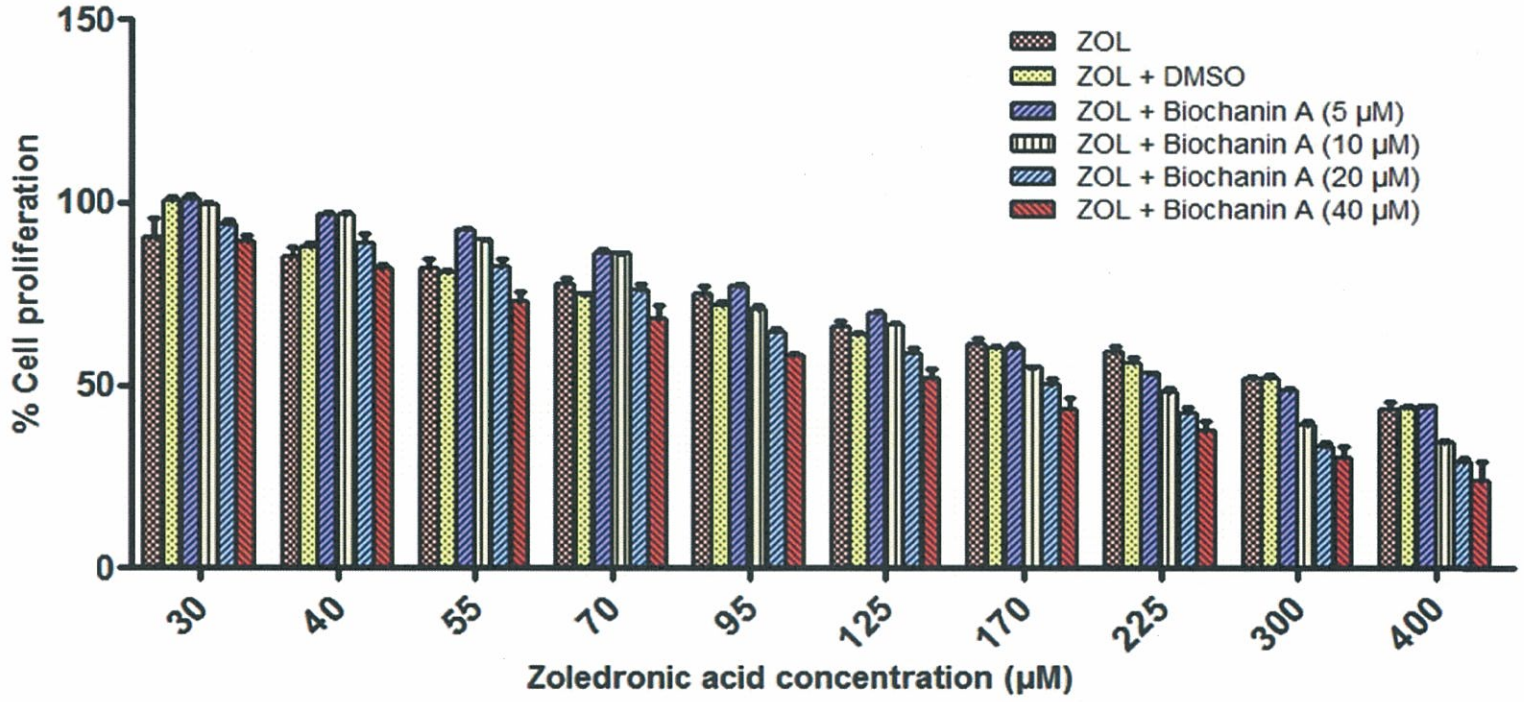


Figure 3.12 Profile of cell proliferation of untreated, DMSO or Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid

As represented in Figure 3.12, the proliferation of 5 μM of Biochanin A treated MCF7/Zol cells did not demonstrate a decrease in cellular proliferation at increasing concentrations of zoledronic acid when compared to non-treatment control group. DMSO treated MCF7/Zol cells were used as negative control and no change was observed in cell proliferation when cells were treated with DMSO for 72 hours. On the other hand, proliferation of MCF7/Zol cells showed significant decreases when they were treated with 10, 20 and 40 μM of Biochanin A. The decrease in % cell proliferation was increased at higher concentrations of Biochanin A, indicating that the inhibitory effect of Biochanin A on BCRP-mediated zoledronic acid cytotoxicity was dose-dependent.

Cell proliferation assay results showed that IC50 value of zoledronic acid was remarkably decreased due to Biochanin A treatment (Table 3.3). The results demonstrated that Biochanin A caused a reduction in IC50 value of zoledronic acid in a dose-dependent manner. At 5 μM, IC50 of zoledronic acid in MCF7/Zol cell line did not show statisticaly significant change. As the concentration of Biochanin A increased, IC50 values of zoledronic acid demonstrated a significant decrease. When the concentration of Biochanin A increased to 10 and 20 μM, nearly 35% and 47% decrease was obtained in IC50 of zoledronic acid, respectively. Approximately 60% reduction was achieved in IC50 of zoledronic acid when MCF7/Zol cells were exposed to 40 μM of Biochanin A.

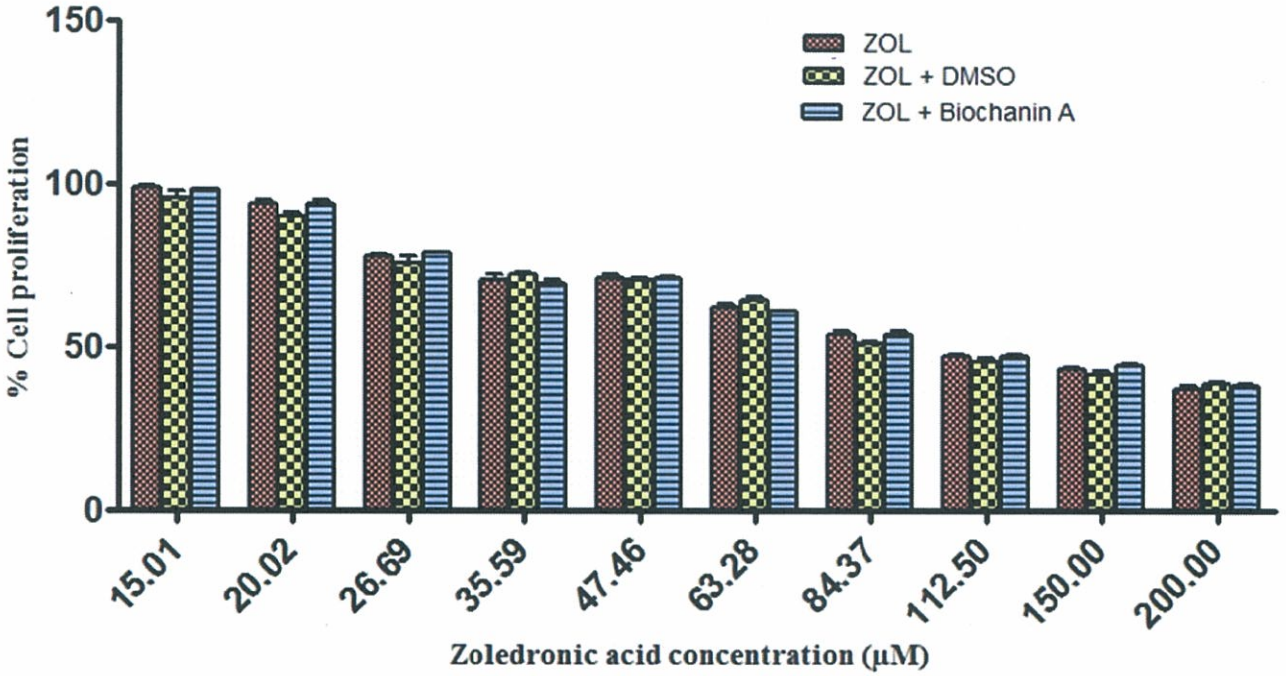


Figure 3.13 Profile of cell proliferation of untreated, DMSO or Biochanin A treated MCF7/S cells at increasing concentrations of zoledronic acid

MCF7/S cells were treated with 4 μM Biochanin A to detect the effect of Biochanin A in non-BCRP expressing cells. DMSO was used as negative control. As showed in Figure 3.13, cell proliferation profiles of MCF7/S cells did not demonstrate any significant difference between non-, DMSO and Biochanin A treatment. The IC50 value of zoledronic acid in MCF7/S cell line after Biochanin A treatment did not show a statistically significant change, compared to non-treatment and negative control groups (Table 3.3). The cell proliferation assay results indicated that Biochanin A did not affect the resistance level of the cells which do not express *BCRP*. The results showed a correlation with previous findings.

Gene expression studies by qPCR analysis demonstrated that MCF7/S cell line did not express *BCRP*, but relatively high *MRP1* expression levels were detected in MCF7/S cells. However, XTT cell proliferation assay results showed that Biochanin A treatment did not cause a significant change in cell proliferation profile of MCF7/S cells. On the other hand, proliferation of BCRP-overexpressing MCF7/Zol cells showed a significant decrease at increasing concentrations of zoledronic acid. The results indicated that Biochanin A selectively reversed BCRP-mediated drug resistance but did not affect MCF7/S cells which expressed high amounts of *MRP1*.

Table 3.3 IC50 values of zoledronic acid in untreated, DMSO treated and Biochanin A treated MCF7/S and MCF7/Zol cells

Cell Line	Treatment	IC50 ± SEM†	R*
MCF7/S	ZOL alone	95.64 ± 2.35	----
	ZOL + DMSO	104.81 ± 0.68	1.09
	ZOL + Biochanin A (4 μM)	107.72 ± 3.08	1.12
MCF7/Zol	ZOL alone	327.01 ± 11.28	3.42***
	ZOL + DMSO	321.38 ± 11.59	3.36***
	ZOL + Biochanin A (5 μM)	293.74 ± 9.12	3.07***
	ZOL + Biochanin A (10 μM)	215.29 ± 10.48	2.26**
	ZOL + Biochanin A (20 μM)	173.36 ± 8.47	1.81**
	ZOL + Biochanin A (40 μM)	139.96 ± 8.85	1.46*

Maximum zoledronic acid concentration was 200 μM for MCF7/S cells and 400 μM for MCF7/Zol cells.

† SEM values were obtained from three independent experiments.

*** $p < 0.001$ compared to ZOL alone in MCF7/S, ** $p < 0.01$ compared to ZOL alone in MCF7/S, * $p < 0.05$ compared to ZOL alone in MCF7/S

Fold reversal values for DMSO and different concentrations of Biochanin A treated MCF7/Zol cells calculated by Equation 2.5 and represented in Table 3.4.

Table 3.4 Fold reversal values for DMSO and Biochanin A treated MCF7/Zol cells

Cell Line	Treatment	Fold Reversal (FR)
MCF7/Zol	ZOL + DMSO	1.02
	ZOL + Biochanin A (5 μM)	1.11
	ZOL + Biochanin A (10 μM)	1.52
	ZOL + Biochanin A (20 μM)	1.88
	ZOL + Biochanin A (40 μM)	2.34

Maximum zoledronic acid concentration was 400 μM.

As demonstrated in Table 3.4, fold reversal (FR) was determined as 1 in DMSO treated MCF7/Zol cells, indicating that DMSO had no effects on zoledronic acid resistance. Biochanin A reversed zoledronic acid resistance in a dose-dependent manner. 5 μM of Biochanin A did not change resistance level of zoledronic acid significantly. At 10 and 20 μM Biochanin A, MCF7/Zol cells were re-sensitized to zoledronic acid 1.5 and 2 fold, respectively, compared to non-treatment control. MCF7/Zol cells were found to be approximately 2.5 fold more sensitive to zoledronic acid after Biochanin A treatment at 40 μM concentration.

Earlier studies showed the effectiveness of Biochanin A treatment on mitoxantrone (a well-known BCRP substrate) cytotoxicity. In a study conducted by Zhang *et. al*, it was shown that the intracellular mitoxantrone accumulation in mitoxantrone selected MCF7 (MCF7/MX100) cell line increased 4 fold more when the cells were treated with 50 μM of Biochanin A, compared to non-treatment control cells. The Biochanin A treatment was found not to be effective on MCF7/S cells. Similarly, 50 μM treated mitoxantrone selected NCI-H460 (NCI-H460/MX20) large cell lung cancer cell line was shown to accumulate 4 fold more mitoxantrone inside the cells. Consequently, 50 μM of Biochanin A treatment completely restored mitoxantrone cytotoxicity in MCF7/MX100 cells (Zhang *et. al*, 2004). The intracellular accumulation of mitoxantrone was increased up to 65% in BCRP-transfected Madin-Darby canine kidney (MDCK/BCRP) cells due to Biochanin A treatment at 25 μM. On the other hand, MDCK/Mock cells, which did not express BCRP, were not affected by Biochanin A treatment (An and Morris, 2010). Accordingly, the Biochanin A treatment affected the *in vivo* concentrations of mitoxantrone. When co-administered with Biochanin A, the mitoxantrone accumulation was decreased in spleen and kidney, where BCRP is constitutively expressed. However, plasma levels of mitoxantrone did not change due to 10 mg/kg IV injection of Biochanin A (An and Morris, 2004). The results showed that Biochanin A treatment lowered mitoxantrone accumulation and cytotoxicity in BCRP-expressing cells *in vitro* and *in vivo*.

In the current study, it was revealed that Biochanin A treatment successfully increased zoledronic acid cytotoxicity up to approximately 4-fold and reversed drug resistance up to 2.5-fold in MCF7/Zol cells. Therefore, it may be efficiently used as a part of adjuvant chemotherapy in breast cancer patients along with zoledronic acid. The increased cytotoxicity of zoledronic acid after treatment with a BCRP modulator may also imply that zoledronic acid was a substrate of BCRP.

In this study, even though 60% reversal was achieved in zoledronic acid resistance, the complete restoration in zoledronic acid cytotoxicity was not obtained. Since zoledronic acid dominantly affects metastatic and apoptotic pathways, it is likely that the resistance to zoledronic acid may alter the genes and proteins in these pathways. The alterations in both mechanisms may also contribute to the zoledronic acid resistance in MCF7 cell line. Kars *et. al* previously showed that *Bcl-2/Bax* ratio was increased when the MCF7 cells developed resistance to zoledronic acid. The increase in the expression of anti-apoptotic Bcl-2 gene and the decrease in expression of pro-apoptotic Bax gene may further imply that the alterations in apoptotic pathways could contribute the zoledronic acid resistance by providing survival advantage to MCF7/Zol cells (Kars *et. al*, 2007).

CHAPTER 4

CONCLUSION

1. Zoledronic acid resistant (MCF7/Zol) cell line had significantly higher IC50 value for zoledronic acid, compared to parental sensitive MCF7 (MCF7/S) cell line. The results indicated that MCF7/Zol cell line was 3.5-fold resistant to zoledronic acid, relative to MCF7/S cells.

2. MCF7/S cells did not have intrinsic *MDR1* and *BCRP* expression, however, high levels of *MRP1* expression was detected in MCF7/S cell line.

3. Gene expression analyses demonstrated that expression of *BCRP* was significantly upregulated in zoledronic acid resistance. *BCRP* was found to be expressed 92-fold more compared to MCF7/S cell line. Expression levels of *MDR1* and *MRP1* did not show a significant change while the cells developed resistance to zoledronic acid. It may be concluded that overexpression of BCRP could be the predominant transport based multidrug resistance mechanism in development of zoledronic acid resistance.

4. Expression of *BCRP* was not affected by Biochanin A treatment at any concentrations. It may indicate that Biochanin A could not display its effects on transcriptional level.

5. Biochanin A treatment did not affect *MDR1* expression in MCF7/Zol cells.

6. Expression of MRP1 did not change after Biochanin A treatment at 5, 10 and 20 μM concentrations. However, upregulation was detected in *MRP1* expression at 40 μM Biochanin A treatment. MCF7/Zol cells could shift the resistance mechanism to MRP1-dependent drug efflux in order to avoid increased zoledronic acid cytotoxicity due to Biochanin A treatment.

7. Biochanin A did not show toxic effects on both MCF7/S and MCF7/Zol cells up to high concentrations in micromolar range.

8. Approximately 4-fold Biochanin A was required to kill half of MCF7/Zol cells than that of MCF7/S cells. The higher IC50 value of Biochanin A could indicate a physical interaction between Biochanin A and BCRP.

9. Treatment of MCF7/Zol cells with 5 μM of Biochanin A did not significantly affect zoledronic acid cytotoxicity. Significant percentages of reduction in IC50 value of zoledronic acid were achieved due to Biochanin A treatment at different concentrations. Biochanin A treatment at 40 μM resulted in 2.5 fold reversal in zoledronic acid resistance.

10. Biochanin A treatment did not show any significant effects on zoledronic acid cytotoxicity in MCF7/S cell line. It may be concluded that Biochanin A was not effective in cells which do not express BCRP.

Treatment with Biochanin A, the selected BCRP modulator, is efficient to reverse BCRP-mediated multidrug resistance and may consequently result in increased success of chemotherapy with reduced side effects.

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APPENDIX A

CELL CULTURE MEDIUM

Table A. 1 Formulation of RPMI 1640 Medium (Biochrom AG, Germany)

Substance	Concentration (mg/l)	Substance	Concentration (mgl/l)
NaCl	6000	L-methionine	15
KCl	400	L-phenylalanine	15
$Na_2HPO_4 \cdot 7H_2O$	1512	L-proline	20
$MgSO_4 \cdot 7H_2O$	100	L-serine	30
$Ca(NO_3)_2 \cdot 4H_2O$	100	L-threonine	20
D-glucose	2000	L-tryptophane	5
Phenol red*	5	L-tyrosine	20
$NaHCO_3$	2000	L-valine	20
L-arginine	200	Glutathione	1
L-asparagine	50	Biotin	0.2
L-aspartic acid	20	Vitamin B_{12}	0.005
L-cystine	50	D-Ca-pantothenate	0.25
L-glutamine	300	Cholin chloride	3
L-glutamic acid	20	Folic acid	1
Glycine	10	Myo-inositol	35
L-histidine	15	Nictoninamid	1
L-hydroxyproline	20	p-amino benzoic acid	1
L-isoleucine	50	Pyridoxin·HCl	1
L-leucine	50	Riboflavin	0.2
L-lysine·HCl	40	ThiamineHCl	1

Biochrom: RPMI 1640, retrieved from http://www.biochrom.de/en/products/cell-culture-media/rpmi-1640/. Last accessed date: 2012, February 1.

APPENDIX B

BUFFERS AND SOLUTIONS

- **Freezing medium:**

DMSO (Cell culture grade)	1 mL
FBS (Heat-inactivated)	9 mL

- **Diethylpyrocarbonate (DEPC) treated dH_2O (1 L):**

DEPC	1 mL
dH2O	1 L

1 mL DEPC was vigorously mixed with 1 mL dH_2O. It was autoclaved at 121°C for 20 min after overnight incubation.

- **Ethidium bromide (EtBr) solution:**

EtBr	10 mg
dH_2O	1 mL

EtBr was dissolved in dH_2O and stored at 4°C in dark.

- **50X Tris-Acetate-EDTA (TAE) buffer (1L):**

Tris base (molecular weight: 121.14 g/mol)	242 g
Acetic acid	57.1 mL
0.5 M EDTA disodium dehydrate (molecular weight: 372.24 g/mol)	100 mL

Volume was completed to 1 L with dH_2O and pH was adjusted to 8.5. Solution was diluted to 1X with dH_2O after it was autoclaved at 121°C for 20 min. The solution was stored at 4°C.

- **2X RNA loading dye (Fermentas, Lithuania):**
  0.5 mM EDTA
  95% Formamide
  0.025% SDS
  0.025% Bromophenol blue
  0.025% Xylene cyanol FF
  0.025% Ethidium bromide

- **6X DNA loading dye (Fermentas, Lithuania):**
  60 mM ETA
  10 mM Tris-HCl (pH 7.6)
  0.03% Xylene cyanol FF
  60% Glycerol

APPENDIX C

THRESHOLD CYCLE VALUES

Table C. 1 Threshold cycle (C_t) values of qPCR

	BCRP	**MRP1**	**MDR1**	**β-actin**
No treatment	15.55	18.26	25.79	8.33
5 µM Biochanin A	15.67	19.45	26.22	8.29
10 µM Biochanin A	16.73	18.29	25.54	9.55
20 µM Biochanin A	16.44	18.01	26.39	9.53
40 µM Biochanin A	16.28	16.83	26.86	9.23

APPENDIX D

EXPRESSIONS OF *BCRP, MRP1, MDR1* AND *β-actin*

Expression levels of *BCRP*, *MRP1*, *MDR1* and *β-actin* were determined by RT-qPCR. The products of RT-qPCR were checked by agarose gel electrophoresis. 2% agarose gel was prepared as described in Section 2.2.2.4 and qPCR products were run at 100V for 60 min (Figure D.1, D.2, D.3 and D.4, respectively).

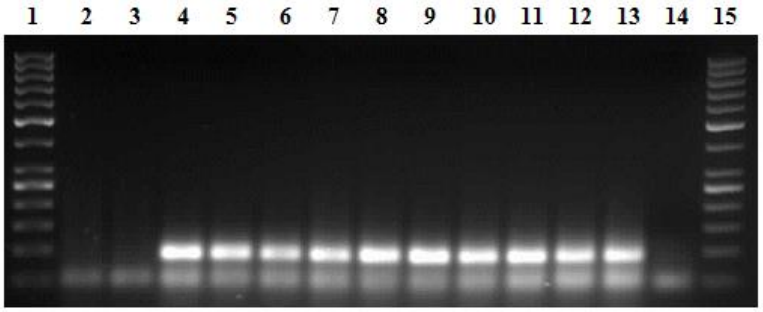

Figure D.1 Expression levels of BCRP*;* Lanes 1 and 15: 50 bp DNA ladder (Fermentas, Lithuania), Lanes 2-3: *BCRP* in MCF7/S cell line, Lanes 4-5: *BCRP* in MCF7/Zol cell line (no treatment), Lanes 6-7: *BCRP* in 5 μM Biochanin A treated MCF7/Zol cell line, Lanes 8-9: *BCRP* in 10 μM Biochanin A treated MCF7/Zol cell line, Lanes 10-11: *BCRP* in 20 μM Biochanin A treated MCF7/Zol cell line, Lanes 12-13: *BCRP* in 40 μM Biochanin A treated MCF7/Zol cell line, Lane 14: Negative control

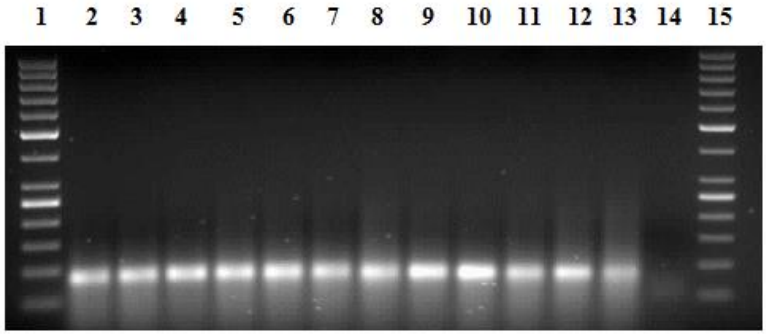

Figure D. 2 Expression levels of MRP1; Lanes 1 and 15: 50 bp DNA ladder (Fermentas, Lithuania), Lanes 2-3: *MRP1* in MCF7/S cell line, Lanes 4-5: *MRP1* in MCF7/Zol cell line (no treatment), Lanes 6-7: *MRP1* in 5 μM Biochanin A treated MCF7/Zol cell line, Lanes 8-9: *MRP1* in 10 μM Biochanin A treated MCF7/Zol cell line, Lanes 10-11: *MRP1* in 20 μM Biochanin A treated MCF7/Zol cell line, Lanes 12-13: *MRP1* in 40 μM Biochanin A treated MCF7/Zol cell line, Lane 14: Negative control

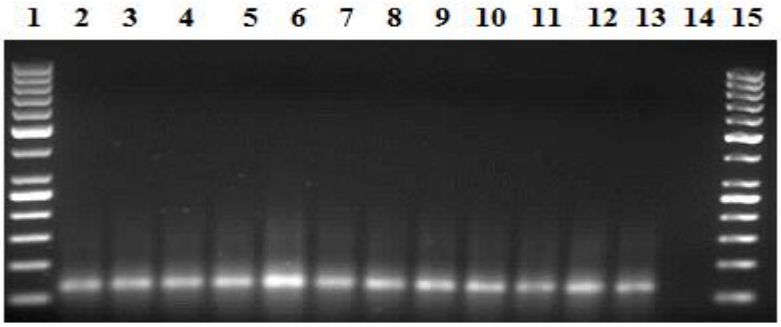

Figure D. 3 Expression levels of MDR1; Lanes 1 and 15: 50 bp DNA ladder (Fermentas, Lithuania), Lanes 2-3: *MDR1* in MCF7/S cell line, Lanes 4-5: *MDR1* in MCF7/Zol cell line (no treatment), Lanes 6-7: *MDR1* in 5 μM Biochanin A treated MCF7/Zol cell line, Lanes 8-9: *MDR1* in 10 μM Biochanin A treated MCF7/Zol cell line, Lanes 10-11: *MDR1* in 20 μM Biochanin A treated MCF7/Zol cell line, Lanes 12-13: *MDR1* in 40 μM Biochanin A treated MCF7/Zol cell line, Lane 14: Negative control

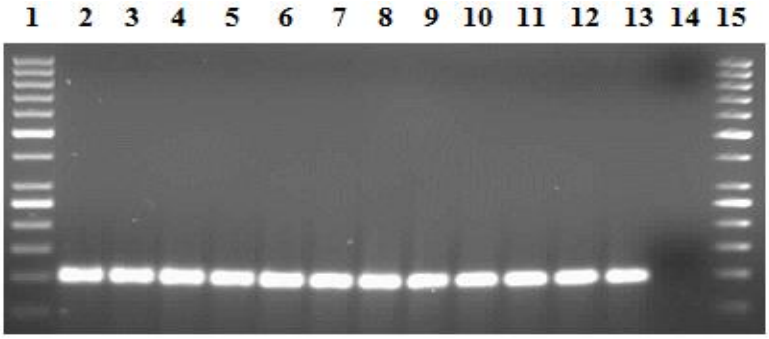

Figure D. 4 Expression levels of β-actin; Lanes 1 and 15: 50 bp DNA ladder (Fermentas, Lithuania), Lanes 2-3: *β-actin* in MCF7/S cell line, Lanes 4-5: *β-actin* in MCF7/Zol cell line (no treatment), Lanes 6-7: *β-actin* in 5 μM Biochanin A treated MCF7/Zol cell line, Lanes 8-9: *β-actin* in 10 μM Biochanin A treated MCF7/Zol cell line, Lanes 10-11: *β-actin* in 20 μM Biochanin A treated MCF7/Zol cell line, Lanes 12-13: *β-actin* in 40 μM Biochanin A treated MCF7/Zol cell line, Lane 14: Negative control

APPENDIX E

CELL PROLIFERATION GRAPHS AND LOGARITHMIC EQUATIONS

All cytotoxicity experiments were carried out in triplicate 96 well plates. Cytotoxicity of Biochanin A and zoledronic acid (before and after Biochanin A treatment at different concentrations) were assessed by plotting % cell proliferation versus concentration graphs for each plate in triplicates. The data were represented as mean ± standart error of the mean (SEM).

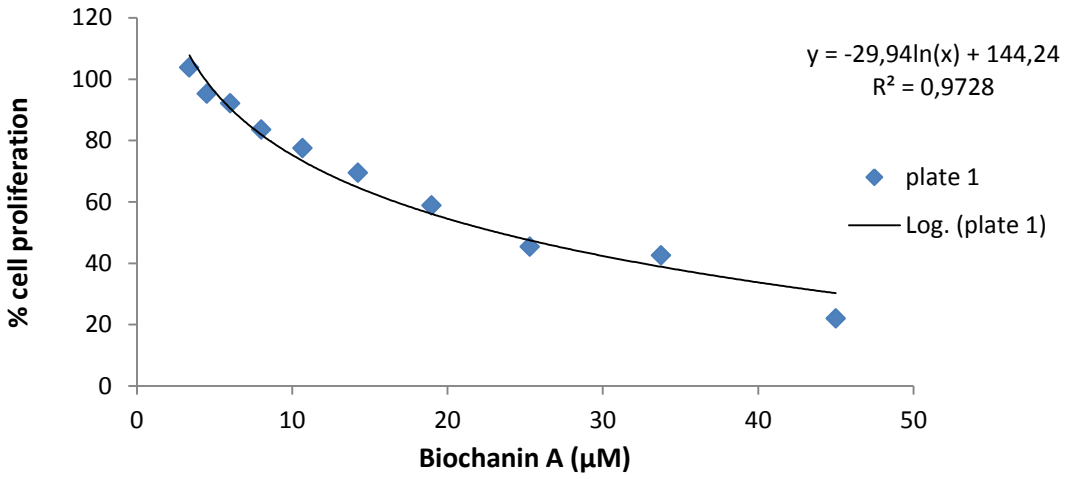

Figure E. 1 Cell proliferation of MCF7/S cells at increasing concentrations of Biochanin A (plate 1)

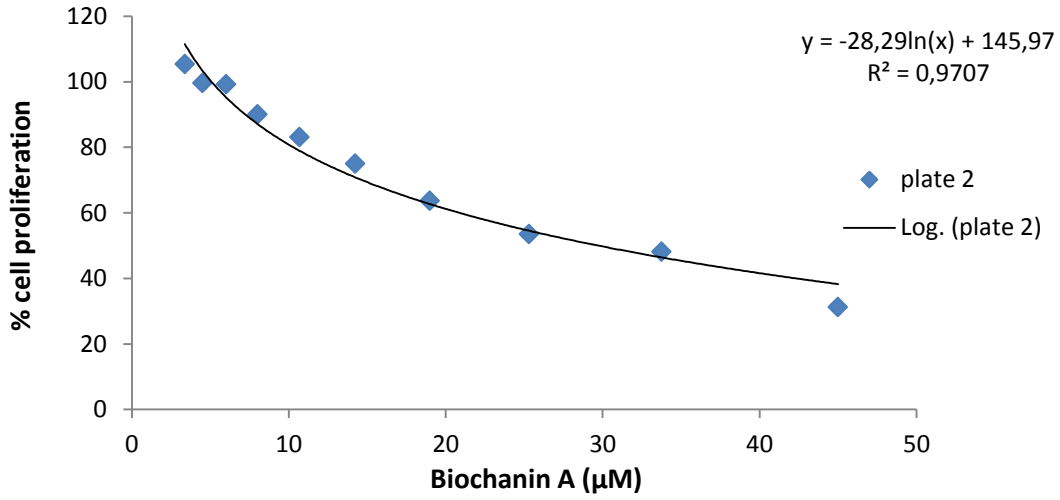

Figure E. 2 Cell proliferation of MCF7/S cells at increasing concentrations of Biochanin A (plate 2)

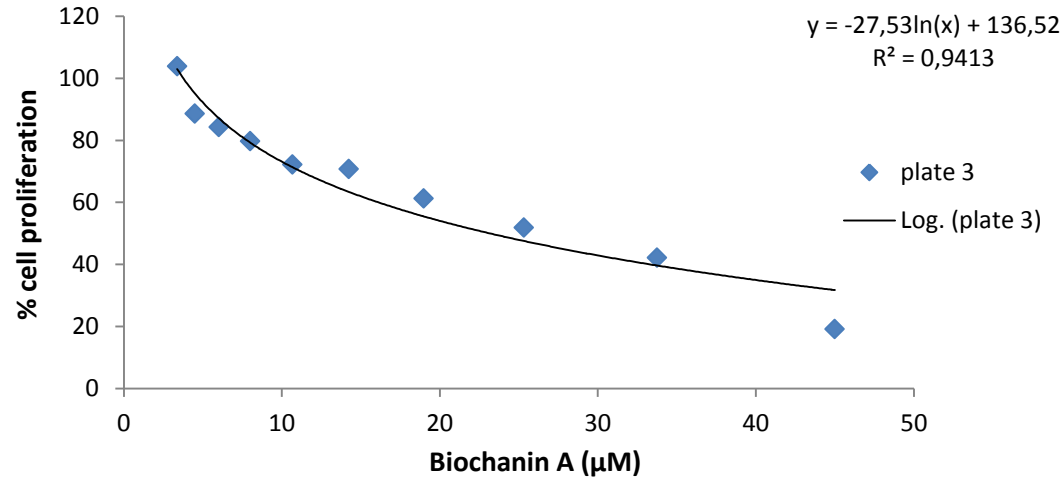

Figure E. 3 Cell proliferation of MCF7/S cells at increasing concentrations of Biochanin A (plate 3)

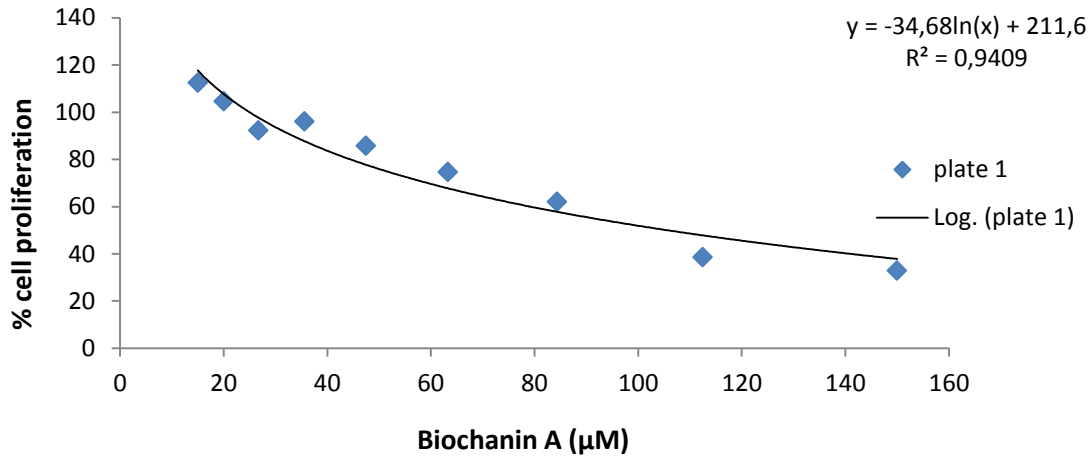

Figure E. 4 Cell proliferation of MCF7/Zol cells at increasing concentrations of Biochanin A (plate 1)

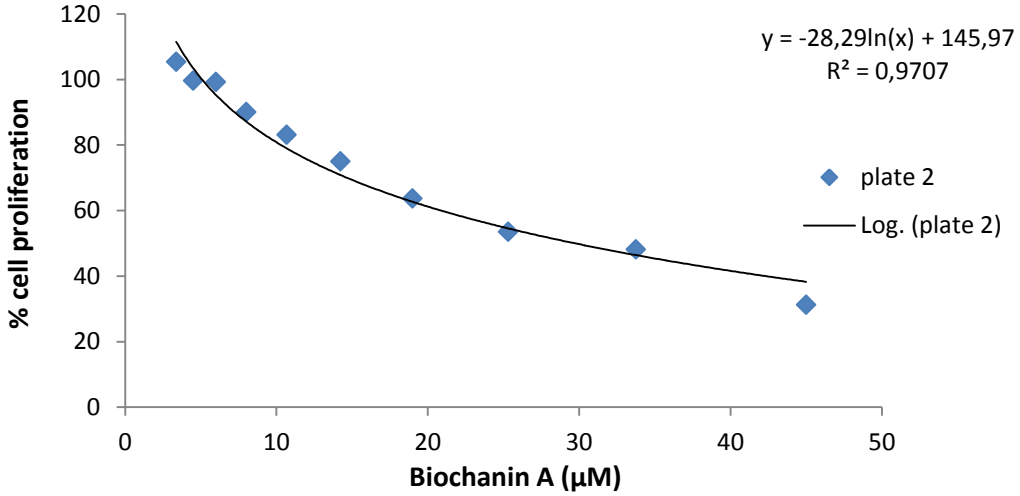

Figure E. 5 Cell proliferation of MCF7/Zol cells at increasing concentrations of Biochanin A (plate 2)

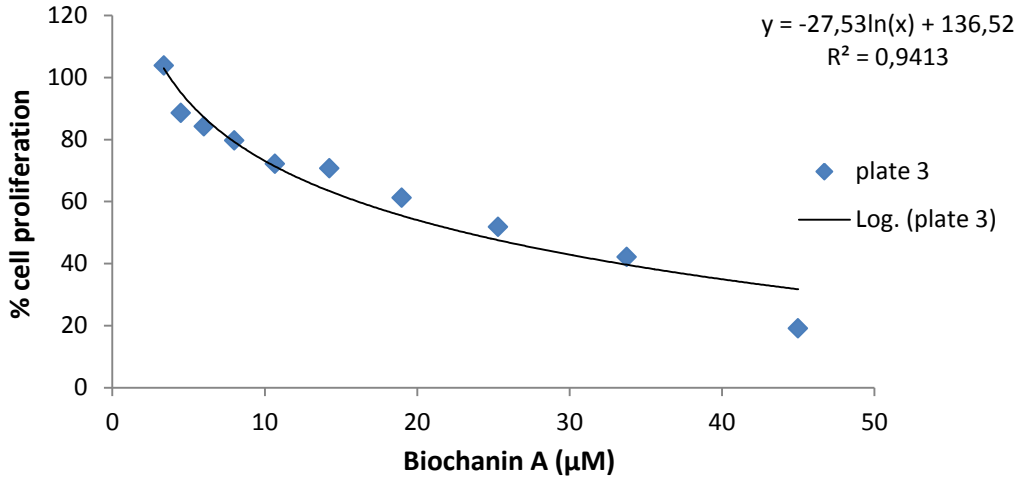

Figure E. 6 Cell proliferation of MCF7/Zol cells at increasing concentrations of Biochanin A (plate 3)

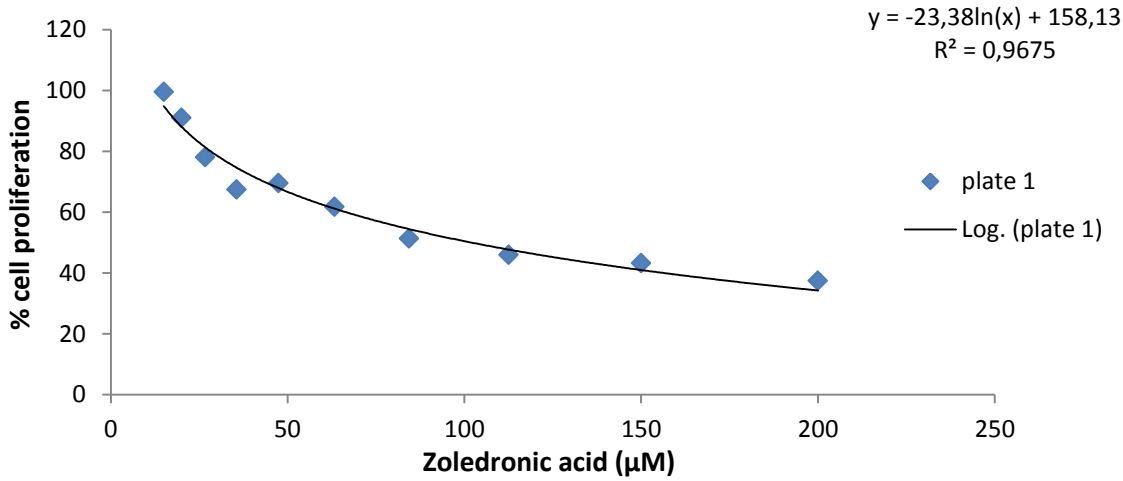

Figure E. 7 Cell proliferation of MCF7/S cells at increasing concentrations of zoledronic acid (plate 1)

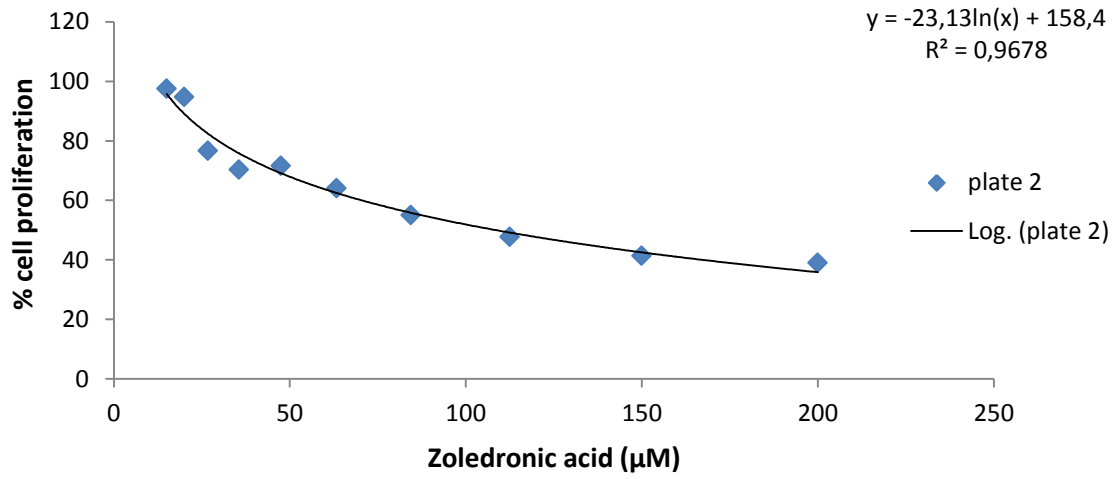

Figure E. 8 Cell proliferation of MCF7/S cells at increasing concentrations of zoledronic acid (plate 2)

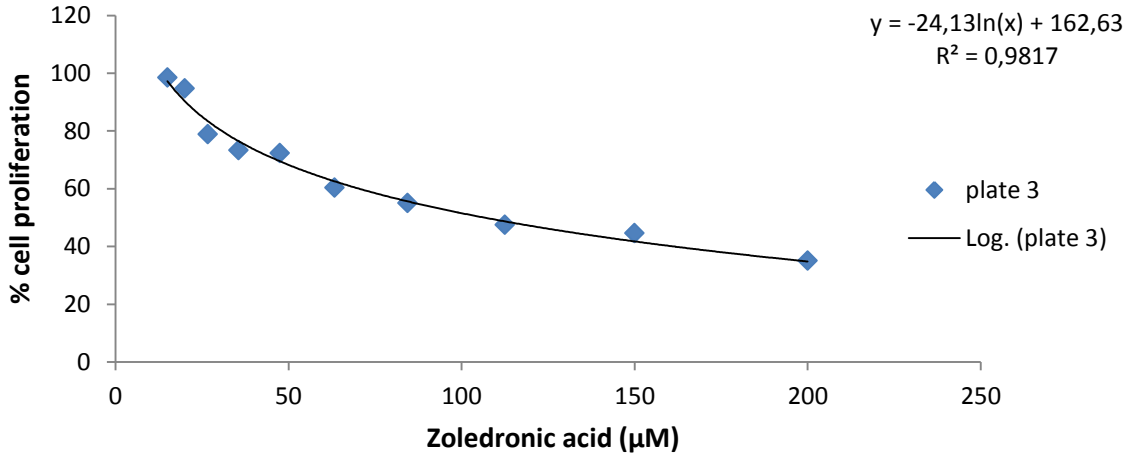

Figure E. 9 Cell proliferation of MCF7/S cells at increasing concentrations of zoledronic acid (plate 3)

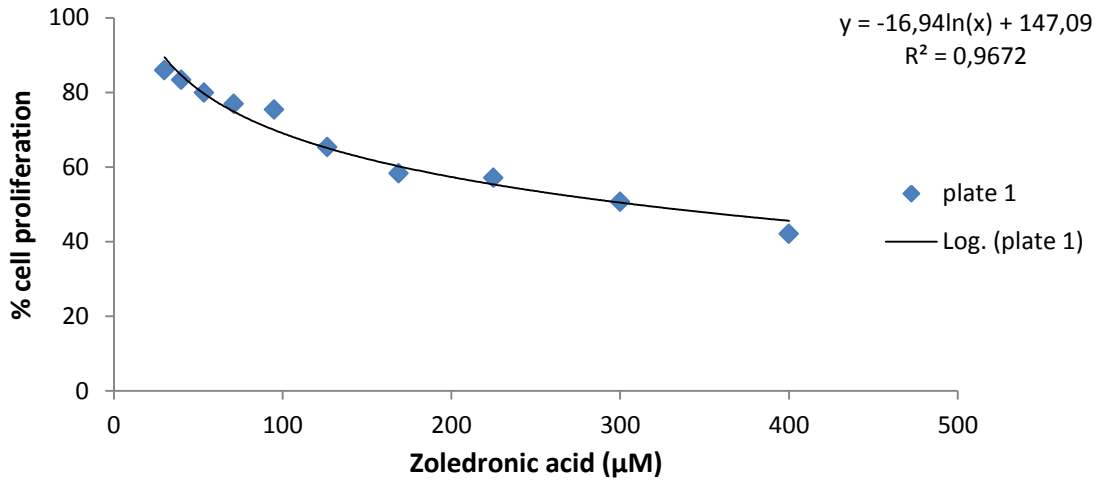

Figure E. 10 Cell proliferation of MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 1)

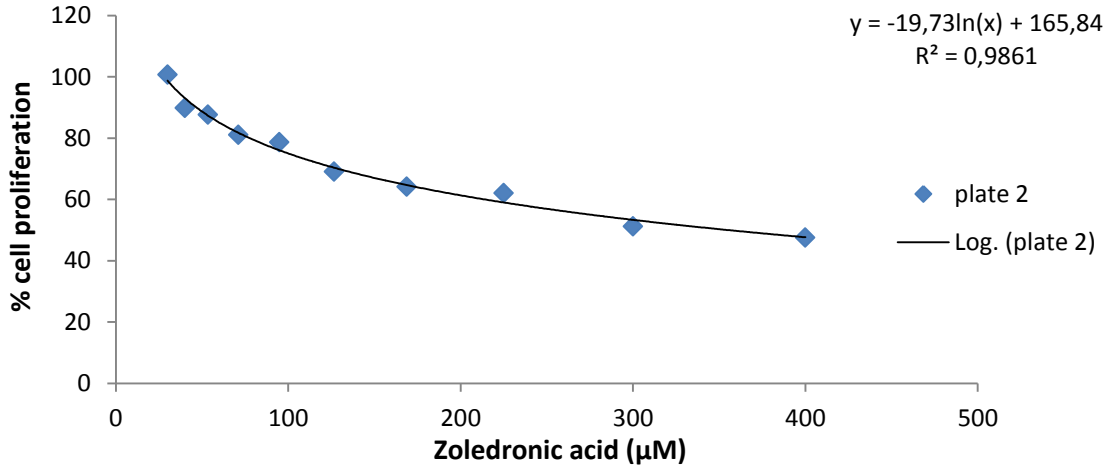

Figure E. 11 Cell proliferation of MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 2)

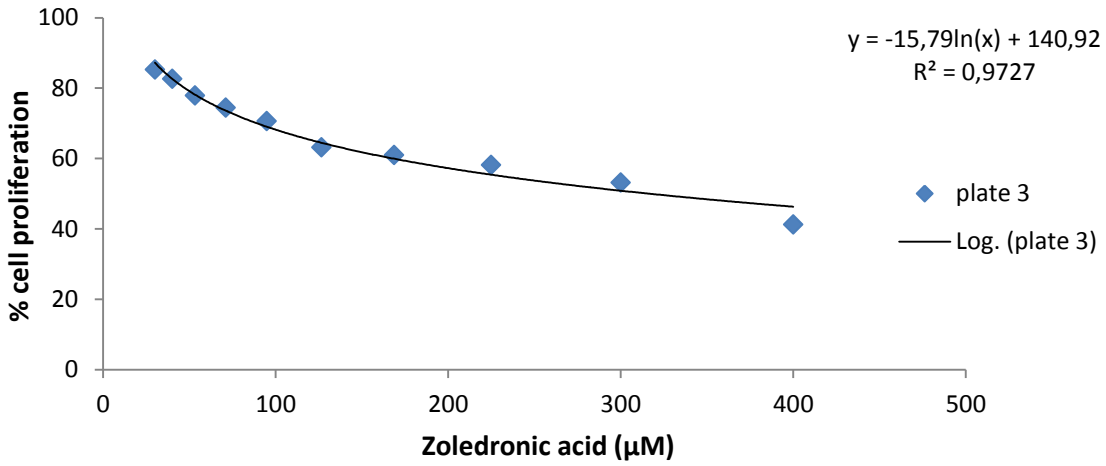

Figure E. 12 Cell proliferation of MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 3)

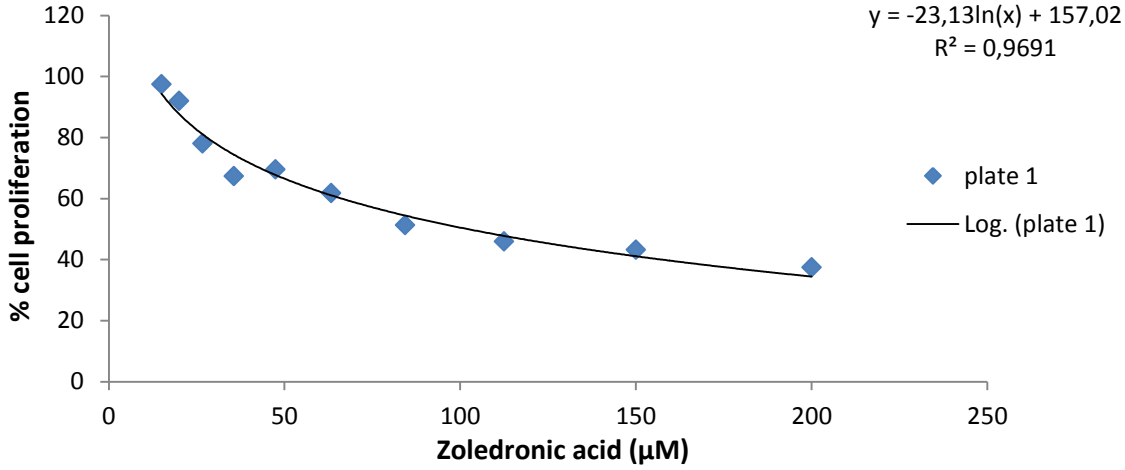

Figure E. 13 Cell proliferation of 4 µM Biochanin A treated MCF7/S cells at increasing concentrations of zoledronic acid (plate 1)

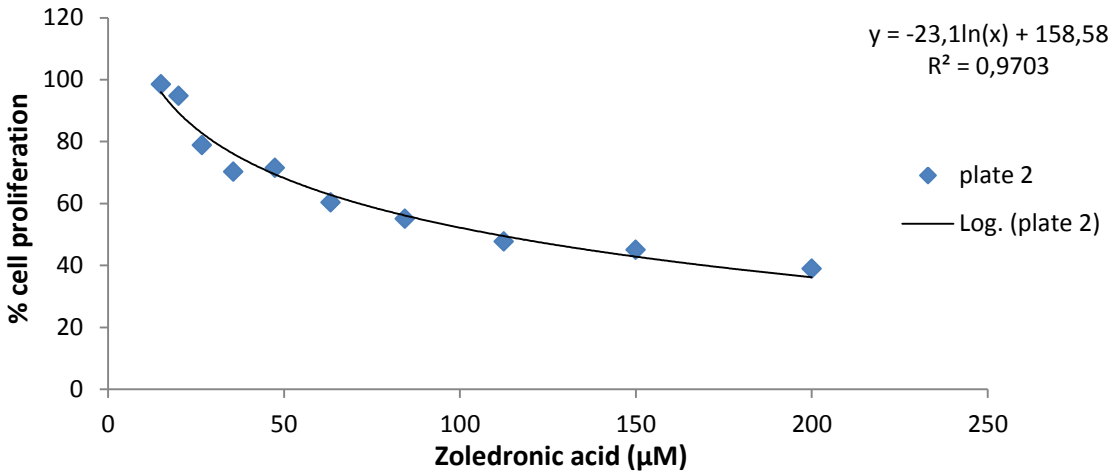

Figure E. 14 Cell proliferation of 4 µM Biochanin A treated MCF7/S cells at increasing concentrations of zoledronic acid (plate 2)

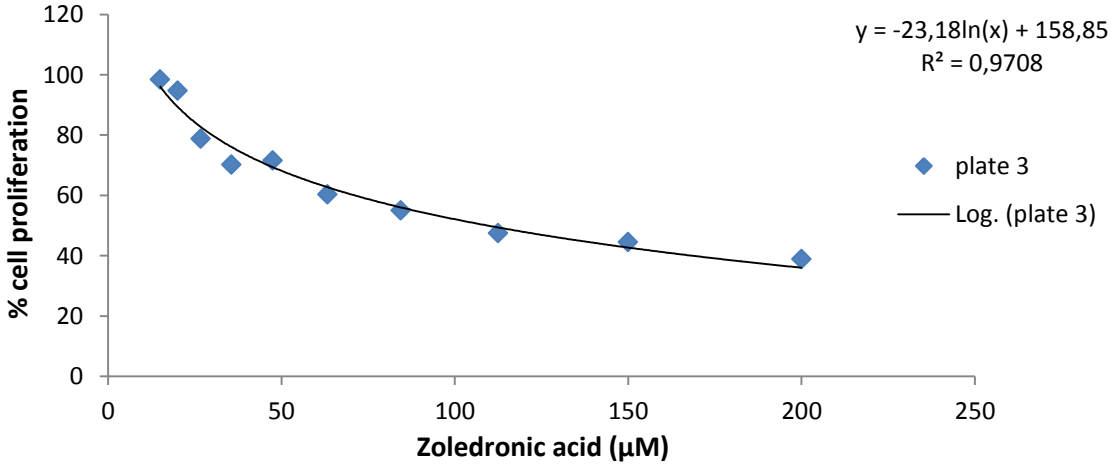

Figure E. 15 Cell proliferation of 4 µM Biochanin A treated MCF7/S cells at increasing concentrations of zoledronic acid (plate 3)

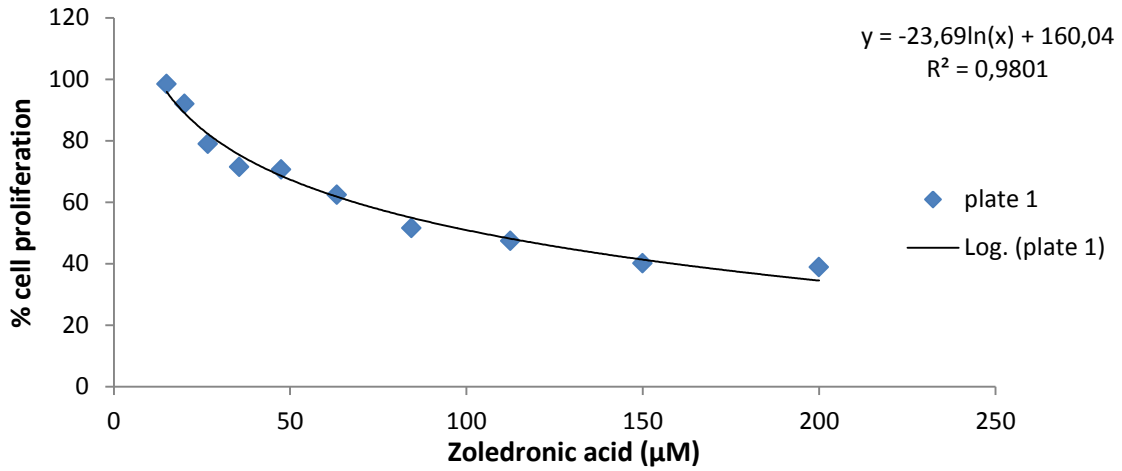

Figure E. 16 Cell proliferation of DMSO treated MCF7/S cells at increasing concentrations of zoledronic acid (plate 1)

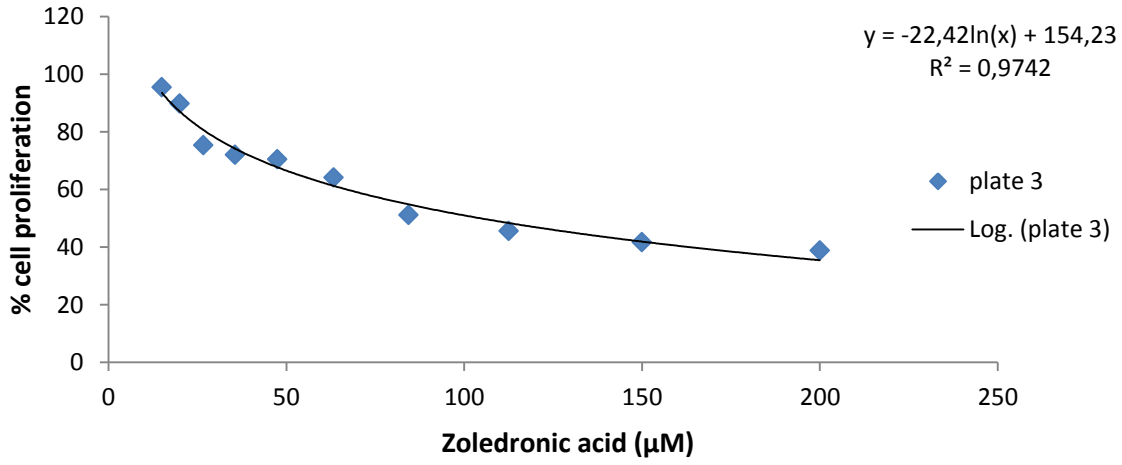

Figure E. 17 Cell proliferation of DMSO treated MCF7/S cells at increasing concentrations of zoledronic acid (plate 2)

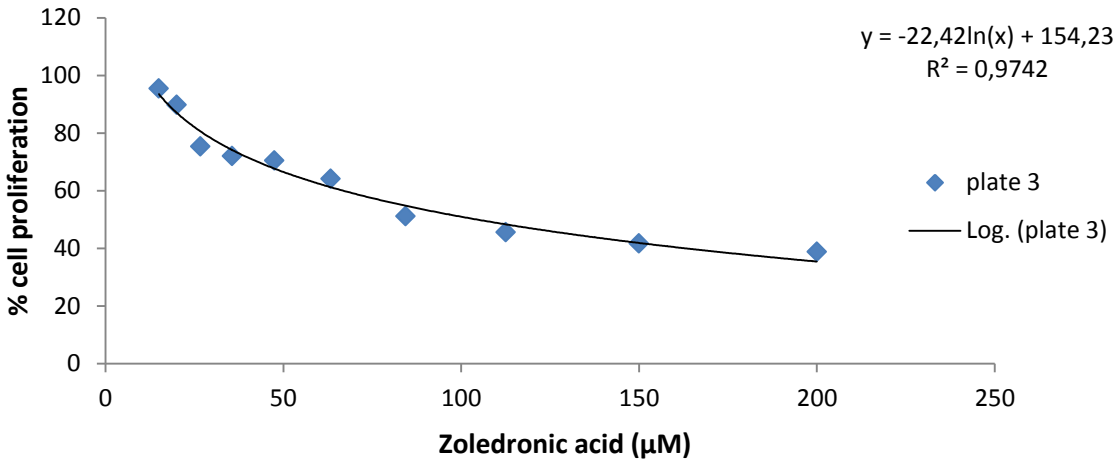

Figure E. 18 Cell proliferation of DMSO treated MCF7/S cells at increasing concentrations of zoledronic acid (plate 3)

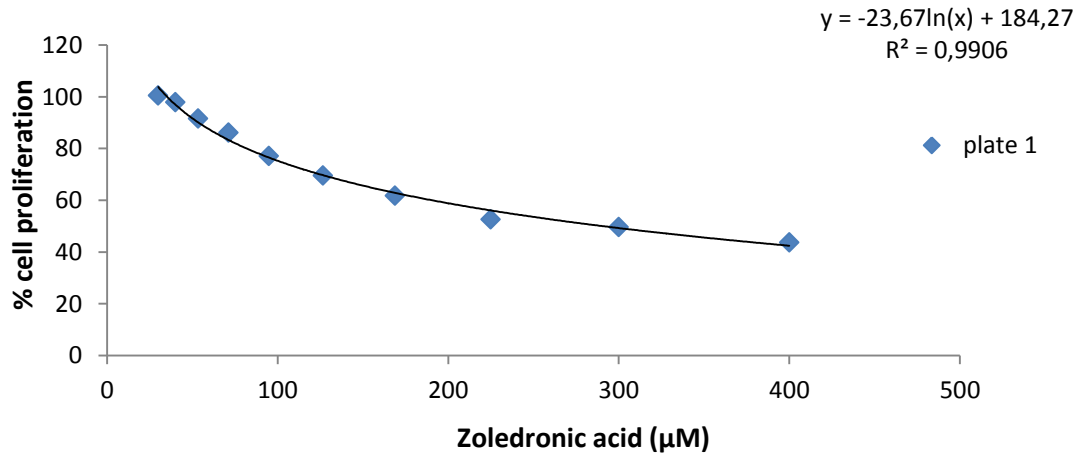

Figure E. 19 Cell proliferation of 5 µm Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 1)

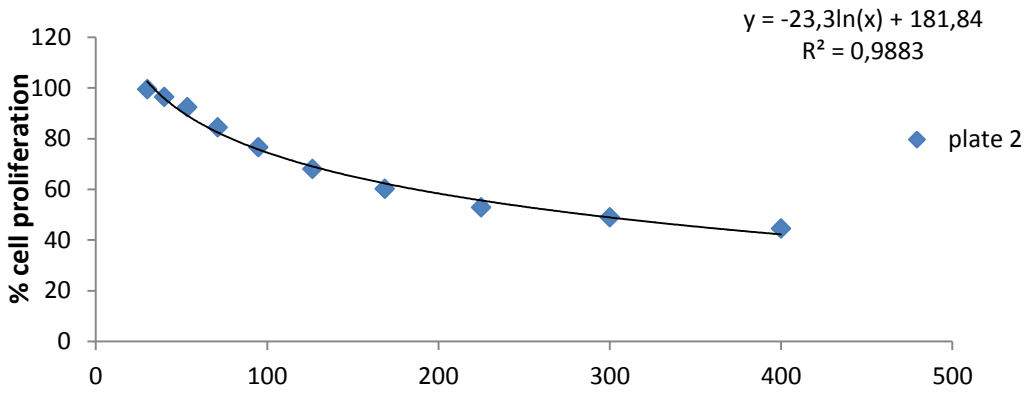

Figure E. 20 Cell proliferation of 5 µm Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 2)

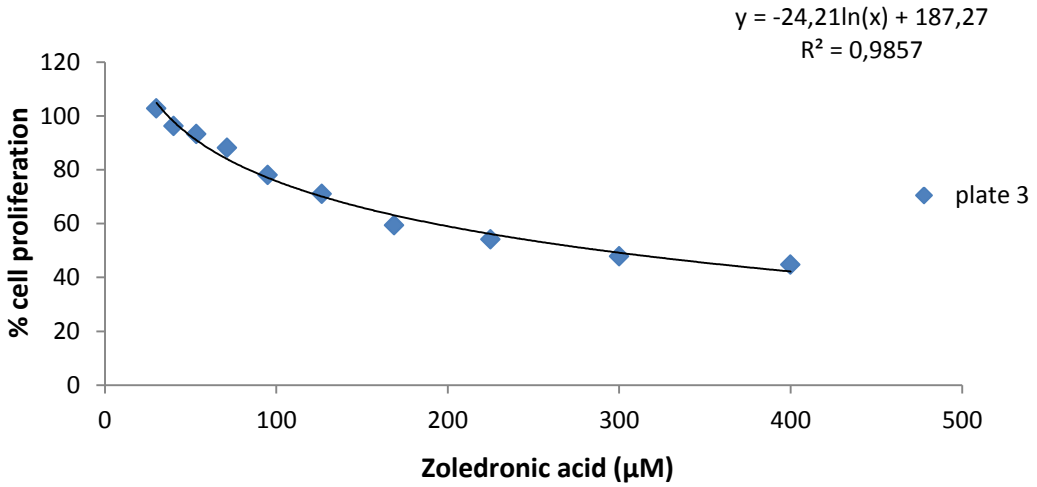

Figure E. 21 Cell proliferation of 5 µm Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 3)

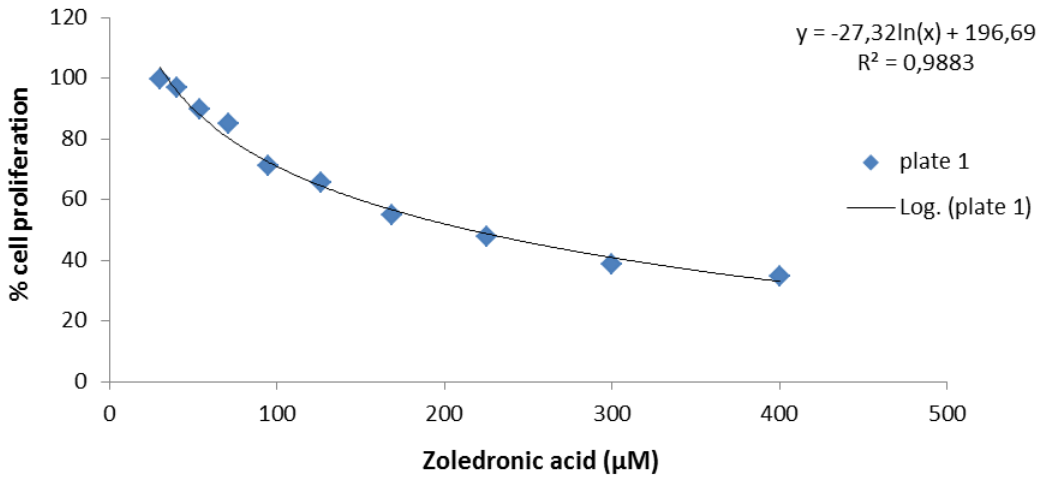

Figure E. 22 Cell proliferation of 10 µm Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 1)

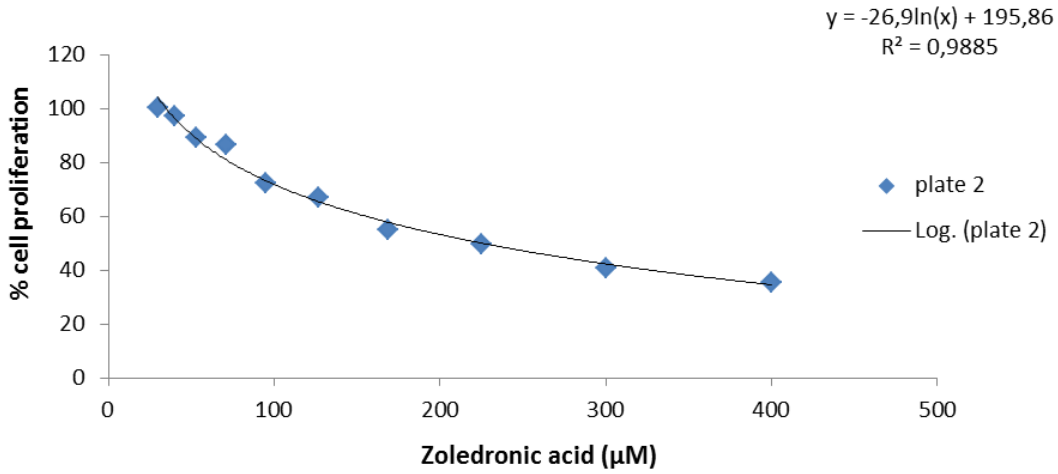

Figure E. 23 Cell proliferation of 10 µm Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 2)

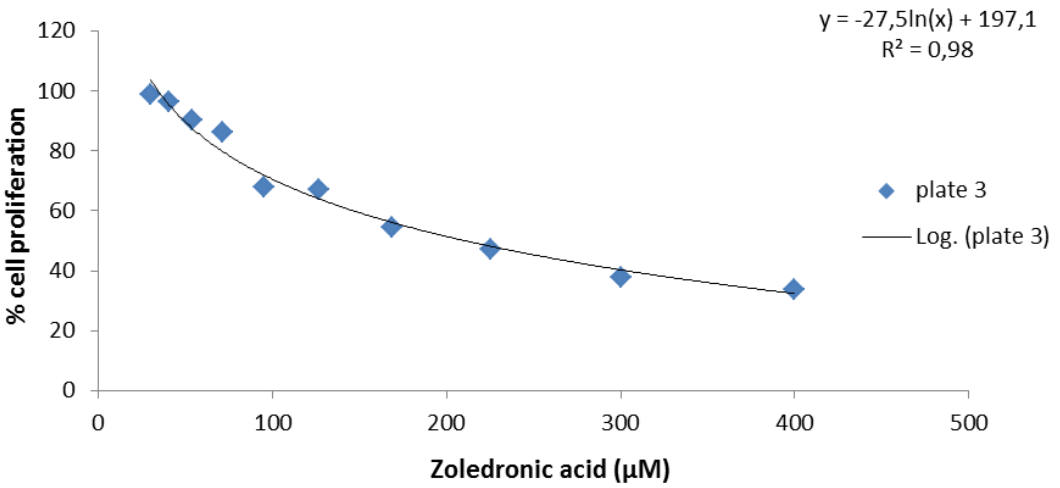

Figure E. 24 Cell proliferation of 10 µm Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 3)

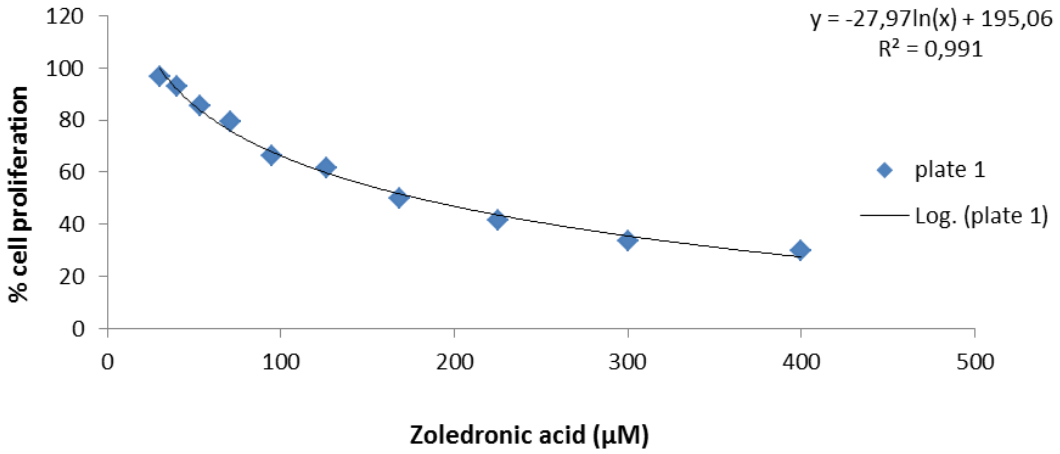

Figure E. 25 Cell proliferation of 20 µm Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 1)

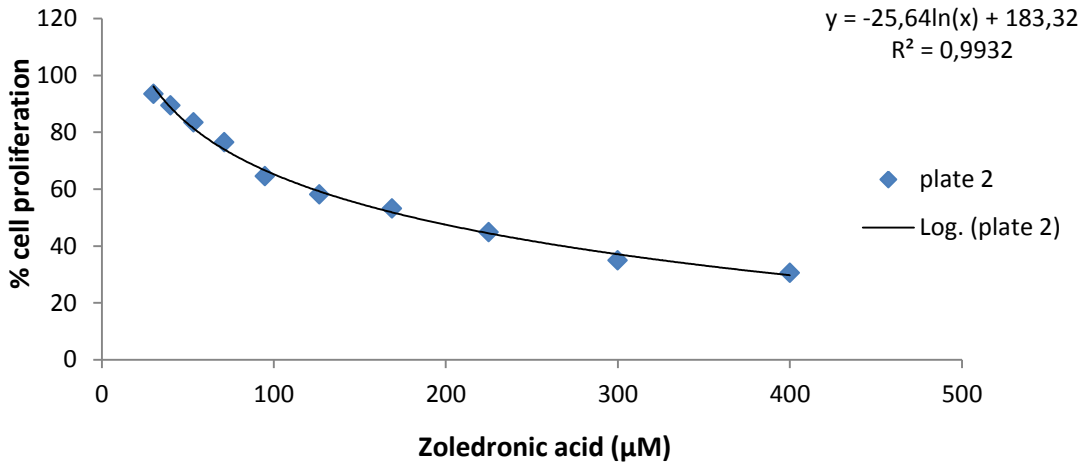

Figure E. 26 Cell proliferation of 20 µm Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 2)

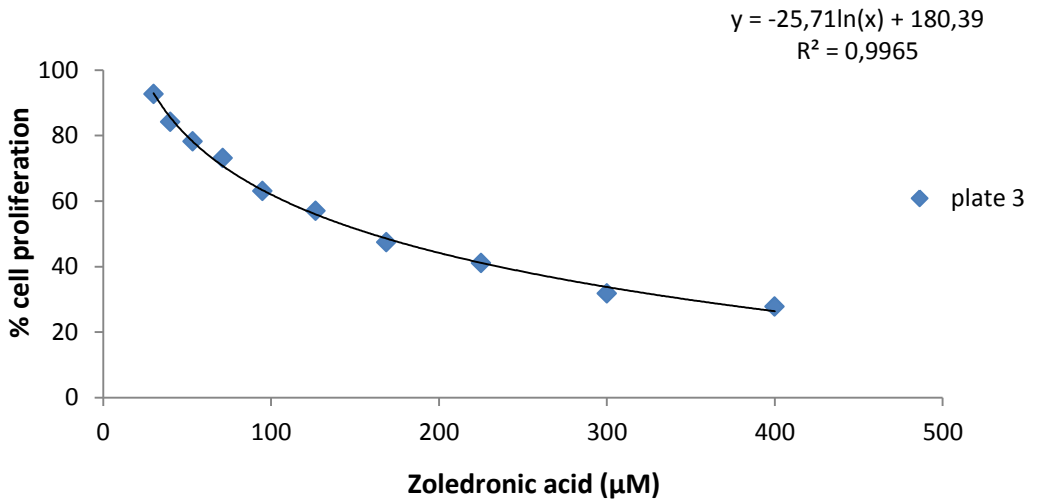

Figure E. 27 Cell proliferation of 20 µm Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 3)

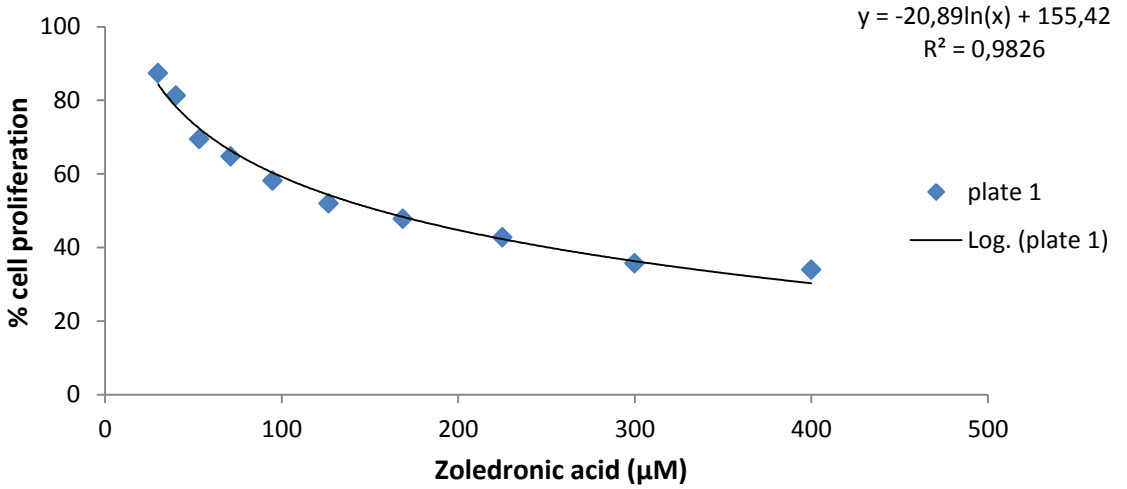

Figure E. 28 Cell proliferation of 40 µM Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 1)

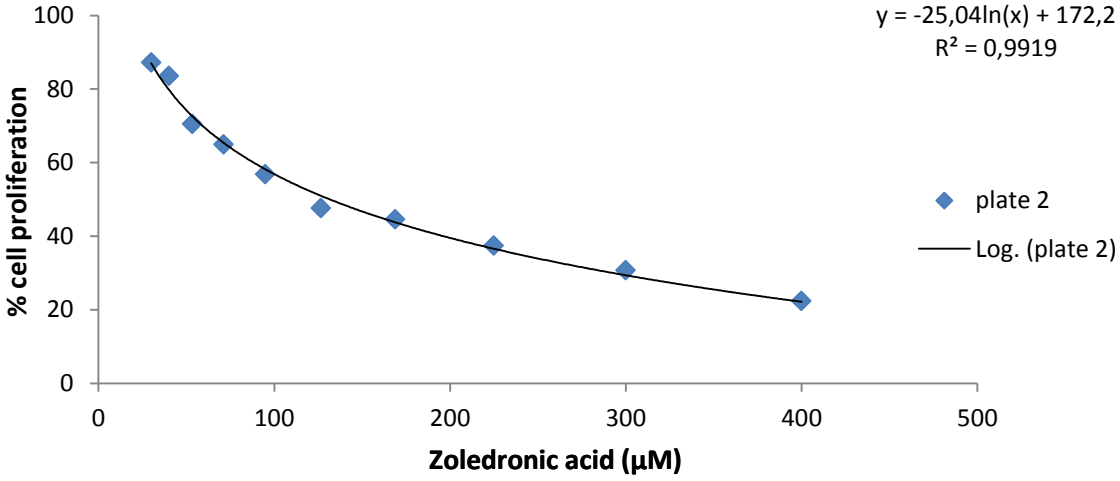

Figure E. 29 Cell proliferation of 40 µM Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 2)

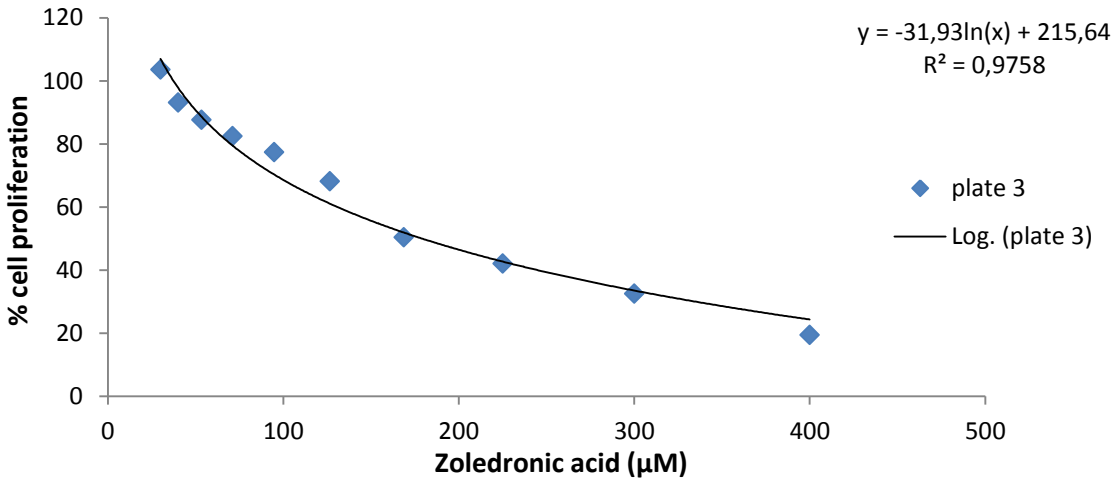

Figure E. 30 Cell proliferation of 40 µM Biochanin A treated MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 3)

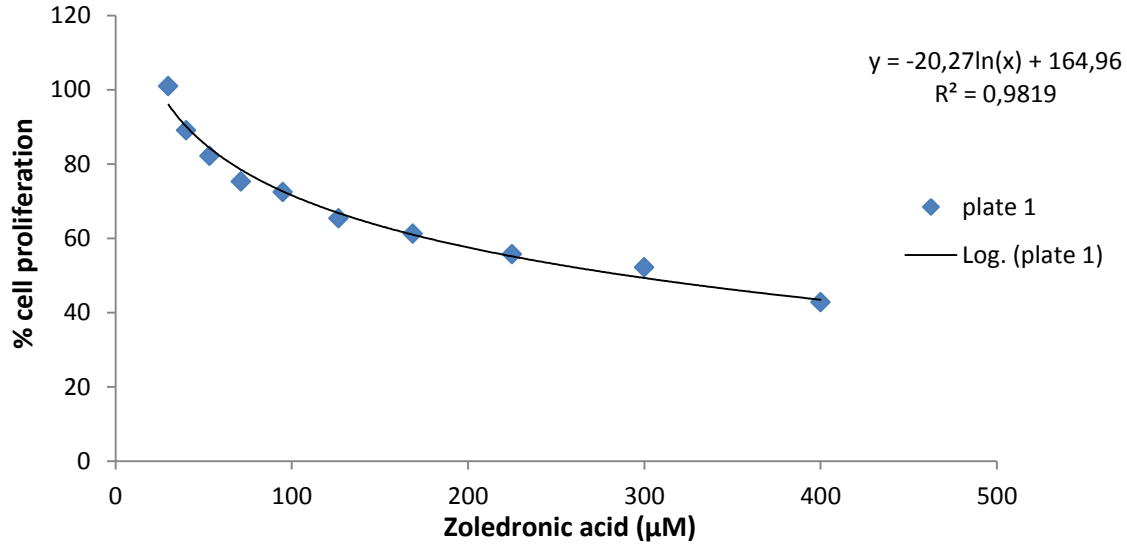

Figure E. 31 Cell proliferation of DMSO MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 1)

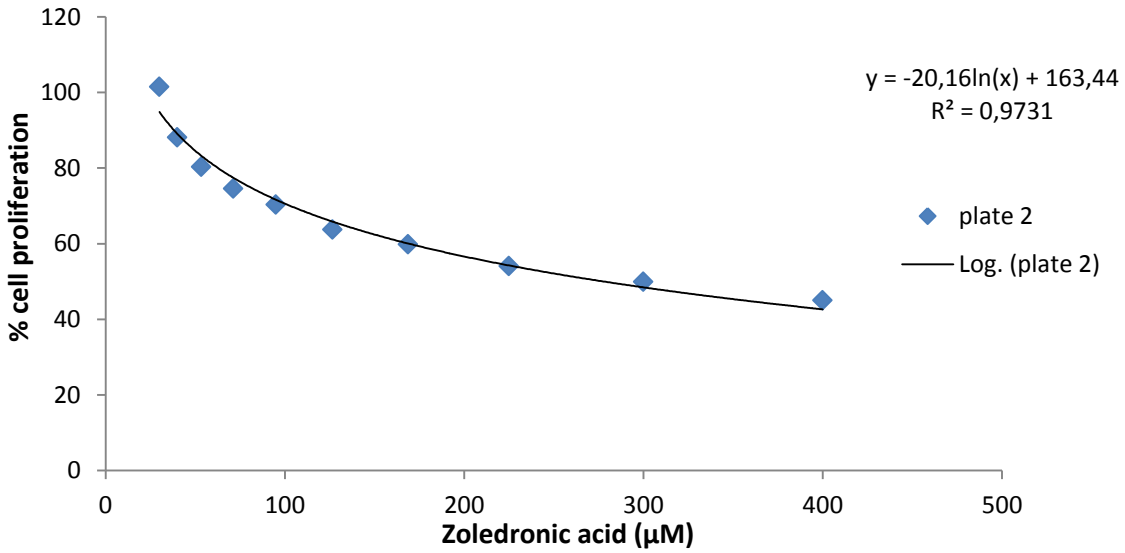

Figure E. 32 Cell proliferation of DMSO MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 2)

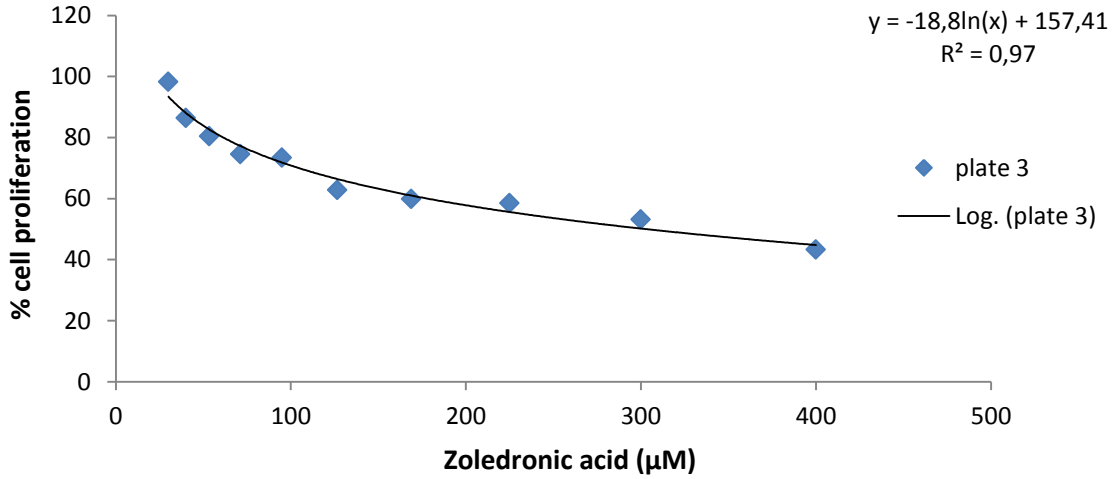

Figure E. 33 Cell proliferation of DMSO MCF7/Zol cells at increasing concentrations of zoledronic acid (plate 3)