SEQUENCING OF TYPE I INSULIN-LIKE GRWOTH FACTOR RECEPTOR
INHIBITION WITH CHEMOTHERAPY IN BREAST CANCER CELLS

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ABSTRACT

The type-I insulin like growth factor (IGF-IR) is either activated and/or overexpressed in a wide range of tumor types and contributes to tumorigenicity, proliferation, survival, metastasis and drug resistance. Disruption of type I insulin-like growth factor receptor (IGF-IR) signaling alone or in combination with other cytotoxic agents has emerged as an important strategy in cancer therapy. Recent findings suggest that the combination of chemotherapy and targeted therapy may be sequence-dependent. Since several anti-IGF-IR antibodies and small kinase inhibitors are being evaluated in phase I, II or III clinical trials, some in combination with cytotoxic chemotherapy, it is important to determine the optimal schedule for antibodies in combination with chemotherapy. Here, we sought to determine the optimal sequence of combining anti-IGF-IR inhibition using anti-IGF-IR antibody or small-molecule inhibitor with chemotherapeutic drugs in human cancer cells *in vitro* and *in vivo*.

Data presented in this study provide direct evidence that anti-IGF-IR antibody with chemotherapy is sequence-dependent. DOX followed by anti-IGF-IR antibody (scFv-Fc or EM164) was the most effective combination strategy to inhibit cell monolayer growth and anchorage-independent growth. This sequential combination triggered increased Poly (ADP-ribose) polymerase (PARP) cleavage compared to other treatment sequences. The reverse sequence, antibody followed by DOX treatment, protected cells from chemotherapy by decreasing apoptosis, arresting cells in S phase, and inhibiting the mRNA, protein and activity of topoisomerase IIα. Finally, our *in vivo* data show that recovery of IGF-IR prior to DOX therapy resulted in the best therapeutic

responses. Low doses of AVE1642 that allowed IGF-IR expression to recover at one week were more effective in combination with DOX than higher antibody doses.

In addition, we show that a novel small-molecule IGF-IR kinase inhibitor, cis-3-[3-(4-methyl-piperazin-l-yl)-cyclobutyl]-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-8-ylamine (PQIP) inhibits both the IGF-IR and insulin receptor (IR) with similar potency by blocking Akt and MAPK activation in human cancer cell lines. At doses that inhibit proliferation, the compound also inhibits the cell motility and induces cell apoptosis. PQIP enhances the cytotoxicity of doxorubicin in inhibiting cell proliferation but not in inducing cell apoptosis. Furthermore, our sequencing study shows that both combining PQIP with doxorubicin simultaneously and doxorubicin followed by PQIP significantly inhibits the anchorage-independent growth, while PQIP followed by doxorubicin does not enhance the cytotoxicity of doxorubicin.

In summary, the timing of IGF-IR inhibition affects responses to chemotherapy. The optimal sequence was DOX followed by anti-IGF-IR antibody, while the opposite sequence inhibited DOX effects. Thus, short course suppression in combination with DOX might be the optimal combination and provides a rationale for the design of future clinical trials. PQIP can be used alone or in combination with chemotherapy for breast cancer treatment.

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Chapter 1.

INTRODUCTION

Introduction

Breast cancer is the most common cancer in women, which is responsible for almost 20 percent of all cancer deaths in women[1]. The treatment of breast cancer has progressed rapidly over the last decade. However, though cytotoxic therapy and endocrine therapy produce objective regression, both treatments are far from completely effective[2]. Identifying new methods to enhance the efficacy of current therapy are clearly needed. Since normal and cancer cells receive survival and proliferative signals from their extracellular environment, targeting of these signals could enhance the clinical benefit of chemotherapy. Indeed, trastuzumab, an antibody directed against the human epidermal growth factor receptor-2 (HER2), is commonly used in combination with chemotherapy for women with advanced cancer[3, 4]. The specific targeting therapy decreases some of the adverse side effects seen with general chemotherapy and radiation therapy. Population, preclinical, and basic data suggest the insulin-like growth factor (IGF) system functions to maintain the malignant phenotype in breast cancer[5-8].

The IGF System

The IGF system is composed of three ligands (IGF-I, IGF-II and insulin), cell surface receptors (IGF-IR, IGF-IIR, the insulin receptor (IR) and Hybrid IGF-IR/IR), six high affinity binding proteins (IGFBP-1 to 6), IGFBP proteases, and the downstream proteins involved in intracellular signaling distal to IGF-IR (Fig.1).

IGF-I and IGF-II are single-chain 7.5 kDa polypeptide growth factors with a high degree of homology to insulin. The physiological function of IGF-I is to act as an effecter of growth hormone (GH)[9], which is produced in the pituitary and controlled by

Figure 1. The IGF system.
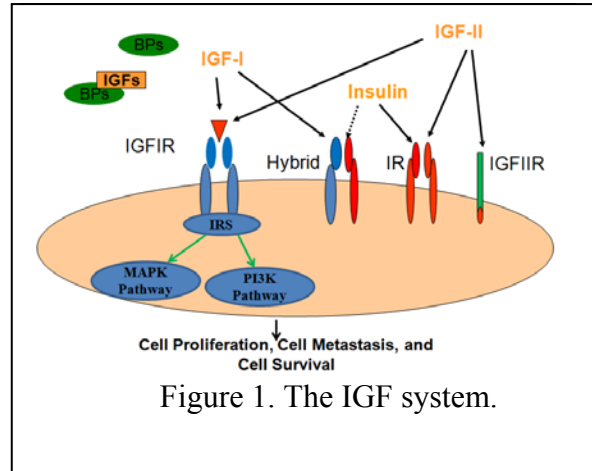
hypothalamic growth hormone-releasing hormone. During puberty, pulsatile GH release from the pituitary stimulates expression of IGF-I in the liver. In addition to its endocrine role, it has been suggested that IGF-I has an important role in prenatal growth. Mice with a homozygous deletion of the IGF-I gene have birth weight less than 60% of their wildtype littermates, these mice have a high post-natal mortality rate[10]. Besides its endocrine role, IGF-I plays an important paracrine and autocrine role during normal development and growth of the organism[9]. IGF-II expression is not regulated by GH. However, IGF-II has proliferative and antiapoptotic actions similar to IGF-I[11]. In addition, IGF-II plays a fundamental role in embryonic and fetal growth, which was proven by IGF-II gene knockout mice. These mice survive but remain smaller than their wildtype littermates[12].

The actions of IGFs can be modulated by interaction with a family of six IGF binding proteins, IGFBP-1 to IGFBP-6, which share 40-60% amino acid identity. IGFBP3 is the largest and most abundant IGFBP, more than 75% IGF is confined to the vascular compartment as a ternary complex with IGFBP3 and the acid labile subunit. By binding IGF-I and IGF-II, IGFBPs regulated the bioavailability of IGFs in the

circulation. The IGFBPs can prolong the half-lives of IGFs but also can compete with receptor for free ligands. In addition, IGFBP-3 and IGFBP-5 have also been shown to competitively inhibit IGF action at the cellular level in the absence of IGF binding and exert IGF-independent proapoptotic and antiproliferative effects through the activation of caspases involved in a death receptor-mediated pathway[13].

The primary signaling receptor through which both IGF-I and IGF-II exert their biological actions is the type I insulin-like growth factor receptor (IGF-IR), which is a tyrosine kinase receptor that shares a 60% homology at the amino acid sequence with the IR[8]. The type II insulin-like growth factor (IGF-IIR) lacks tyrosine kinase activity and exerts antiproliferative and proapoptotic activities by sequestration of IGF-II, reducing its availability for interaction with the IGF-IR[14]. Hybrid IGF-IR/IR also exist, and the IGF-IR /IR-A hybrids have higher affinity for both IGF-I and IGF-II but lower affinity for insulin, whereas IGF-IR/IR-B binds to IGF-I and IGF-II but does not interact with insulin[15-17]. The presence of the hybrid receptor adds an additional layer of complexity to IGF signaling.

Signal Transduction by the IGF-IR

IGF-IR is a type 2 tyrosine kinase receptor which is a heterotetrameric protein composed of two extracellular α-subunits and two transmembrane β-subunits linked by disulfide bonds[18, 19]. Each α-subunit contains the ligand binding site, whereas each β-subunit contains the tyrosine kinase domain. Unlike other classical tyrosine kinase receptors, such as epidermal growth factor receptor, IGF-IR activity is dependent on ligand-binding. Binding of ligand to IGF-IR induces receptor autophosphorylation and

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activation of multiple downstream cell survival and proliferation signaling pathways, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-phosphate kinase (PI3K)[20-22]. Activation of PI3 kinase pathway has been linked to cell survival[22]. One major mechanism by which PI3K/AKT signaling promotes cell survival is through phosphorylation the pro-apoptotic protein BAD[23, 24]. Induction of cell growth and proliferation has been linked to both the PI3 kinase pathway and the MAP kinase pathway downstream of IGF-IR[25, 26].

Insulin Receptor Structure and Signaling

The insulin receptor (IR) is highly homologous to the IGF-I receptor. In various mammalian species, homology between IR and IGF-IR ranges from 45-65% in the ligand binding domains to 60-85% in the tyrosine kinase and substrate recruitment domains. Two splice variants of IR exist (IR-A and IR-B). Similar to IGF-IR, IR is also a heterotetrameric protein composed of two extracellular α-subunits and two transmembrane β-subunits linked by disulfide bonds. Each α-subunit contains the ligand binding site, whereas each β-subunit contains the tyrosine kinase domain. Upon ligand binding, the receptor undergoes trans-autophosphorylation. The activated IR phosphorylates several substrates such as IRS-1, IRS-2 and other adaptor proteins, leading to the activation of MAPK and PI3K pathways.

The Role of IGF in Cancer

Abundant data from cell culture, animal and human epidemiologic studies have suggested that the IGFs and IGF-IR functions to promote proliferation, inhibit apoptosis and stimulate transformation in some cancer cells[27, 28]. High levels of circulating IGF-I are associated with an increased risk of developing breast, prostate, colon and lung cancer[29, 30]. There is also substantial evidence that IGF-I and IGF-II expression is increased in many cancers including breast cancer and multiple myelomas. A very recent report showed that the loss of imprinting of the IGF-II gene is frequently observed in the colorectal carcinoma patients[31].

Studies in transgenic mice have also revealed an important role of IGF-I in mammary tumorigenesis. Transgenic mice expressing human des(1-3) IGF-I display an increased incidence of mammary tumors[32, 33]. Furthermore, data in a transgenic mouse system suggest that mice deficient in liver-expressed IGF-I have a reduced ability to develop mammary tumors[34]. In human studies, circulating IGF-I levels are higher in breast cancer patients than in controls[35].

The IGF-IR, the primary mediator of the biological actions of IGF-I, is also implicated in maintenance of the malignant phenotype. Overexpression of IGF-IR causes transformation of fibroblasts[36]. In one *in vivo* study by Carboni *et al.*, mice which were engineered to express a human CD8α –IGF-IR fusion protein under the control of the mouse mammary tumor virus promoter developed salivary and mammary adenocarcinomas as early as 6 week after birth[37]. IGF-IR has been detected in a majority of primary breast tumor samples with overexpression in 30% to 40% of breast cancers[38]. In several cancers, increased tyrosine kinase activity of IGF-IR has been

reported[39]. In addition, a high level of IGF-IR in patients with breast cancer is associated with a greater risk of recurrence after local radiation therapy[40].

Insulin receptor substrate-1 (IRS-1), the primary downstream signaling molecule of IGF-IR, is reported to be overexpressed in some primary breast tumors and a high IRS-1 is associated with a decreased disease-free survival in a subset of patients with all tumors[41, 42]. Activation of specific IRS species are associated with distinct biological effects[43], activation of IRS-1 signaling was associated with cell growth, whereas insulin receptor substrate-2 (IRS-2) signaling was associated with cell motility[44, 45].   Nagle *et al.* showed that mammary tumor cells obtained from IRS-2 knock-out mice were less invasive and more apoptotic in response to growth factor deprivation than their WT counterparts. In contrast, IRS-1(-/-) tumor cells, which express only IRS-2, were highly invasive and were resistant to apoptotic stimuli[46].

Breast cancer cells also produce several IGFBPs that could modulate IGF action. Low level of IGFBP or high IGF: IGFBP ratio has been reported to increase cancer risk[30, 47]. Overexpression of IGFBP-2 has been reported in glioblastoma multiforme (GBM), the most malignant brain tumor[48]. Higher levels of IGFBP-1 were associated with reduced risk of distant recurrence and higher levels of IGFBP-3 were associated with an increased risk of distant recurrence in breast cancer patients[49].   In addition to the indirect modulation of IGF's mitogenic and antiapoptotic signals by ligand sequestration, IGFBPs also exert IGF-independent effects on cell survival. For example, IGFBP-4 and IGFBP-5 can rescue cells from ceramide or integrin-mediated apoptosis, which may account for the poor prognosis in breast cancers with high

IGFBP-4 expression[50-52] whereas IGFBP-3 has been found to directly inhibit breast cancer cell growth without interacting with IGFs[51, 53].

The Role of the IGF Axis in Cancer Metastasis

Besides regulating tumor growth, IGF-IR can also regulate other phenotypes associated with malignancy such as metastasis. Work by Brodt *et al.* showed that highly metastatic H-59 cells overexpressing a soluble IGF-IR molecule had significantly liver metastases [53]. Several other lines of evidence also support that the IGF system plays a role in liver metastases. Immunohistochemical analysis of hepatic resections of colorectal carcinoma patients showed that the invasive margins of liver metastases were highly positive for IGF-II and IGF-IR[54]. In addition, several studies have reported that inhibition of IGF-IR can inhibit metastasis of various cancer cells. Our lab showed that mice harboring LCC6 cells overexpressing a COOH-terminally truncated dominant negative IGF-IR did not form lung metastasis compared with the parent LCC6 cells[55]. Furthermore, in a transgenic model, overexpression of both IGF-II and the IGF-IR in the RIPI-Tag2 mice, in which SV40-T antigen was overexpressed under the control of an insulin promoter, resulted the rapid development of lymph-node metastases[56]. All these reports suggest that IGF-IR plays an important role in metastasis of cancer cells.

Common and Different Biological Roles of IR and IGF-IR

Both IR and IGF-IR have evolved by a common ancestor gene and are part of a system, and co-ordinate metabolic and growth responses in multicellular organisms. Although, the structure and the signaling pathways of the two receptors are similar, IR

and IGF-IR have different biological roles. In normal physiological conditions, the IR pathway plays a key role in glucose homeostasis, while the IGF-IR pathway is crucial to mediate body growth in response to pituitary GH. One basis of this different role is that IR and IGF-IR have different cell distribution: IR is expressed at high levels only in adipose tissue, muscle and liver, while IGF-IR is expressed at certain levels in almost all tissues[57]. Second, activated IR and IGF-IR recruit different intracellular substrates. For instance, the adapter protein Grb10[58] and the membrane protein CEACAM-2 involved in receptor down-regulation preferentially interact with IR[59]. In contrast, other substrates involved in cell proliferation or apoptosis appear to preferentially associate with IGF-IR, such as CrkII and 14-3-3[60]. Mastick *et al.* compared some of the downstream signaling pathways activated by IR and IGF-IR in NIH-3T3 cells transfected with IR or IGF-IR[61]. They found that both receptors activate theMAPK pathway and stimulate DNA synthesis to a similar extent whereas the IRS-1 pathway was more sensitive to insulin than to IGF-I[61]. Another study obtained similar results; IGF-IR was more efficient than IR in activating the MAPK pathway in transfected Rat fibroblasts[62].

IGF-IR has more potent ability to induce cell transformation than IR. R- rat fibroblasts (in which IGF-IR is knocked-out by homologous recombination) are refractory to oncogene driven cell transformation[36]. Moreover, IGF-IR, but not IR overexpression causes a ligand-dependent transformed phenotype[36]. cDNA microarray technology identified both different and similar genes that were regulated by insulin and IGF-I. One study showed that mouse fibroblast NIH-3T3 cells overexpressing either IGF-IR or IR stimulated with IGF-I or insulin, 27 of 30 genes

were up-regulated by IGF-I but not by insulin and 9 genes were up-regulated by insulin but not by IGF-I. Genes responding to IGF-I were primary involved in regulation of mitogenesis or differentiation, while genes responding to insulin spans a broader spectrum[63]. Taken together, these data indicate that these two receptors have both common and distinct effects on cell proliferation, differentiation, morphogenesis and these effects may be relevant in cancer.

The Insulin Receptor in Cancer

It is well known that several cancer cell lines require insulin for optimal cell growth. Insulin binding sites were described to be present in human breast cancer cells and several other cancer cell lines. Moreover, IR has been shown in most normal and neoplastic hematatopoietic cells to regulate proliferation and differentiation. A murine T-cell lymphoma cell (LB cells) has been described to depend on IR activation for growth but not IGF-IR[64]. Besides of the cell line studies, some animal models provide evidence for a direct role of insulin in cancer. One study showed that MCF-7 human breast cancer cells do not form tumors in diabetic nude mice but do form tumors in these mice treated with insulin[65]. In contrast, breast tumors induced by the carcinogen 7,12 dimethylbenz(a)anthracene (DMBA) in the rat were inhibited when the rats were made diabetic by alloxan administration, which destroys pancreatic β-cells and causes insulin deficiency. Exogenous insulin can restore tumor growth in these animals[66]. Moreover, mice transplanted with LB lymphoma cells are refractory to lymphoma growth when made diabetic by streptozotocin[67]. All these studies indicated that insulin may be involved in the growth of some malignancies.

Increased IR content was found in many kinds of carcinomas, such as breast, colon, lung, ovary and thyroid cancers. Papa *et al.* reported that approximately 80% of breast cancers showed an IR content higher than the mean value found in normal breast tissues using ELISAs analysis[68]. Immunostaining data indicated that IR was predominantly overexpressed in neoplastic cells and not in stromal adipocytes and inflammatory cells. The later study demonstrated that the binding affinity of IR was similar in cancer and normal tissues. However, IR expression in breast cancer was more sensitive to insulin than in normal breast tissues[69]. Moreover, studies on the possible prognostic significance of IR overexpression indicated that patients with tumors of high IR content had a lower 5-year disease-free survival (DFS) than patients with tumors with moderate IR level[70].

Insulin elicits biological effects in cancer cells by acting on its own receptor but not by activating IGF-IR. Several experiments confirmed this finding. For example, Milazzo *et al.* reported that anti-IR specific antibody but not anti-IGF-IR antibody significantly inhibited insulin stimulated cell growth in some estrogen responsive breast cancer cell lines (MCF-7, ZR-75 and T47-D)[71]. Insulin is also able to stimulate directional cell motility like other growth factors[72]. Since cell motility is relevant to tumor metastases, breast cancer cells overexpressing IR may have increased metastatic potential. Moreover, studies with insulin analogues further support that insulin and IR play a role in cancer. Treatment with the insulin analogue AspB10 induced tumors of the mammary gland in female rats[73]. In addition, IR could be activated not only by insulin but also by IGF-II. Autocrine IGF-II could stimulate cell proliferation through

IR activation in MDA-MB-157 breast cancer cells, which express 5-fold more IR than IGF-IR[74].

Since extensive studies have indicated that IR is involved in several aspects of the cancer phenotype, the role of IR in cancer needs to be considered. Agents which target both receptors may be a good choice for cancer therapy.

Anti-IGF-IR therapy

Given the role of IGF signaling in many aspects of the malignant phenotype, it would be valuable to have reagents to disrupt IGF action. Several strategies to interrupt IGF signaling are currently under investigation, including endocrine maneuvers to suppress IGF production; antisense oligonucleotides to reduce functional IGF-IR levels; monoclonal antibodies, dominant negative receptors, and tyrosine kinase inhibitors to IGF-IR activation; and neutralization of IGF action using IGFBPs[6, 75-77].

Suppression of IGF production

The majority of circulating IGF-I is produced by the liver in response to growth hormone (GH) released from the pituitary gland, acting through hepatic growth hormone receptors (GHR). GH-releasing hormone antagonists disrupt the pituitary production of GH and reduce circulating levels of GH and have been shown to inhibit the growth of a variety of cancers in animal model systems, including breast cancer[78, 79]. Somatostatin and its analogues inhibit the release of GH and thyroid-stimulating

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hormone from the pituitary gland. Preclinical studies on the anticancer activity of the somatostatin analog octreotide showed 50% reduction in tumor growth using two in vivo breast cancer models, ZR-75-1 breast xenografts and DMBA induced mammary tumors in rats[80]. However, octreotide administered with tamoxifen did not improve response or survival in patients with metastatic breast cancer compared to tamoxifen alone[81]. While octreotide was able to reduce serum IGF-I levels, it was possible that this reduction was insufficient to block IGF-IR signaling.

More potent methods to disrupt endocrine IGF-I have been developed. GH-RH antagonists MZ-5-156 or JV-1-36 induced the growth-arrest of estrogen-independent MDA-MB-468 human breast cancer xenografts in nude mice[82]. Pegvisomant, a competitive antagonist of GHR, is the most potent therapy for reducing serum IGF-I levels in acromegalic patients and may have a role in cancer treatment[82]. However, these strategies to disrupt endocrine IGF-I do not address paracrine or autocrine sources of IGF-I. Furthermore, IGF-II is not under GH control, and merely suppressing serum IGF-I levels may be insufficient to block all IGF ligands.

Ligand neutralization using IGFBPs

Since IGF ligands are required to activate IGF-IR, disruption of ligand-receptor interactions is an attractive method to disrupt IGF signaling. Blockade of IGF-mediated cellular effects can be accomplished with overexpression of IGFBPs or by treatment with exogenous IGFBPs. IGFBP-1, either exogenously added or endogenously produced, has been observed to inhibit IGF-IR function resulting in inhibition of IGF-I mediated growth of MCF-7 breast cancer cells[83, 84]. However, very short half life

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limits IGFBP1 application *in vivo*, and several approaches can be used to increase the half life of IBFBP1, including conjugation to polyethylene glycol and fusion it to the Fc domain of human immunoglobulin G. This approach has been used by some groups. For example, treatment with polyethylene glycol-conjugated recombinant IGFBP-1, inhibited growth of MDA-MB-231 breast tumor xenografts and malignant ascites formation in MDA-MB-435/LCC6 cells[83]. Silibinin, a concentrated extract of Milk Thistle, has been shown to have anti-proliferative action against some malignant cell lines by increasing IGFBP3 mRNA and protein levels[85]. Similar neutralization of IGF ligands has been accomplished using the extracellular domain of IGF-IR[53, 86] and with neutralizing antibodies against IGF-I and IGF-II[87]. Thus, several methods to neutralize IGF ligand interaction with IGF-IR have been tested. Neutralization strategies have the advantage of targeting both IGF-I and IGF-II without the need to identify a specific receptor subtype.

Decreasing Expression of IGF-IR

Several approaches have been developed to decrease IGF-IR expression, including antisense oligodeoxynucleotides (ODNs), antisense RNA, and small interfering RNA (siRNA). Antisense oligodeoxynucleotides and RNA forms heteroduplexes with the target mRNA to inhibit mRNA translation or cause mRNA degradation by RNase H. Several studies reported that anti-IGF-IR ODNs decreased IGF-IR expression at nanomolar concentrations, reduced cell proliferation, and induced apoptosis in various human and rodent cancer cell types[88, 89]. Moreover, in some instances, treatment with ODNs also induced tumor regression in animal models. Small

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interfering RNAs against IGF-IR have been shown to decrease IGF-IR levels and

inhibit IGF-IR signaling in cancer cells[90]. However, the delivery of antisense RNA

and siRNA is still inefficient and new strategies designed to deliver these reagents need

to be developed.

Inhibition of IGF-IR Activation

Abundant evidence implicating IGF-IR is essential for the transformed

phenotype and inhibition of apoptosis in breast cancer; targeting this receptor directly

may be an effective cancer therapy. Antibody blockade of growth factor receptors is a

proven strategy to inhibit receptor-mediated effects, with the effectiveness of

trastuzumab against HER2 overexpressing breast cancers being a prime example[91].

Several anti-IGF-IR antibodies have been developed and tested in preclinical model

systems. α-IR3, the first monoclonal antibody directed against IGF-IR, inhibited clonal

growth and blocked the mitogenic effects of exogenous IGF-I in breast cancer cells *in

vitro*[92]. Interestingly, α-IR3 inhibited MDA-MB-231 tumor formation in athymic

mice when administered at the time of tumor cell inoculation, but was ineffective

against MCF-7 tumor xenografts. Since MCF-7 cells are sensitive to IGF-IR blockade

in vitro, it is possible that the pharmacokinetic properties of the antibody are an

important determinant of anti-tumor activity. A chimeric humanized single chain

antibody(scFv-Fc), a partial agonist of IGF-IR, exhibited dose-dependent growth

inhibition of IGF-IR-overexpressing NIH-3T3 cells, and significantly suppressed MCF-

7 breast tumor growth in athymic mice[93-95]. EM164, a purely antagonistic anti-IGF-

IR antibody, displayed potent inhibitory activity against IGF-I and IGF-II, and serum-

stimulated proliferation and survival of MCF-7 breast cancer cells[96]. A high-affinity

fully human monoclonal antibody, A12, blocked IGF-I and IGF-II signaling and

exhibited strong anti-tumor cell activity against MCF-7 xenograft tumors by enhancing

apoptosis[97]. Another fully human antibody CP-751,871 inhibited xenograft tumor

growth of multiple cancer types, including breast cancer, lung cancer, and colorectal

cancer[98]. In addition, two other fully human antibodies against IGF-IR, H7C10 and

19D12 have also been reported to inhibit multiple cancer cell growth[98-100]. All these

antibodies can down-regulate IGF-IR over a similar time course. Currently, the

antibodies A12, CP-751,871 and h7C10 are in phase I, II / III clinical trials.

An alternative strategy to inhibit IGF action is to target the tyrosine kinase

activity of the receptor. Several members of the tyrphostin tyrosine kinase inhibitor

family (eg, AG10124, AG1024, AG538, and I-OMe AG538) were shown to

competitively inhibit IGF-IR autophosphorylation and kinase activity in intact IGF-IR-

overexpressing NIH-3T3 cells and to inhibit growth of MDA-MB468 and MCF-7 breast

cancer cells in monolayer and colony formation in soft agar[101, 102]. However, cross-

reactivity of these compounds with the insulin receptor tyrosine kinase was reported due

to the high degree of homology between the two receptors. Newer agents have been

developed with apparently more selective IGF-IR activity[90, 103]. However, it is

uncertain if a highly selective IGF-IR tyrosine kinase inhibitor is desirable. Since

insulin receptor may mediate some of the biological effects of the IGFs, it is possible

that both IGF-IR and insulin receptor will need to be inhibited in tumors. Using anti-

sense oligonucleotides, Salatino *et al.* have shown that specific targeting of the IGF-IR

in mice inhibits tumor growth[88], supporting the idea that specific inhibition of IGF-IR

may block tumor growth. However, since mice have very low serum levels of IGF-II after birth[12], it remains to be seen if targeted disruption of IGF-IR alone is sufficient to inhibit tumor growth in humans.

Conventional Chemotherapy for Breast Cancer

It is well established that most chemotherapeutic agents eliminate cells by triggering apoptosis. Most currently approved agents target specific molecules required for a cell to traverse the cell cycle. Targets range from DNA itself, to enzymes (topoisomerases), or structural proteins (tubulin) required for cell division. There are three types of cells in a solid tumor: dividing cells that are continuously cycling, resting cells which may potentially enter the cell cycle, and those cells no longer able to divide. In breast cancer, Clarke *et al.* have suggested that only a minority of cells contained within a tumor have the capacity to contribute to all these subpopulations of tumor cells leading to the idea that cancer stem cells exist[104]. Essentially only dividing cells are susceptible to the main currently available chemotherapy drugs. It is the existence of resting or stem cells that makes cancer chemotherapy unable to completely eradicate advanced tumors by chemotherapy; even after a substantial clinical response, a population of cells may still exist that have full capacity to enter the cell cycle.

The primary therapy of localized-early stage I and II- breast cancer is either breast-conserving surgery and radiation therapy or mastectomy with or without reconstruction[105]. Systemic adjuvant therapies designed to eradicate clinically undetectable microscopic deposits of cancer cells which may have spread from the primary tumor, result in decreased recurrences and improved survival[106, 107].

Adjuvant therapies include chemotherapy and hormonal therapy. In the adjuvant setting, chemotherapy is usually given in combination for 4-6 months. A wide variety of agents have been effective in breast cancer including DNA alkylators (cyclophosphamide), topoisomerase inhibitors/DNA intercalators (doxorubicin), anti-metabolites (5-fluorouracil, methotrexate), and tubulin interacting agents (paclitaxel)[107]. Adjuvant chemotherapy effectively reduces the odds of recurrence and death by approximately 20% to 60% of patients. However, since this reduction of risk is not complete, substantial research effort is directed toward improving the benefits of chemotherapy and overcoming the chemotherapy resistance. New target discovery and combination of new agents with "conventional" agents represent an active area of investigation.

Doxorubicin is one of the most widely used anticancer drugs in the clinic to treat breast cancer and a variety of other cancers. The exact mechanism of action of doxorubicin is complex and still somewhat unclear, though it is known to interact with DNA by intercalation and inhibition of macromolecular biosynthesis[108] . The primary cellular target for doxorubicin is topoisomerase II. This essential enzyme removes knots and tangles from the genome by introducing transient double-stranded breaks in the DNA backbone[109]. Doxorubicin kills cells by stabilizing the topoisomerase II-DNA cleavage complex that is a transient intermediate in the catalytic cycle of the enzyme. The accumulation of cleavage complexes in treated cells leads to produce permanent DNA double stand breaks. If these breaks overwhelm the cell, they can initiate death pathways. Doxorubicin is considered to be the most effective agent in the treatment of breast cancer patients. Unfortunately, resistance to this agent is

common[110]. Developing Resistance is also common for other cytotoxic agents. The pursuit of a strategy that overcomes resistance remains an interesting field. Accumulating evidence indicates that a number of mechanisms known to contribute to clinical drug resistance might be relevant to breast cancer.

IGF Signaling Confers Resistance to Chemotherapy

Drug resistance is the most important cause of cancer treatment failure. Anticancer drugs fail to kill cancer cells for a number of reasons, including failing to reach tumors, poorly absorbing or metabolically deactivating. Drug resistance are either innate or acquired after extended treatment[111].

The IGF signaling system regulates multiple levels of tumor progression. Besides promoting mitogenic activity, IGF-I also protects breast cancer cells from drug-induced apoptosis[112-114]. In fibroblasts, protection from apoptosis requires the tyrosine kinase activity of IGF-IR, as kinase defective receptors do not protect fibroblasts from apoptosis[115]. In addition to drug-induced apoptosis, IGF-IR activation appears to block other stimuli as well. For example, BNIP3 (Bcl-2/E1B 19 kDa interacting protein) is a proapoptotic member of the Bcl-2 family expressed in hypoxic regions of tumors. Treatment of the breast cancer cell line MCF-7 cells with IGF effectively protected these cells from BNIP3-induced cell death[116] likely via activation of PI3K and the Akt/PKB pathways[115, 117]. Akt phosphorylates BAD, a member of the Bcl-2 family of proapoptotic proteins, phosphorylated BAD cannot heterodimerize with Bcl-2 or Bcl-Xl, remains in the cytosol and cell death is inhibited[24]. It has also been suggested that IGF can inhibit apoptosis by increasing

the expression of Bcl-XL at both the mRNA and protein level[118]. Thus, signaling from IGF-IR to multiple proteins involved in the intrinsic apoptotic pathways suggest a mechanism for protection from cell death signals.

IGFs provide resistance of breast cancer cells to chemotherapeutic agents[113]. IGF-I alters drug sensitivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs. It increased cell survival of HBL100 cells treated with 5-FU, methotrexate, tamoxifen or camptothecin, but no changes were observed in Bcl-2 protein or Bax mRNA levels[119]. IGF-I signaling is also associated with resistance to the growth-inhibitory actions of trastuzumab by upregulation of ubiquitin-related p27kip1 degradation and activation of the PI3K signaling pathway[120]. In breast cancer cells, IGF-I can activate JNK which is generally associated with a pro-apoptotic response. However, activation of Akt seems to override pro-apoptotic effects of JNK activation[121]. Thus targeting IGF-IR could enhance a pro-apoptotic response initiated by many different agents.

Combination of Anti-IGF Strategy with Chemotherapy, Hormonal Therapy and Targeted Therapy in Cancer

Combination of different types of therapy is commonly used in cancer treatment, because it simultaneously interrupts different targets of cancer growth, enhances the chemotherapy response, and decreases the toxicity and resistance of the drug.

In theory, inhibition of survival pathways by blocking IGF-IR signaling while enhancing apoptotic stimuli has appeal. Combination of anti-IGFIR antibody αIR3 with doxorubicin resulted in increased cytotoxicity in IGF-I stimulated cells than with

chemotherapy alone[122]. Similar enhancement of chemotherapy effects have been

shown in Ewing's sarcoma cells[123]. Tyrphostin AG1024 (a tyrosine kinase inhibitor

of IGF-IR) demonstrated a marked enhancement in radiosensitivity and amplification of

radiation-induced apoptosis which was associated with increased expression of Bax,

p53 and p21, and decreased expression of Bcl-2[124]. Another study demonstrated that

co-targeting IGF-IR and c-kit synergistically inhibited proliferation and induction of

apoptosis in H209 small cell lung cancer cells[125]. There is also evidence that

somatostatin analogues may enhance the effect of tamoxifen in animal models by

suppressing plasma IGF-I and IGF–II levels[126]. Transfection of IGF-IR antisense

oligonucleotides enhanced sensitivity to doxorubicin and cisplatin in human central

nervous system atypical teratoid/rhabdoid cells[127]. Another study showed that the

IGF-IR kinase inhibitor NVP-ADW742 could sensitize small cell lung cancer cells to

etoposide and carboplatin[128]. Recent preclinical studies showed that CP-751,871 in

combination with 5-fluorouracil significantly inhibited Colo-205 colon cancer xenograft

growth compared with 5-fluorouracil alone[98]. Furthermore, combining anti-IGF-IR

antibody h7C10 with either a chemotherapeutic agent vinorelbine or an anti-EGFR

antibody is superior to inhibit the A549 non-small-cell lung cancer tumors than either

agent alone[99].

In addition to conventional agents, it is also possible that anti-growth factor

receptor strategies can be combined. Recent evidence shows that increased levels of

IGF-IR signaling appears to interfere with the action of trastuzumab in breast cancer

cell models that overexpress HER2/neu[126], Her2 gene is amplified in approximately

30% of invasive breast cancer and is associated with poor prognosis and disease-free

survival[27]. Thus, strategies that target IGF-IR signaling may prevent or delay development of resistance to trastuzumab. All these reports implied that anti-IGF-IR therapy has tremendous potential when used in combination with other therapy.

Sequencing of Targeted Therapy or hormonal therapy with Chemotherapy in Cancer

A pilot trial of chemo-hormonal therapy in 1991 showed that androgen priming before chemotherapy demonstrated a higher response rate and modestly increased actuarial survival in prostate cancer patients[129, 130]. This observation provides the rationale for the design of new therapeutic schemes in which chemotherapy given at the time of estrogen-induced tumor cell proliferation might achieve a greater degree of tumor cell kill. Tamoxifen, an anti-estrogen agent is widely used in the treatment of breast cancer and recently was evaluated in large clinical trials as a preventative agent for estrogen-receptor positive tumors[106]. However, only half of patients with ER positive tumors respond to tamoxifen treatment. Subsequent reports suggested that a strong sequence-dependent interaction exists between tamoxifen and methotrexate in MCF-7 breast cancer cells; sequential 24 hours exposure to tamoxifen followed by methotrexate led to antagonism of the methotrexate effect[131, 132]. In addition, specifically, recent data from a large cooperative group trial demonstrated an estimated disease-free survival (DFS) advantage of 18% for sequential rather than concurrent chemotherapy and tamoxifen, suggesting an antagonistic effect of tamoxifen on cytotoxic chemotherapy with concurrent administration[133]. Questions have been raised regarding the optimal sequencing of tamoxifen and chemotherapy in patients

treated after breast-conserving surgery. Tamoxifen exerts a cytostatic effect on estrogen-positive breast cancer cells. Thus, when cells are arrested at G1 by tamoxifen first, fewer cells will progress to the S phase which will result in a decrease in the effect of methotrexate which targets S-phase cells. Taken together, these considerations suggest that combining chemotherapy with targeted therapy may be sequence-dependent.

Trastuzumab (Herceptin), an anti-HER2 antibody, is widely and successfully used to treat patients overexpressing the HER2 growth factor receptor as a single agent or in combination with other chemotherapeutic drugs. However, one recent report found that inhibition of HER-2 by trastuzumab or low molecular weight inhibitors first would increase paclitaxel resistance of SKOV-3 ovarian carcinoma cells[134]. Thus, it is important to decide the appropriate sequence for the use of combination anti-growth factor antibodies with chemotherapy in preclinical phase

Purpose of this study

Here, our goal is to find the optimal sequence for combining the anti-IGF-IR therapy (anti-IGF-IR antibody and IGF-IR tyrosine kinase inhibitor) with chemotherapy in inhibiting cancer cell proliferation, inducing cell apoptosis, and inhibiting mouse xenograft growth and tumor metastasis.

Hypothesis

Sequencing of anti-IGF-IR inhibition with chemotherapy could enhance the cytotoxicity of chemotherapy *in vitro* and *in vivo*.

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Summary of Key Findings

Data presented here provide direct evidence that combining of anti-IGF-IR antibodies with chemotherapy is sequence-dependent. The sequential treatment of doxorubicin followed by anti-IGF-IR antibodies, including either the agonistic antibody scFv-Fc or the fully antagonistic antibody AVE1642, significantly inhibits cell proliferation, induces cell apoptosis and inhibits tumor growth and metastasis, while the opposite sequence with anti-IGF-IR antibody followed by doxorubicin protects cancer cells from the cytotoxicity of doxorubicin. This sequence-dependent effect is specific to DNA damaging agents but not anti-metabolism chemotherapeutic agents. In addition, we also provide evidence that combining doxorubicin with a small dose of AVE1642 is much more effective to inhibit LCC6 mouse xenograft growth and lung metastasis than combining with a high dose of AVE1642. Furthermore, it is shown that pretreatment with anti-IGF-IR antibody followed by doxorubicin decreased mRNA, protein and activity of topoisomoerase II, while the opposite sequence increases topoisomearse II activity.

Using IGF-IR tyrosine kinase inhibitor to block the IGF-IR signaling activation is another important strategy in anti-IGF-IR therapy. cis-3-[3-(4-methyl-piperazin-l-yl)-cyclobutyl]-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-8-ylamine (PQIP), a novel small molecule IGF-IR inhibitor, was reported to display 14-fold cellular selectivity to human IGF1R compared with IR in 3T3/hu IGF1R cell (NIH 3T3 cells overexpressing IGF-IR). In our study, we found that PQIP has equal efficiency in inhibiting IGF-I and insulin stimulated signaling events in MCF-7 breast cancer cells and LCC6 melanoma cells. Importantly, PQIP enhances the cytotoxicity of doxorubicin

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in inhibiting cell proliferation but does not affect cell apoptosis. Moreover, there is no significant difference between cells were treated with doxorubicin in combination with PQIP simultaneously or with doxorubicin followed by PQIP. However, the sequence of PQIP followed by doxorubicin is the least effective. These *in vitro* observations will be tested in *in vivo* mouse xenograft growth models.

In summary, our studies have important implications for IGF1R targeted cancer therapy. First, downregulation of IGF1R may not affect tumor growth but could sensitize cells to apoptotic insults. Secondly, the sequencing of IGF1R inhibition with chemotherapy may distinguish between synergy and interference. This observation is important, as several anti-IGF1R antibodies including AVE1642 are being evaluated in phase I /II/III clinical trials as single agents and in combination with chemotherapy. The antibody dosages used in these trials will likely result in sustained downregulation of IGF1R. While this should clearly extinguish IGF1R signaling, the effects on the response to DNA damaging agents might be counterproductive. Third, given the potential toxicity on glucose metabolism of chronic IGF1R inhibition, it is possible that low doses of antibodies might have reduced toxicity. Our study suggests that careful consideration of sequencing and dose is necessary when considering combining anti-IGF1R strategies with chemotherapy.

CHAPTER 1

Sequencing of Anti-IGF-IR Antibodies with Chemotherapy *in vitro* and *in vivo*

INTRODUCTION

Although chemotherapy produces objective responses in patients with breast cancer, it is far from completely effective. Cytotoxic agents also may cause severe and dose-limiting systemic toxicities. Thus, identifying new methods to enhance the beneficial effects of chemotherapy while decreasing systemic toxicities are clearly needed[2, 77]. Recent progress suggests that combination of chemotherapy with targeted therapy is superior to either one alone. For example, patients with metastatic colon cancer treated with both the anti-EGFR antibody, cetuximab, and the chemotherapy drug irinotecan have superior results, even if the patients progressed on irinotecan alone[135]. In addition, trastuzumab (Herceptin), an anti-HER2 antibody, is widely used to treat patients overexpressing the HER2 growth factor receptor in combination with chemotherapeutic drugs[3, 4].

Among the new potential cancer targets, the type I insulin-like growth factor receptor (IGF-IR) has emerged as a relevant pathway. Population, preclinical, and research findings suggest that the insulin-like growth factor (IGF) system functions to maintain the malignant phenotype in cancer[5]. Disruption of IGF-IR activation has been shown to inhibit cancer cell growth and motility *in vitro* and *in vivo*[6, 7, 94, 136]. Various approaches of disrupting IGF1R activity have been developed as potential interventions in the treatment of malignancies in the past several years. Antibodies that disrupt IGF-IR function have been developed. scFv-Fc, a chimeric humanized single chain antibody, causes initial receptor biochemical signaling followed by receptor down-regulation, and exhibits dose-dependent growth inhibition of some breast cancer

cell lines[94, 95]. EM164, a full antagonistic anti-IGF-IR antibody, did not stimulate

IGF-IR autophosphorylation, but downregulated IGF-IR *in vitro* and *in vivo*. It also

displays inhibitory activity against IGF-I and IGF-II induced survival of MCF-7 breast

cancer cell[96]. The humanized version of EM164, AVE1642 (sanofi-aventis) is

currently in clinical trials against various type of solid cancer.

Several studies have shown that activation of IGF-IR protects breast cancer cells

from apoptosis induced by chemotherapy and radiation, and receptor activation

mediates resistance to chemotherapy and radiation[119, 120, 137]. Therefore,

inhibiting IGF-IR signaling may enhance the sensitivity of cancer cells to

chemotherapy. Indeed, several groups have shown that combining anti-IGF-IR

antibody with chemotherapy enhances chemotherapy responses in human cancer

cells[96, 98, 99].

Recent findings suggest that combination of chemotherapy and targeted therapy

may be sequence-dependent. A large cooperative group trial demonstrated an estimated

disease-free survival advantage of 18% for sequential rather than concurrent

chemotherapy and tamoxifen treatment when given in the adjuvant setting[133]. In

addition, inhibition of HER2 by trastuzumab first, but not in the reverse order, increased

paclitaxel resistance of ovarian cancer cells[134]. Since several anti-IGF1R antibodies

are being evaluated in phase I /II/III clinical trials, some in combination with cytotoxic

chemotherapy[138], it is be important to determine the optimal schedule for the

antibodies in combination with chemotherapy.

Hypothesis and Approach

Sequentially combining anti-IGF-IR antibodies with chemotherapy enhances the response of chemotherapy *in vitro* and *in vivo*. To directly test this hypothesis, MCF-7 and LCC6 cells were treated with sub-cytotoxic concentrations of doxorubicin (DOX) with or without anti-IGF-IR antibodies (scFv-Fc or EM164 and its humanized version AVE1642). Treatments were given simultaneously, DOX followed by anti-IGF-IR antibody, or anti-IGF-IR antibody followed by DOX, with measurement of in vitro proliferation, apoptosis, and anchorage-independent growth. Effects of sequencing on LCC6 xenograft growth and metastasis were studied.

RESULTS

scFv-Fc, but not EM164 stimulated MCF-7 cell entry into S phase of the cell cycle as IGF-I. Several antibodies have been developed as a strategy to inhibit IGF-IR activation. Two different anti-IGF-IR antibodies were chosen in these studies. scFv-Fc , a chimeric humanized single chain antibody, stimulates IGF-IR activation and cell growth *in vitro* yet partly inhibits MCF-7 xenograft growth in mice by causing IGF-IR downregulation[94]. EM164, a full antagonistic anti-IGF-IR antibody, does not stimulate IGF-IR antophosphorylation, but downreguates IGF-IR *in vitro* and *in vivo*[96]. Since most chemotherapeutic drug is cell cycle specific, only proliferating cells are killed. Thus, we measured entry of cells into S phase of the cell cycle following scFv-Fc and EM164 treatment. Cells were plated in serum-containing media, serum-starved for 48 hours to arrest cells in G_0/G_1 phases of the cell cycle, and treated with IGF-I, scFv-Fc and EM164 for different time points. Cells were then stained with

propidium iodide and analyzed by flow cytometry to determine the fraction of cells in each phase of the cell cycle. IGF-I treatment stimulates the MCF-7 cells to enter into S Phase of the cell cycle, and the peak is at 24 hours (Figure 1.1A). Surprisingly, anti-IGF-IR antibody scFv-Fc, which inhibits the binding of IGF-I and IGF-II to IGF-IR, also stimulates the MCF-7 cells entering into S Phase of the cell cycle same as IGF-I. In contrast, the antagonistic anti-IGF-IR antibody EM164 does not stimulate MCF-7 cells to enter S phase (Figure 1.1B). Together, these two antibodies have different biological features. scFv-Fc stimulates cell entering into S phase, and it maybe benefit the S phase specific chemotherapeutic drugs.

Sequence-dependent anti-proliferation effects of combining scFv-Fc or AVE1642 with doxorubicin in MCF-7 cells. To assess whether the IGF-IR antibody, scFv-Fc can synergize or inhibit the cytotoxicity of doxorubicin, the IC_{50} of doxorubicin were studied in a monolayer growth using MTT assays. These assays were done in the absence (1%FBS) or presence of IGF-I (5 nM). Whether in the presence of IGF-I or in the 1% FBS condition, simultaneous treatment with scFv-Fc and doxorubicin or the sequence of scFv-Fc followed by doxorubicin, decreased cytotoxicity was observed (Figure 1. 2A, B). The IC50 of doxorubicin was increased when antibody was given before or simultaneously with the drug. Interestingly, doxorubicin followed by scFv-Fc, resulted in enhanced doxorubicin cytotoxicity and the IC50 2-4 times lower in MCF-7 cells.

Next, we wanted to know whether the sequence-dependent inhibition was specific to scFv-Fc, a partial agonistic antibody of IGF-IR. We examined the effect of

doxorubicin in combination with EM164, an antibody that does not stimulate IGF-IR biochemical activation and displayed potent inhibitory activity against IGF-I, IGF-II and serum stimulated proliferation of diverse cancer cell lines[96]. As shown in Figure 1. 3A and B, similar results were obtained, as the sequence of doxorubicin followed by EM164 significantly inhibited the cell proliferation in MCF-7 cells. These results suggest that the cytotoxic effects of combining doxorubicin with scFv-Fc or EM164 are sequence-dependent with doxorubicin followed by antibody demonstrating the optimal anti-proliferative effect even though they have different effects to the cell cycle.

Doxorubicin and etoposide, but not 5-FU, followed by scFv-Fc or EM164, significantly inhibited the anchorage-independent growth of LCC6 cells. In MCF-7 cells, IGF-IR activation stimulates proliferation complicating the interpretation of this type of sequencing experiment[113], because we have to consider the time and dose of adding the IGF-I into the system. In order to further evaluate the effects of inhibition of IGF1R on chemotherapy and confirm the sequence-dependent inhibition effects, without the added complexity of the growth regulatory effects of IGF1R on the cell, we concentrated on the LCC6 cell line in anchorage-independent growth assays. This cell line, derived from the MDA-MB-435 cell line, expresses IGF1R yet is not growth regulated by its activation[55]. Recently, this cell line has been shown to originate from a malignant melanoma cell[139], but still is an excellent model for studying the effects of IGF1R inhibition on cancer cell biology.

In this experiment, we used a dose of doxorubicin (100 ng/ml) that by itself, it did not inhibit colony formation. Cells were either treated simultaneously with

doxorubicin and scFv-Fc or treated sequentially. As shown in Figure 1.4A, similar to the result of the MTT assay in MCF-7 cells, the sequential treatment of doxorubicin followed by scFv-Fc significantly inhibited colony growth compared to the other sequences. Since scFv-Fc is a partial agonistic antibody of IGF1R, we examined the effect of doxorubicin in combination with EM164 in anchorage-independent growth. As shown in Figure 1.4B, similar results were obtained, as the sequence of doxorubicin followed by EM164 significantly inhibited the colony growth in LCC6 cells.

Rochester *et al.* showed that silencing of the IGF-IR gene enhances sensitivity to DNA damaging agents in human prostate cancer cells, but not with other cytotoxic agents[140]. Doxorubicin is a toposomerase II inhibitor and also intercalates into DNA to cause DNA damage[141]. To investigate whether the sequence-dependent inhibition of doxorubicin in combination with anti-IGF-IR antibodies was specific to DNA damaging drugs, the effect of combining anti-IGF-IR antibodies with etoposide and 5-FU were examined. Etoposide is another topoisomerase II inhibitor and induces double strand DNA breaks[142], while 5-FU is an anti-metabolite which undergoes biotransformation to 5-fluoro-2'-deoxyuridine- 5'-phosphate and forms a covalently bound ternary complex with the enzyme thymidylate synthase and its cofactor to inhibit DNA synthesis[143]. As expected, etoposide followed by scFv-Fc or EM164 significantly inhibited colony growth (Figure 1.5A, B). In contrast, sequence-dependent inhibition was not observed with 5-FU in combination with scFv-Fc or EM164 (Figure 1.6A, B).

Doxorubicin followed by scFv-Fc or AVE1642 increased PARP cleavage and pretreatment with scFv-Fc or EM164 rendered cell resistant to doxorubicin induced apoptosis. IGF-I protects MCF-7 breast cancer cells from doxorubicin and paclitaxel by induction of proliferation and inhibition of apoptosis[113]. In LCC6 cells, mono-layer cell growth is not affected by IGF-I; we used these cells to examine whether anti-IGF-IR antibody scFv-Fc or AVE1642 sequencing affected doxorubicin induced apoptosis in LCC6 cells. LCC6 cells were treated with doxorubicin and scFv-Fc or AVE1642 simultaneously or sequentially. After treatment, both adherent and nonadherent cells were collected and subjected to Western blotting with antibodies to PARP, a caspase substrate that is cleaved in cells undergoing apoptosis. As shown in figure 1.7, doxorubicin followed by scFv-Fc or AVE1642, but not in other sequences, significantly increased DOX (500 ng/ml) induced PARP cleavage. Neither antibody by itself induced PARP cleavage. In addition, doxorubicin followed by scFv-Fc or AVE1642 significantly increased caspase 7 activation (Figure 1.8), which is an upstream molecule of PARP and causes PARP cleavage.

To address whether these changes in PARP cleavage reflected an increase in induction of apoptosis by chemotherapy, we measured annexin-V staining by flow cytometry analysis. LCC6 cells were treated with doxorubicin in combination with scFv-Fc or EM164 concurrently or sequentially for 48 hours, the adherent and nonadherent cells were analyzed. In this assay, pre-treatment with EM164 significantly decreased the doxorubicin induced apoptosis (Figure 1.9). We did not see DOX followed by EM164 increased cell apoptosis in this experiment. However, these data

indicate that if IGF-IR was inhibited prior to doxorubicin exposure, then cells were protected from the effects of doxorubicin.

Pretreatment with scFv-Fc or AVE1642 followed by doxorubicin arrested LCC6 cells in S phase. To investigate the potential mechanism of this sequence-dependent effect of doxorubicin in combination with anti-IGF-IR antibodies, we studied the cell cycle profile of LCC6 cells treated with doxorubicin and scFv-Fc or AVE1642. In these cells, the antibodies themselves did not affect cell cycle distribution of LCC6 cells (Figure 1.10A, B). Consistent with previous report, doxorubicin treated cells were arrested in G2-M phase of the cell cycle[144]. Simultaneous exposure cells to scFv-Fc or AVE1642 and doxorubicin, doxorubicin followed by scFv-Fc or AVE1642, showed similar effects to doxorubicin alone. In contrast, cells pre-treated with antibody followed by doxorubicin decreased G2-M arrest with an increase in G1 and S-phase fraction (Figure 1,10A, B). These data suggested that inhibition of IGF1R prior to doxorubicin exposure blunted the effects of doxorubicin as reflected by effects on the cell cycle.

Pretreatment of AVE1642 followed by doxorubicin decreased topoisomerase II activity, expression and mRNA level. Since the effects of sequencing were seen with drugs that affect topoisomerase II (topo II), such as doxorubicin and etoposide**,** we hypothesized that blockade of IGF1R may affect topo II activity. We measured topo II activity in nuclear extracts by the decatenation of kDNA assay. This assay measures the ability of topo II to catalyze strand passage between two double-stranded DNA

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segments. Topo II decatenation activity (per identical amounts of nuclear extract proteins) was approximately 5-fold lower in the sequence AVE1642 followed by doxorubicin extracts as compared with doxorubicin alone (Figure 1.11, lane 6 versus lane 9). In contrast, nuclear extracts from cells, with sequential treatment of doxorubicin followed by AVE1642, had significantly increased topo II activity (Figure 1.11, lane 6 versus lane 8). To determine if the altered topo II activity was related to level of topo II protein, we determined topo II levels in cellular extracts by immunoblotting. AVE1642 followed by doxorubicin caused a 2-fold decrease in Topo II protein expression (Figure 1.12). In addition, we consistently observed a marked drop in topo IIα mRNA expression in the group pretreatment with AVE1642 (Figure 1.13). Taken together, these data suggest that decreased topo II activity by pretreatment with AVE1642 led directly to a decreased sensitivity of LCC6 cells to topo II poisons like doxorubicin. In contrast, post-doxorubicin blockade of IGF1R increased the activity of topo II and increased sensitivity of LCC6 cells to doxorubicin.

Sequencing of anti-IGF-IR antibodies with doxorubicin did not affect doxorubicin accumulation in LCC6 cells. Guo *et al.* reported that IGF-I promotes multidrug resistance in MCLM colon cancer cells due to increased mRNA expression of multidrug resistance gene-1(mdr-1) and the p-glycoprotein (P-gp) in cells[145]. Our *in vitro* data showed that pretreatment of cells with scFv-Fc followed by doxorubicin causes some resistance to doxorubicin. Since scFv-Fc has the similar effect as IGF to stimulate cell proliferation *in vitro*, we propose that it may activate the mdr-1 gene, increase the P-gp, and decrease the accumulation of intracellular doxorubicin. Anthracyclines such as

doxorubicin are intrinsically fluorescent; the cellular fluorescence intensity can serve as an indicator of drug accumulation and drug sensitivity[146]. In order to explore the underlying mechanism of this sequence-dependent effect, intracellular doxorubicin accumulation was analyzed by flow cytometry. LCC6 cells were treated with doxorubicin and scFv-Fc, AVE1642 and anti-CD20 simultaneously or sequentially, and then cells were collected to analyze the intracellular fluorescence by flow cytometry. Compared to the control anti-CD20 antibody, combining scFv-Fc or AVE1642 with doxorubicin did not change doxorubicin accumulation (Figure 1.14).

Sequential treatment of doxorubicin followed by a low dose of AVE1642 significantly inhibited xenograft tumor growth and metastasis. Our *in vitro* data suggest that blockade of IGF-IR after doxorubicin was the most effective combination. Moreover, inhibition of IGF-IR prior to doxorubicin might interfere with the cytotoxic effects of topo II inhibitors. Since antibodies have a very long half-life *in vivo,* we next explored whether low doses of antibody would allow IGF-IR to recover over the course of a week. Mice bearing two xenograft tumors implanted in the mammary fat pad of opposite sides were studied. When xenograft tumors were formed, mice were treated with varying doses of AVE1642 by intraperitoneal injection. Tumors were removed from mice at 2 and 7 days after AVE1642 treatments. Figure 1.15 shows that all doses of AVE1642 suppressed IGF-IR levels at 2 days after treatment. Low doses of antibody (25 and 50 μg/mouse) allowed IGF-IR to recover to pre-treatment levels at 7 days. While some individual variation was seen at day 2 (200 μg dose), higher doses of antibody (100, 200 and 400 μg/mouse) suppressed IGF-IR levels for the entire 7 days.

Similar as AVE1642, IGF-IR level was recovered at the seventh day after one time scFv-Fc injection (data not shown).

Since pretreatment with scFv-Fc or EM164 which causes IGF-IR down-regulation protects the cell from the cytotoxicity of doxorubicin, it is important to give the second cycle treatment until the IGF-IR recovered. Figure 1.16 showed that the schedule of the *in vivo* experiment design. In addition, since low doses of AVE1642 suppressed IGF-IR expression immediately after dosing, but allowed recovery after 7 days, we used a low and high dose of antibody to model our *in vitro* findings showing that IGF-IR suppression at the time of doxorubicin was deleterious. After the first cycle of treatment, the low dose of AVE1642 in combination with doxorubicin is similar as the sequence doxorubicin followed by AVE1642 *in vitro*; the high dose of AVE1642 in combination with doxorubicin is similar as the sequence pretreatment with AVE1642 followed by doxorubicin *in vitro*. As shown in Figure 1.17, doxorubicin and AVE1642 alone had little effect on LCC6 xenograft growth. A low dose (50 μg/mouse) and a high dose (800 μg/mouse) of AVE1642 were used in combination with doxorubicin. This high dose of AVE1642 was based on our previously published results demonstrating tumor inhibition of MCF-7 cells[147]. Doxorubicin followed with the low dose of AVE1642 (four repeated weekly cycles) best resembled the *in vitro* optimal sequence and was most effective *in vivo*. Since high dose of AVE1642 (>100 μg/mouse) resulted in sustained IGF-IR downregulation, repeated 4 cycles of doxorubicin followed by 800 μg/mouse AVE1642 mimicked the *in vitro* sequence of antibody pretreatment followed by doxorubicin. This higher dose of antibody was superior to doxorubicin alone, but less effective than the low dose of AVE1642.

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It has been reported that IGF-IR is involved in invasion and metastasis in several

tumor types[8, 55, 148]. LCC6 has been reported to metastasize from the mammary fat

pad to the lungs of mice[55]. Next we investigated whether the sequential combination

doxorubicin with AVE1642 influenced lung metastasis *in vivo*. At the end of the

treatment period, lungs were harvested and examined by histological examination for

metastases. Figure 1.18A demonstrates representative histological specimens of the

treatment groups showing reduced size of pulmonary metastases in the doxorubicin plus

low dose AVE1642 treated animals. Figure 1.18B demonstrates that the numbers of

pulmonary metastases were also reduced in this treatment group.

Anti-IGF-IR antibodies commonly downregulate receptor levels and it is felt

that this is a common mechanism of action[94]. To determine if our dosing scheme

affected IGF-IR levels in a manner we expected, we examined tumor IGF-IR levels one

week after treatment. Figure 1.19 showed that IGF-IR levels were not affected in

control and doxorubicin treated animals; 50 μg AVE1642 only mildly inhibited the

IGF-IR levels 7 days after treatment and doxorubicin followed by 800 μg AVE1642

dramatically inhibited the IGF-IR for at least 7 days. In order to investigate the effects

of doxorubicin in combination with the AVE1642 on IGF-IR signaling *in vivo*, IGF-I

was injected to the mice 45 minutes before the mice were sacrificed. As shown in

Figure 1.19, seven day after the AVE1642 injection, 800 μg AVE1642 still inhibited the

IGF-I stimulated AKT activation, while 50 μg AVE1642 did not. The downstream

IGF1R signaling change is consistent with the IGF-IR level. Since the sequence of

doxorubicin followed 50 μg AVE1642 showed a better anti-tumor effect compared with

800 μg AVE1642, chronic downregulation of IGF1R by high-doses of antibody may not be the optimal strategy when combining with DNA damaging agents.

DISCUSSION

Several lines of experimental evidence validate IGF-IR signaling blockade as an important anticancer target that may prove clinically useful in combination with chemotherapy. Treatment with αIR3 has been shown to enhance the effect of chemotherapeutic agent doxorubicin and vincristine against Ewing's sarcoma cells[123]. An almost complete inhibition of non small lung cancer cells (A549) xenograft growth was observed when mice were treated with anti-IGF-IR antibody H7C10 combined with vinorelbine[99]. Another human anti-IGF-IR antibody CP-751,871 was shown to enhance the anti-tumor growth effect of chemotherapy in several different cell lines[98]. Hug *et. al* reported that tamoxifen (TAM) protected cells from the cytotoxicity of both chemotherapy agents 5-fluorouracil and doxorubicin[149]. Data from recent randomized trials also demonstrated a clinically antagonistic effect of TAM on concurrent cytotoxic chemotherapy and beneficial effect of sequential chemotherapy and TAM[133, 150]. Similarly, inhibition of HER2 by trastuzumab followed by paclitaxel increased resistance[134]. These preclinical and clinical studies demonstrated that sequencing need to be considered when combined the chemotherapy with target therapy. Several anti-IGF-IR antibodies are currently being evaluated in phase I, II or III clinical trials[138, 151]. Although it is possible that combination of anti-IGF-IR antibodies with cytotoxic chemotherapy might be more beneficial than single agents alone, it is also possible that disruption of IGF-IR signaling might

interfere with the cytotoxic effects. Thus, the optimal schedule for the combination therapy remains to be established.

Our study assesses the effect of two anti-IGF-IR antibodies, scFv-Fc and EM164 (humanized version, AVE1642) given concurrently or sequentially, in combination with several widely used cytotoxic drugs. Our study demonstrates several significant considerations for combination therapy. First, there are important sequence-dependent effects when combining some types of chemotherapy with anti-IGF-IR antibodies, with drug followed by anti-IGF-IR antibody as the best sequence. Second, low doses of antibody may be more effective by simulating this sequence dependent effect *in vivo*. Third, inhibition of IGF-IR prior to exposure to doxorubicin may interfere with the cytotoxic effects of the drug.

Doxorubicin exerts its effects on cancer cells via two different mechanisms: first, it intercalates between the bases of DNA and blocks DNA synthesis and transcription; second, it inhibits the activity of topoisomerase II and leads to breaks in the genomic DNA[152]. Topo II has two isoforms, IIα and IIβ, and the topo IIα exhibiting cell cycle regulation of its expression that is elevated during S phase and that peaks at G2/M phase[153]. Furthermore, tumor cell sensitivity to topo II inhibitors has been positively correlated with increased nuclear topo IIα levels[154, 155]. Our cell cycle analysis revealed that, when cells were treated with doxorubicin at sub-toxic concentration, most cells accumulated in the G_2-M phase reflecting doxorubicin's mechanism of action; in contrast, pretreatment with scFv-Fc and AVE1642 caused an increase in G_0/G_1 and S phase accumulation (Fig. 4). Since topo IIα expression is regulated by cell cycle and relates to drug sensitivity, we measured the topo IIα

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expression and activity of different groups. Our experiments demonstrated that pretreatment with anti-IGF1R antibodies decreased topo IIα mRNA, protein level and enzymatic activity, and decreased sensitivity to doxorubicin. In contrast, the sequence doxorubicin followed by AVE1642 increased the topo II activity and was associated with increased sensitivity of doxorubicin (Fig.5). The sequence of pretreatment with anti-IGF-IR antibodies resulted in fewer cells in G2/M phase compared with other sequences, consistent with the reduction in topo IIα levels. Son *et. al.* showed that the activity of topo II was reduced in a doxorubicin-resistant human stomach cancer cell lines[156]. Another report showed that cytotoxicity of doxorubicin was inhibited by anti-ErbB2 antibody trastuzumab and caused by a decrease of topo II protein and activity[157]. These data underscore the correlation of topo II protein level with doxorubicin sensitivity; increases in topo IIα expression is associated with sensitivity to the doxorubicin presumably due to increased target on which the drug acts. Although, a direct mechanism between IGF signaling and topo II has not been demonstrated, our results suggest that alteration in topo II activity may be one possible mechanism to explain the sequence-dependent effect in combination of anti-IGF1R antibody with doxorubicin.

Down-regulation of IGF-IR expression by monoclonal antibodies has been shown *in vitro* and *in vivo*[147]. Our *in vitro* data suggested that down-regulation of the receptor before chemotherapy would attenuate the cytotoxicity of doxorubicin. Extending these findings to an *in vivo* mouse model, LCC6 xenograft growth was significantly inhibited with doxorubicin followed by AVE1642 compared with either treatment alone (Fig. 6B). As predicted by the *in vitro* data, a lower dose of AVE1642

was more effective in combination with doxorubicin than the higher dose. Our data show that transient downregulation of receptor by the low dose of antibody was associated with a more substantial tumor inhibition in combination with doxorubicin. Previous data from our laboratory showed that inhibition of IGF-IR may prevent metastases to lung in LCC6 cells xenograft tumor. Here, we tested effectiveness of combined therapy against lung metastasis. Similar to the growth of the xenograft tumor, the best results were obtained with doxorubicin followed by transient downregulation of IGF-IR. One hypothesis to explain the lack of benefit for the sustained downregulation of receptor might be the decreased topoisomerase II protein and activity *in vivo* seen in these experimental conditions. Our data show that sustained downregulation of IGF-IR impairs sensitivity of doxorubicin by lowering topo IIα level. The status of topoismoerase II in combination therapy also needs further in-depth investigation. It is noteworthy that not all chemotherapies show this sequence dependency. Furthermore, it is uncertain if cells dependent on IGF-IR for proliferation would also have similar sequence-dependent effects. Cohen at al. demonstrated that low doses of a monoclonal antibody were ineffective at suppressing growth of a mouse fibroblast cell line transfected with IGF-IR[98]. These data have been taken to suggest that continuous suppression of IGF-IR is necessary for single agent activity, but lower doses of IGF-IR inhibitors with chemotherapy have not been studied in IGF-IR-dependent cells. Further *in vivo* modeling of these sequence effects with additional classes of chemotherapy and cell lines need to be pursued.

In summary, our studies suggest that scFv-Fc and AVE1642 exhibits marked sequence-dependent synergistic effects when administered after doxorubicin against

LCC6 human breast cancer cell lines *in vitro* and *in vivo,* which provides a rationale for designing future clinical trial of this combination. Several anti-IGF-IR antibodies including AVE1642 is being evaluated in phase I /II clinical trial. However, almost all the trials were done by administering the antibody and chemotherapeutic drugs simultaneously. Our results demonstrated drug followed the antibody is the optimal sequence, and a low dose of antibody may be a better choice for combination therapy. Therefore, this study suggest that careful consideration of sequencing and dose is necessary when combine the chemotherapy with anti-IGF-IR antibody.
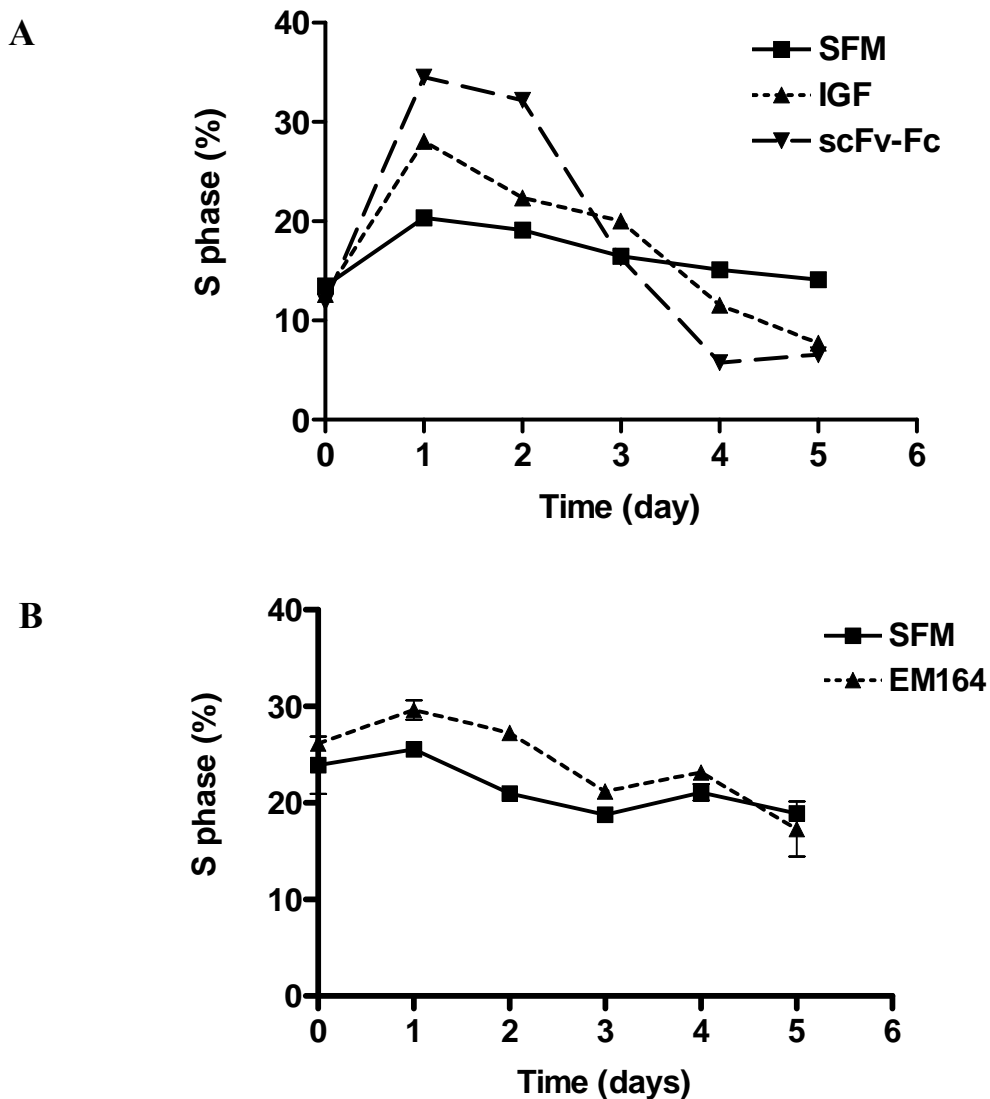
Figure 1.1
scFv-Fc, but not EM164 stimulated MCF-7 cell entry into S phase of the cell cycle as IGF-I.
Cells were plated, serum-starved for 24 hours, and treated with 5 nM IGF, 30 µg/ml scFv-Fc (A), 15 µg/ml EM164 (B) for different days. Cells were collected at different time point and stained with propidium iodide, and then cells were analyzed by flow cytometry.
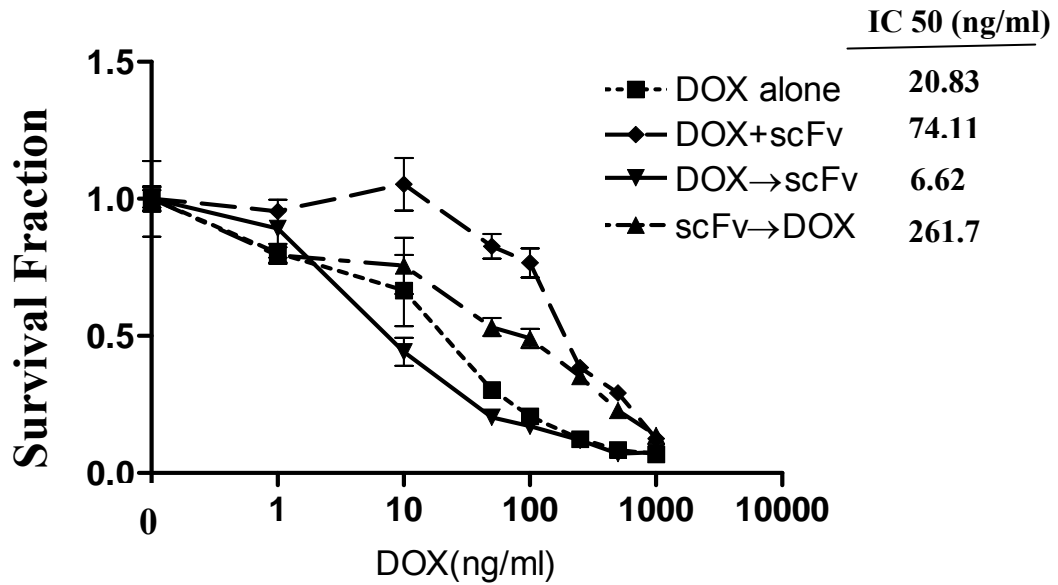
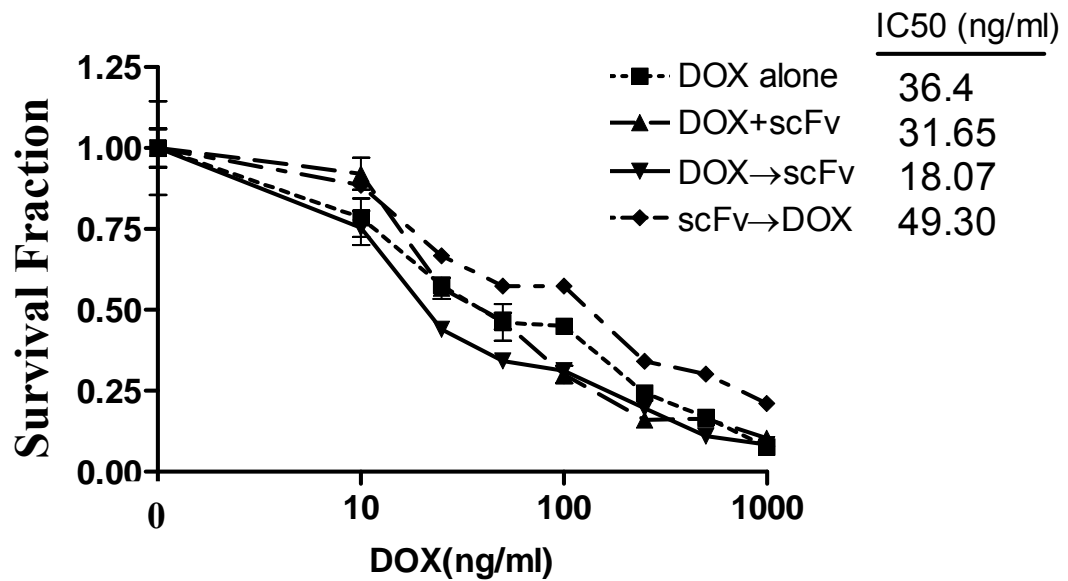|	IC 50 (ng/ml)
DOX alone	20.83
DOX+scFv	74.11
DOX→scFv	6.62
scFv→DOX	261.7

Figure 1.2A
In the presence of IGF, doxorubicin followed by scFv-Fc inhibited cell proliferation in MCF-7 cells.
MCF-7 cells were plated, and, after 24hours, serum-starved overnight, stimulated with 5 nM IGF-I. MCF-7 cell were treated with different concentration of doxorubicin (DOX) , scFv-Fc (scFv, 30 μg/ml) simultaneously or sequentially . MTT assay performed as an indicator of cell numbers.

Figure 1.2B
In the presence of 1%FBS, doxorubicin followed by scFv-Fc inhibited cell proliferation in MCF-7 cells.
MCF-7 cells were plated, and, after 24hours, serum-starved overnight. MCF-7 cell were treated with different concentration of doxorubicin (DOX) , scFv-Fc (scFv, 30 μg/ml) simultaneously or sequentially in 1% FBS condition. MTT assay performed as an indicator of cell numbers.
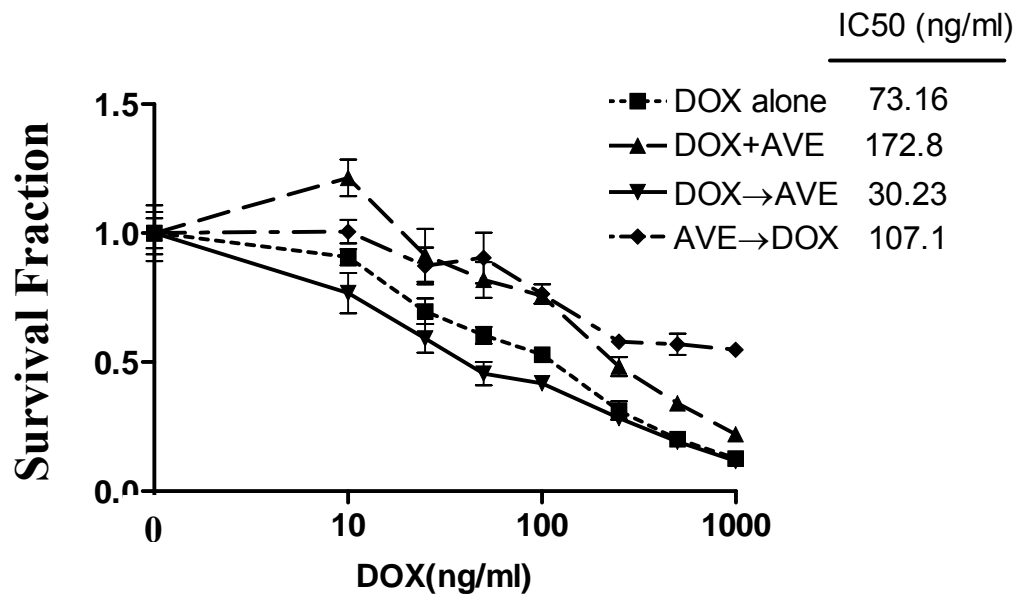
Figure 1.3A
In presence of IGF, doxorubicin followed by AVE1642 inhibited cell proliferation in MCF-7 cells.
MCF-7 cells were plated, and, after 24hours, serum-starved overnight. MCF-7 cell were were treated with different concentration of doxorubicin (DOX) , AVE1642 (AVE, 15 μg/ml) simultaneously or sequentially with 5nM IGF-I stimulation. MTT assay performed as an indicator of cell numbers.
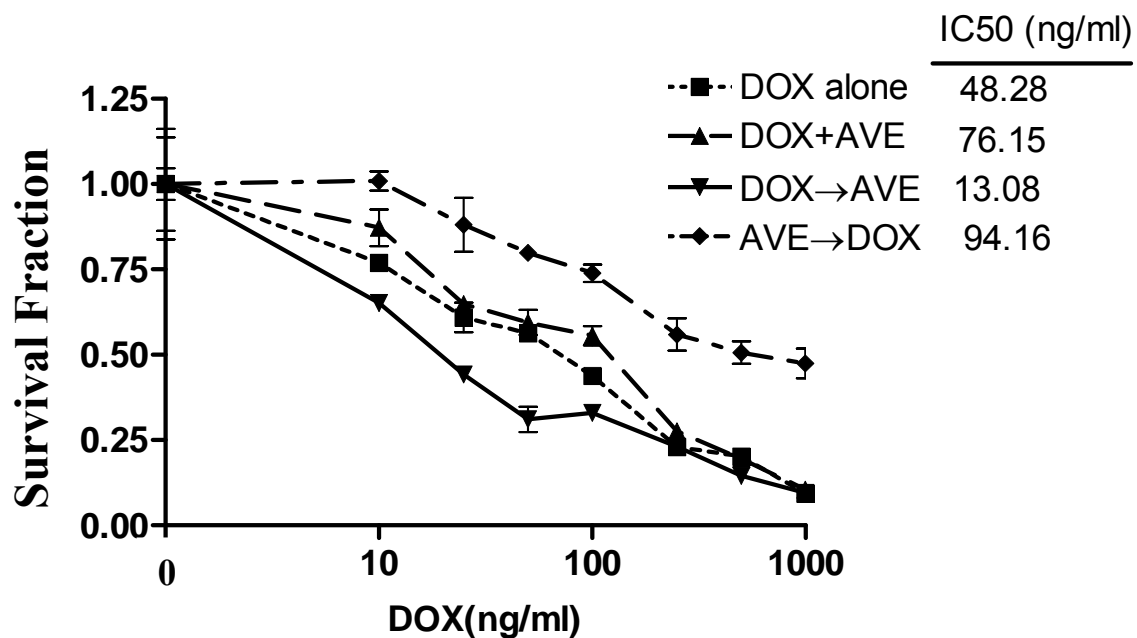
Figure 1.3B
In the presence of 1%FBS, doxorubicin followed by AVE1642 inhibited cell proliferation in MCF-7 cells.
MCF-7 cells were plated, and, after 24hours, serum-starved overnight. MCF-7 cell were were treated with different concentration of doxorubicin (DOX) , AVE1642 (AVE, 15 µg/ml) simultaneously or sequentially in 1% FBS condition. MTT assay performed as an indicator of cell numbers.
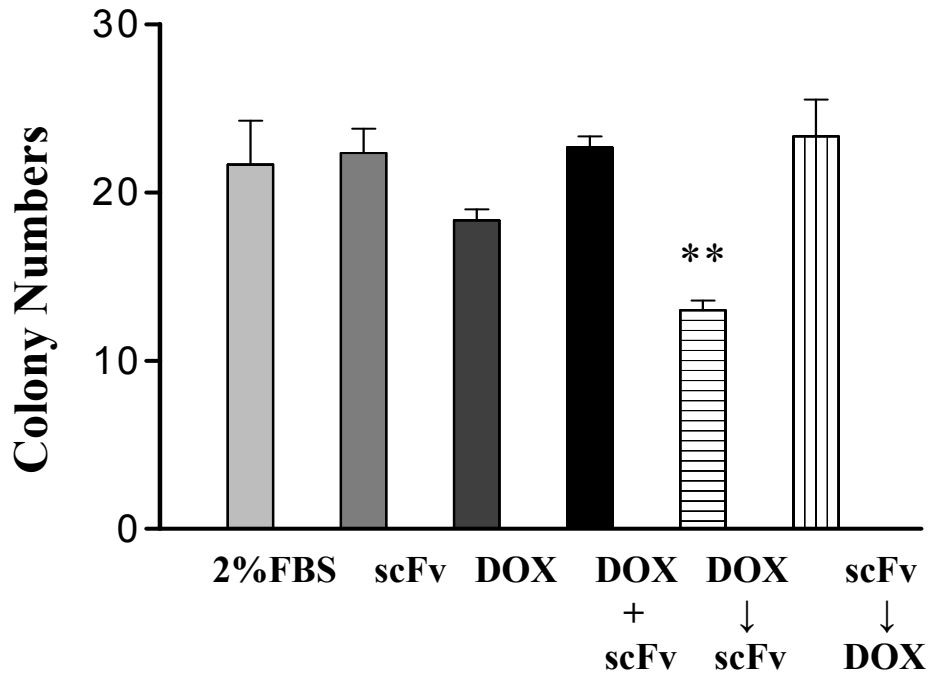
Figure 1.4A
Doxorubicin followed by scFv-Fc significantly inhibited the anchorage-independent growth in LCC6 cells.
LCC6 cells treated with or without antibodies scFv-Fc (scFv) and Doxorubicin in the presence of 2% FBS were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates and incubate for 24 hours. If a second treatment was needed (shown by the arrow), it was added as a solution to the top agar with continued incubation for 10-14 days and the number of colonies formed counted. ** P<0.01 vs DOX alone.
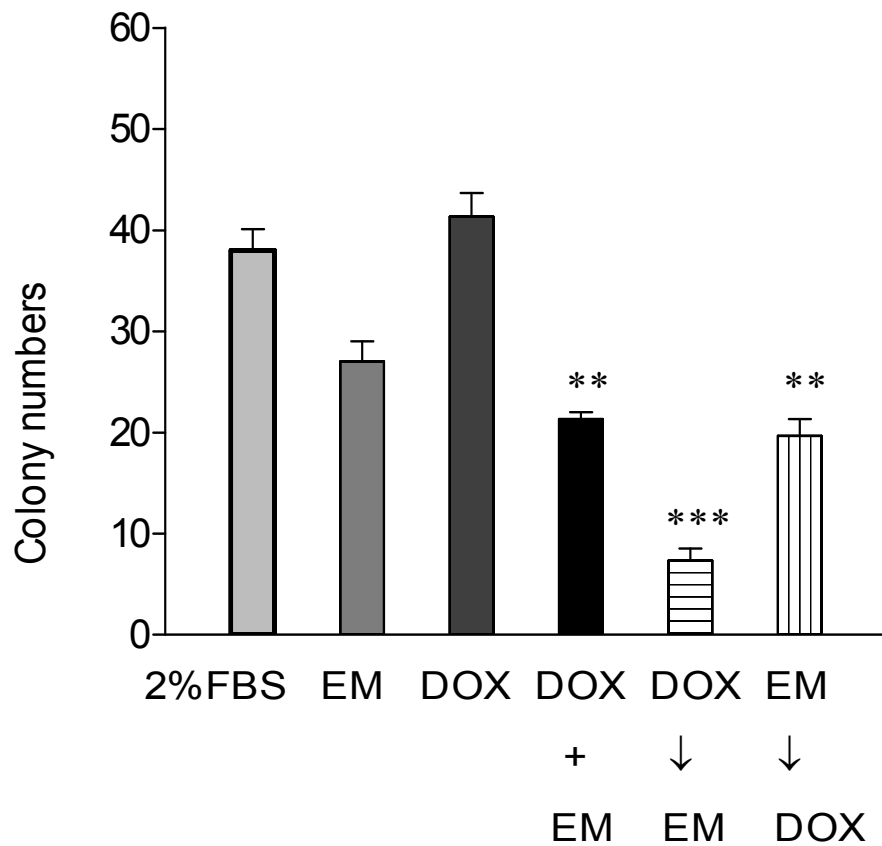
Figure 1.4B
Doxorubicin followed by EM164 significantly inhibited the anchorage-independent growth in LCC6 cells.
LCC6 cells treated with or without antibodies EM164 (EM, 20µg/ml) and Doxorubicin (DOX, 100 ng/ml) in the presence of 2% FBS were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates and incubate for 24 hours. If a second treatment was needed (shown by the arrow), it was added as a solution to the top agar with continued incubation for 10-14 days and the number of colonies formed counted. ** $P<0.01$ vs DOX alone; *** $P<0.001$ vs DOX alone.
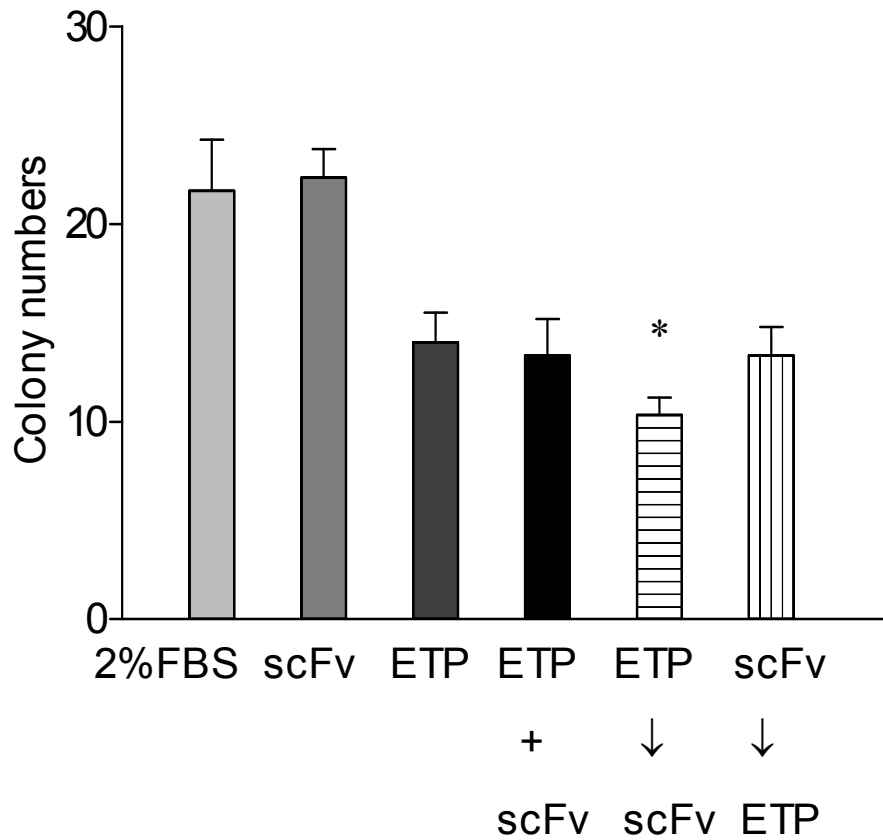
Figure 1.5A
Etoposide followed by scFv-Fc significantly inhibited the anchorage-independent growth of LCC6 cells.
LCC6 cells treated with or without antibodies scFv-Fc (scFv, 30μg/ml) and Etoposide (ETP, 1μm) in the presence of 2% FBS were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates and incubate for 24 hours. If a second treatment was needed (shown by the arrow), it was added as a solution to the top agar with continued incubation for 10-14 days and the number of colonies formed counted. *P<0.05 vs ETP alone.

Figure 1.5B
Etoposide followed by EM164 significantly inhibited the anchorage-independent growth of LCC6 cells.
LCC6 cells treated with or without antibodies EM164 (EM, 20μg/ml) and etoposide (ETP, 1μM) in the presence of 2% FBS were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates and incubate for 24 hours. If a second treatment was needed (shown by the arrow), it was added as a solution to the top agar with continued incubation for 10-14 days and the number of colonies formed counted. ** P<0.01 vs ETP alone.
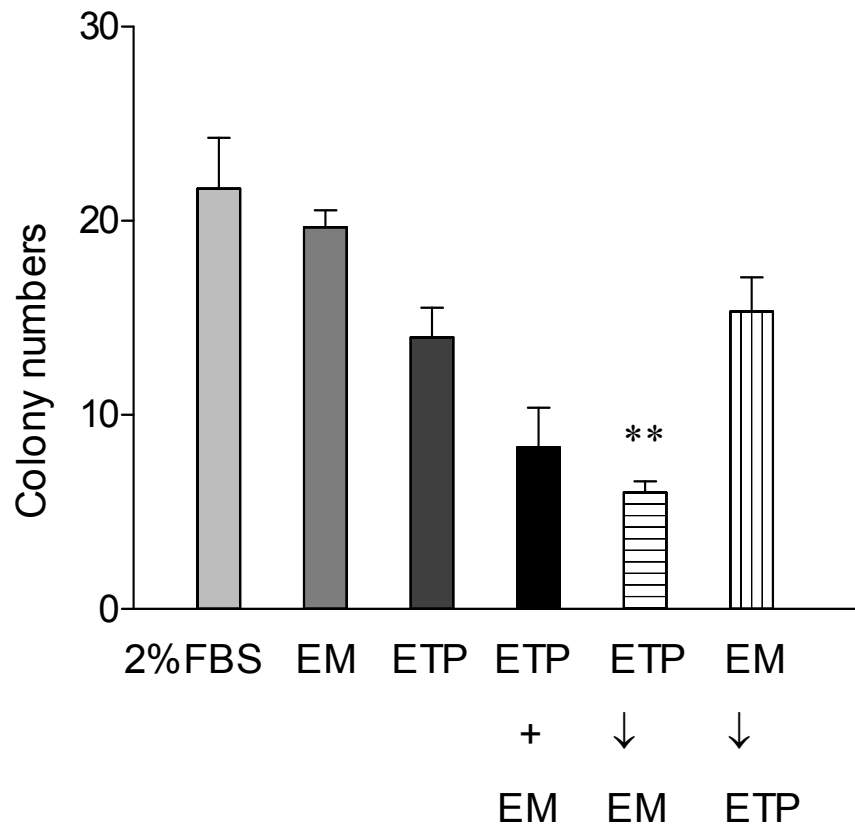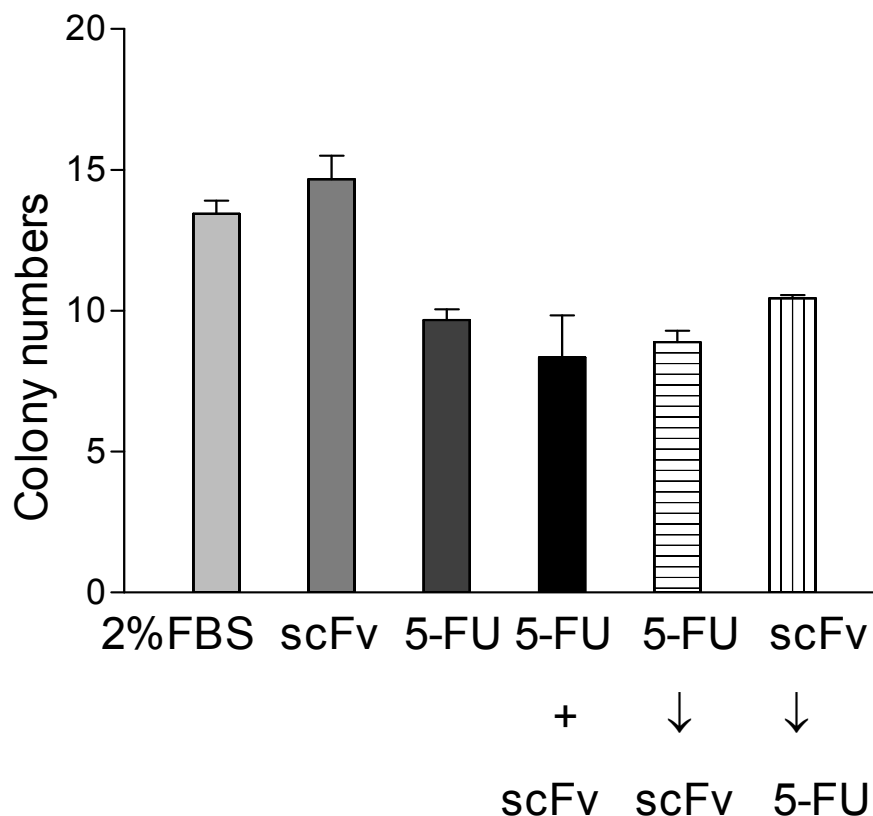
Figure 1.6A
Sequentially combining 5-FU with scFv-Fc did not enhance the inhibition effects of the anchorage-independent growth of LCC6 cells.
LCC6 cells treated with or without antibodies scFv-Fc (scFv, 30μg/ml) and 5-fluorouracil (5-FU, 2.5 μM) in the presence of 2% FBS were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates and incubate for 24 hours. If a second treatment was needed (shown by the arrow), it was added as a solution to the top agar with continued incubation for 10-14 days and the number of colonies formed counted. Error bars represent standard error of the mean.

Figure 1.6B
Sequentially combining 5-FU with EM164 did not enhance the inhibition effects of the anchorage-independent growth of LCC6 cells.
LCC6 cells treated with or without antibodies EM164 (EM, 15μg/ml) and 5-fluorouracil (5-FU, 2.5μM) in the presence of 2% FBS were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates and incubate for 24 hours. If a second treatment was needed (shown by the arrow), it was added as a solution to the top agar with continued incubation for 10-14 days and the number of colonies formed counted. Error bars represent standard error of the mean. * P<0.05 vs 5-FU alone.
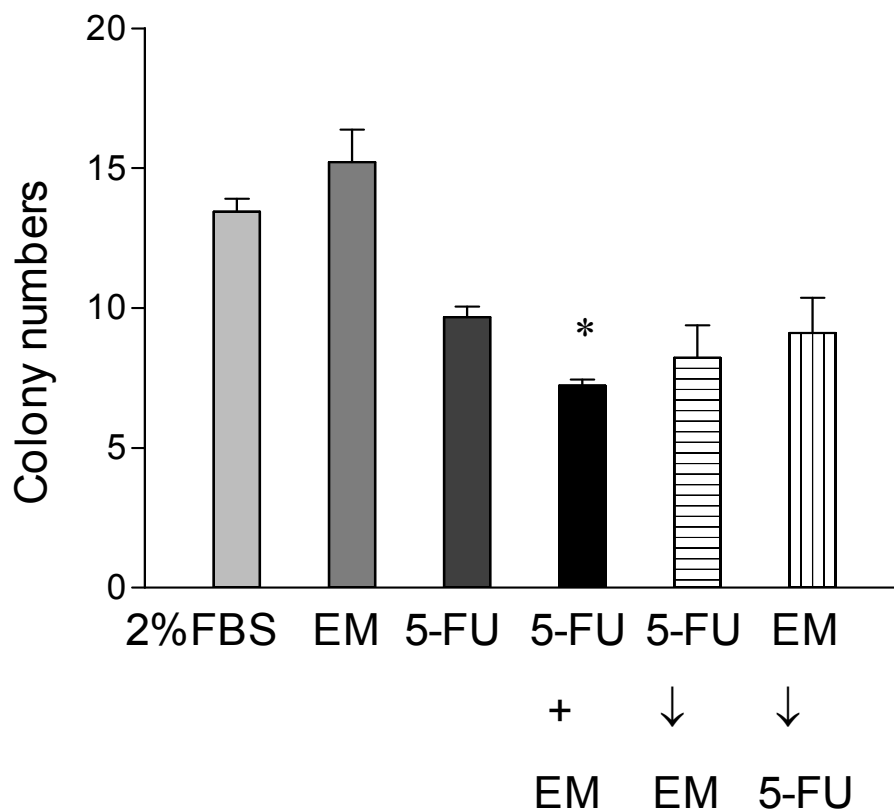
Figure 1.7
Doxorubicin followed by scFv-Fc or AVE1642 significantly increased PARP cleavage.
Serum starved LCC6 cells were treated with 1%FBS (control), doxorubicin, scFv-Fc, AVE1642 or antibody in combination with DOX simultaneously or sequentially for 48 hours totally. PARP cleavage was determined by Western blot analysis of whole cell lysates. Actin signaling was included as loading controls.
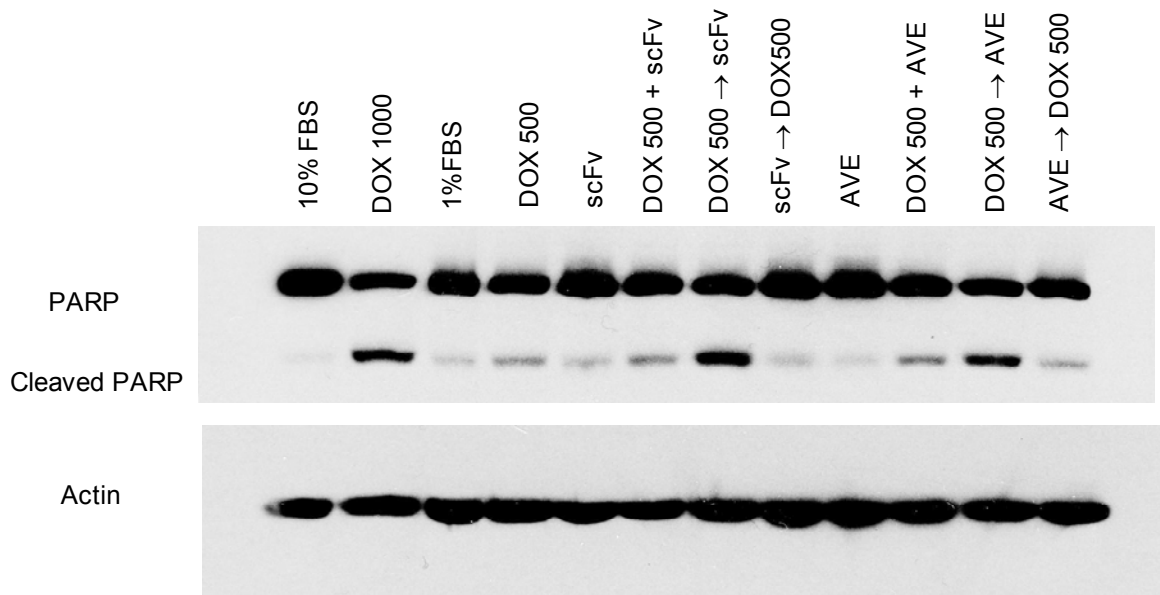
Figure 1.8
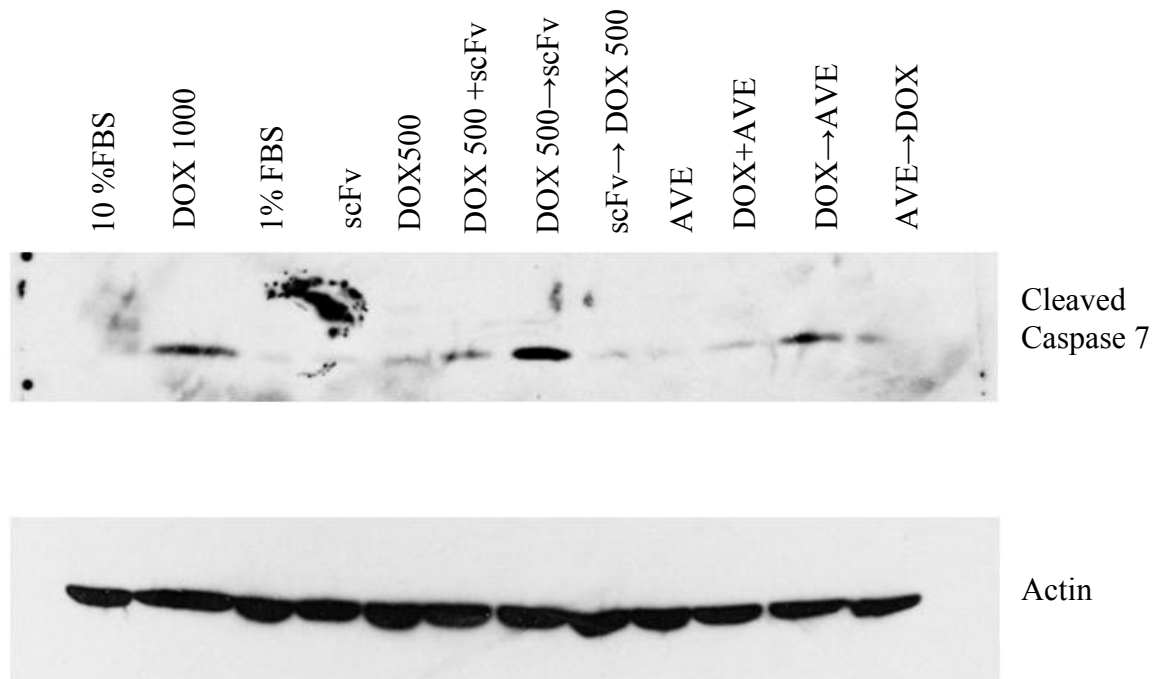**Doxorubicin followed by scFv-Fc or AVE1642 significantly increased caspase 7 cleavage.**
Serum starved LCC6 cells were treated with 1%FBS (control), doxorubicin, scFv-Fc, AVE1642 or antibody in combination with DOX simultaneously or sequentially for 48 hours totally. Cleaved caspase 7 was determined by Western blot analysis of whole cell lysates. Actin signaling was included as controls.
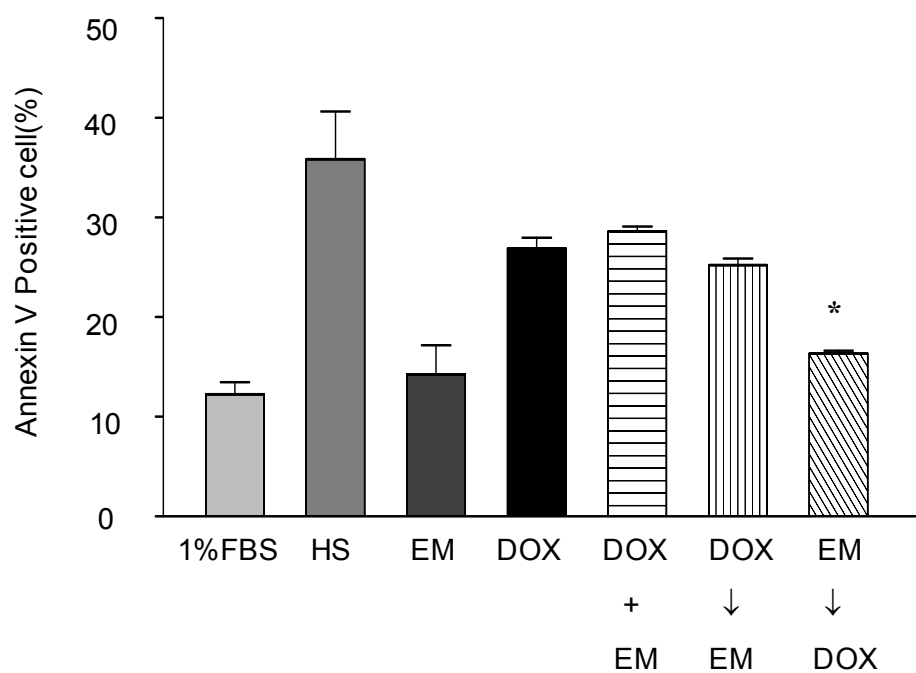
Figure 1.9
Pretreatment of EM164 rendered LCC6 cells resistant to doxorubicin induced apoptosis.
LCC6 cells were treated with different compounds alone or in combination with DOX simultaneously or sequentially, and then cell were collected and stained with APC– Annexin V and 7-AAD and analyzed by flow cytometry. The experiment was repeated three times with similar results; a representative experiment is shown for cells of apoptosis (percentage of all cells that were apoptotic). * P< 0.05 vs DOX alone. Heat shocked cell is the positive control.

Figure 1.10A
Pretreatment with scFv-Fc followed by doxorubicin prevented G2-M phase arrest in LCC6 cells.
Cells were plated, serum-starved for 24 hours. The cells were treated with 100 ng/ml doxorubicin (DOX), 30 µg/ml scFv-Fc (scFv) , or scFv in combination with DOX simultaneously or sequentially for 48 hours as described under Material and Methods, and then cells were stained with propidium iodide and analyzed by flow cytometry. The experiment was repeated three times with similar results, and a representative experiment is shown.
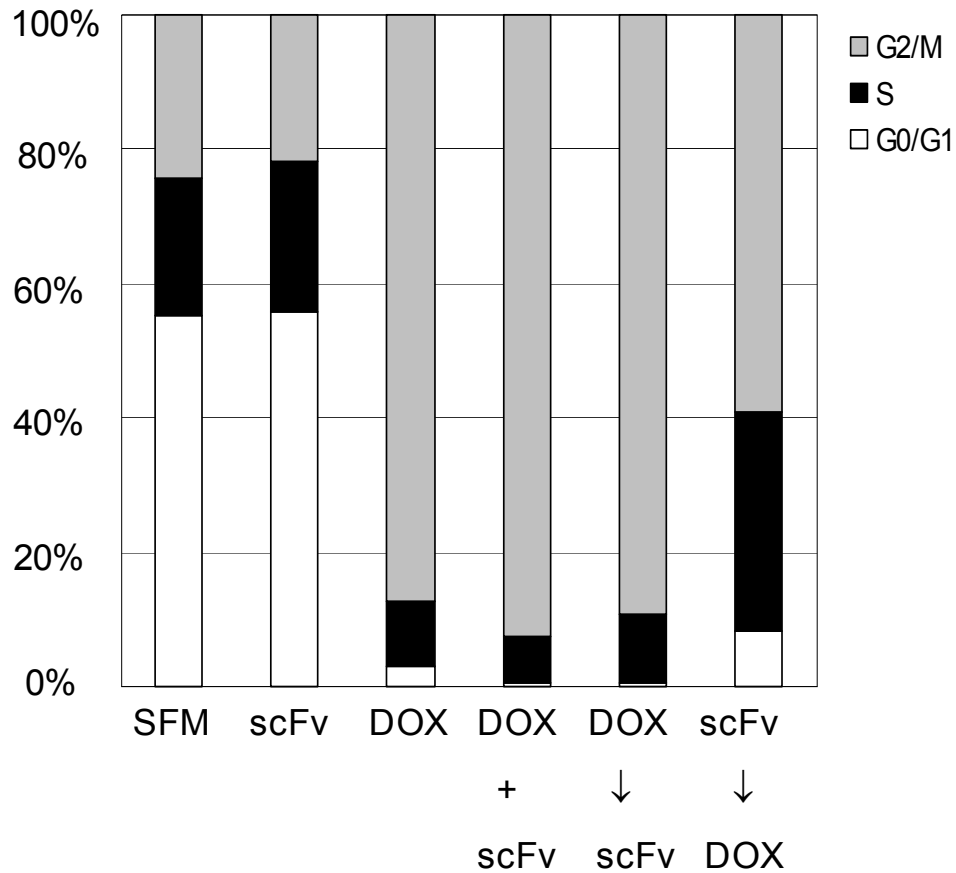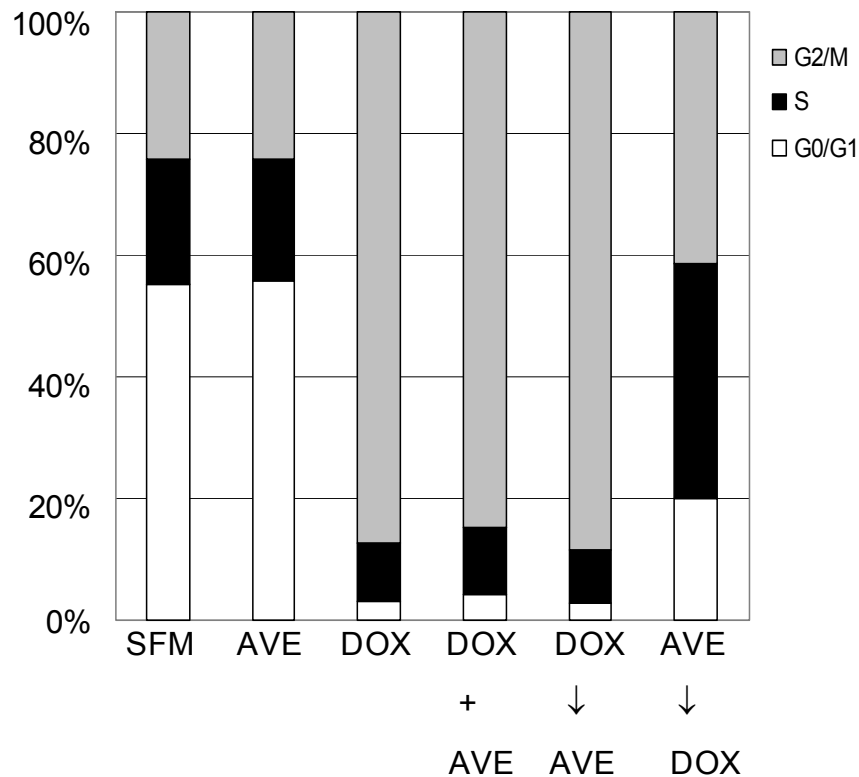
Figure 1.10B
Pretreatment with AVE1642 followed by doxorubicin prevented G2-M phase arrest in LCC6 cells.
Cells were plated, serum-starved for 24 hours. The cells were treated with 100 ng/ml doxorubicin (DOX), 20 µg/ml AVE1642 (AVE), or AVE in combination with DOX simultaneously or sequentially for 48 hours as described under Material and Methods, and then cells were stained with propidium iodide and analyzed by flow cytometry. The experiment was repeated three times with similar results, and a representative experiment is shown.
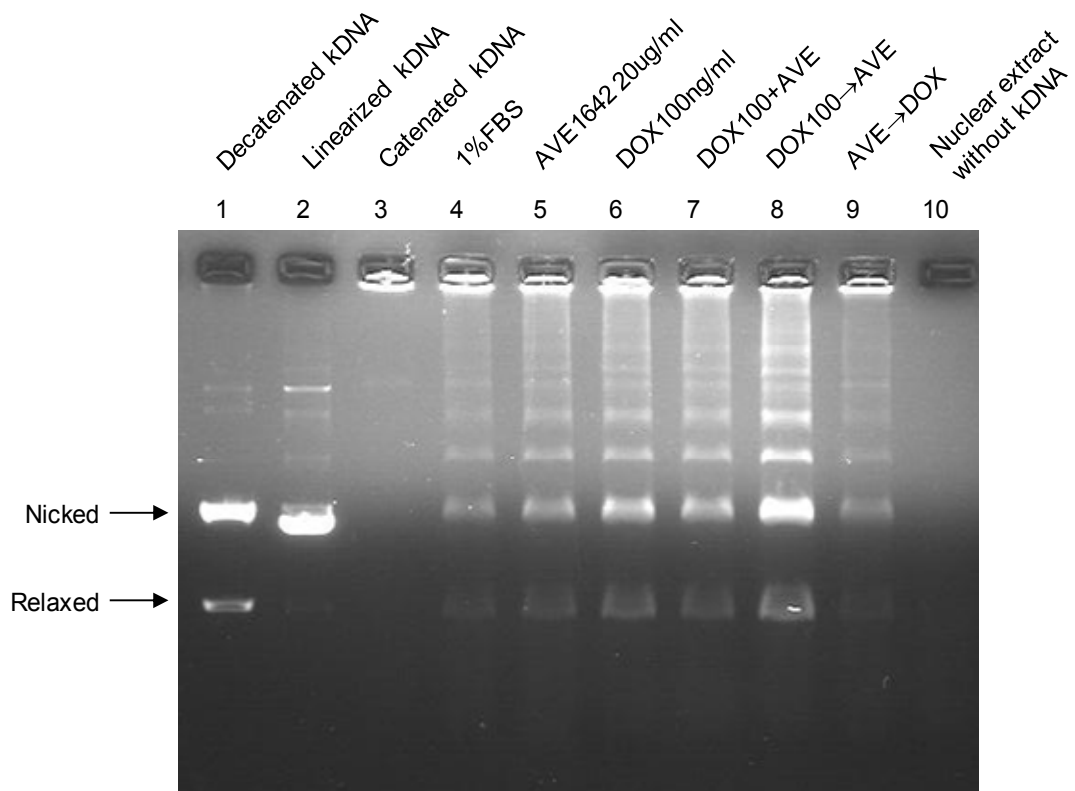
Figure 1.11
Pretreatment of AVE1642 followed by doxorubicin decreased topoisomerase II activity in LCC6 cells.
Decatenation assay. LCC6 cells were starved in serum-free media for 24 hours and then treated with AVE1642 (20µg/ml), DOX (100ng/ml) alone or in combination simultaneously or sequentially for 48 hours. Nuclear extracts were incubated with kinetoplast DNA at 37°C for 15 min. After gel electrophoresis in 1% agarose, the decatenated products containing nicked circular and relaxed circular DNA were determined.
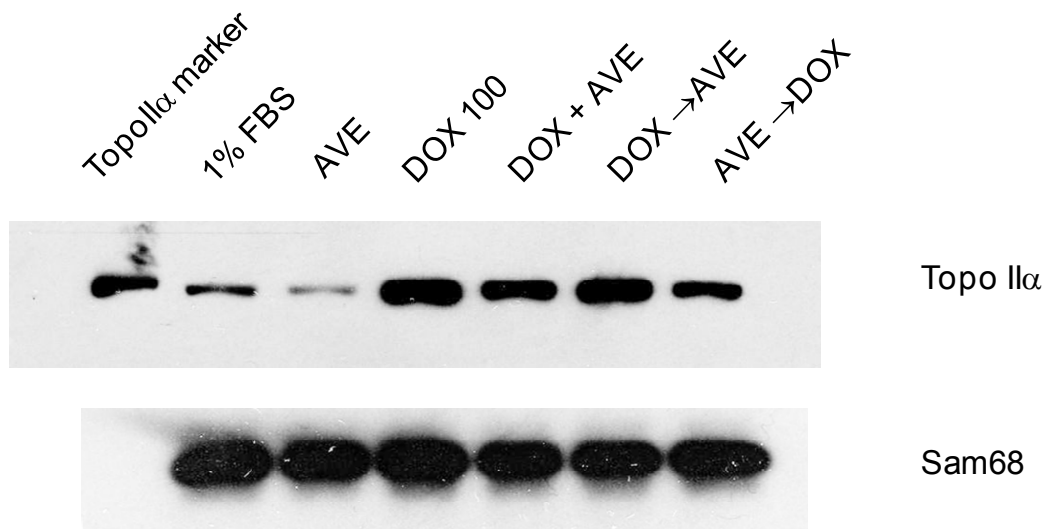
Figure 1.12
Pretreatment of AVE1642 followed by doxorubicin decreased topoisomerase II level in LCC6 cells.
Nuclear extracts were prepared for LCC6 cell treated with AVE1642 (20μg/ml), DOX (100ng/ml) alone or in combination simultaneously or sequentially for 48 hours. After quantification, 50 μg of nuclear extract were immunoblotted for topoIIα with purified topoIIα as control. Sam 68 is the loading control.
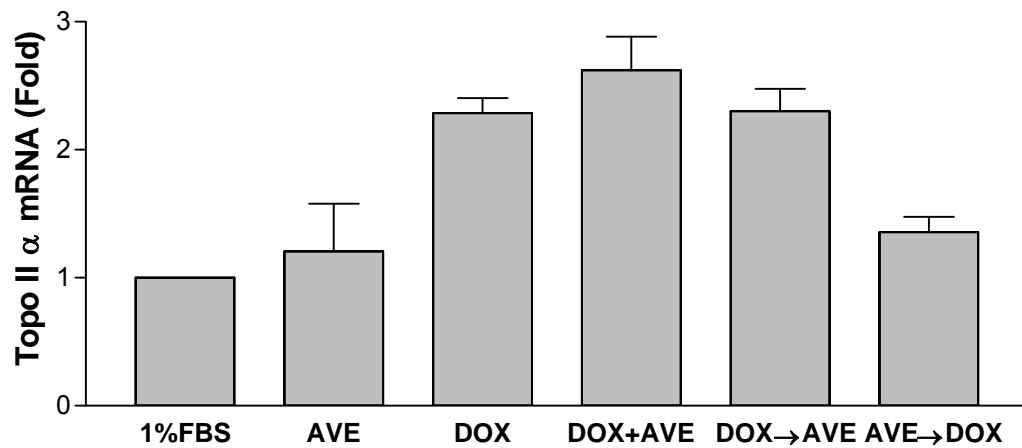
Figure 1.13
AVE1642 followed by Doxorubicin decreased the topoisomerase IIα mRNA level in LCC6 cells.
Serum starved cells were treated with AVE1642 (AVE, 20 μg/ml), Doxorubicin (DOX, 100 ng/ml) and DOX in combination with AVE simultaneously or sequentially for 48 hours. Total RNA was purified and qRT-PCR was performed using Topo IIα specific primers. To adjust for variations in starting template, the gene expression was normalized with an internal reference gene (β-actin).
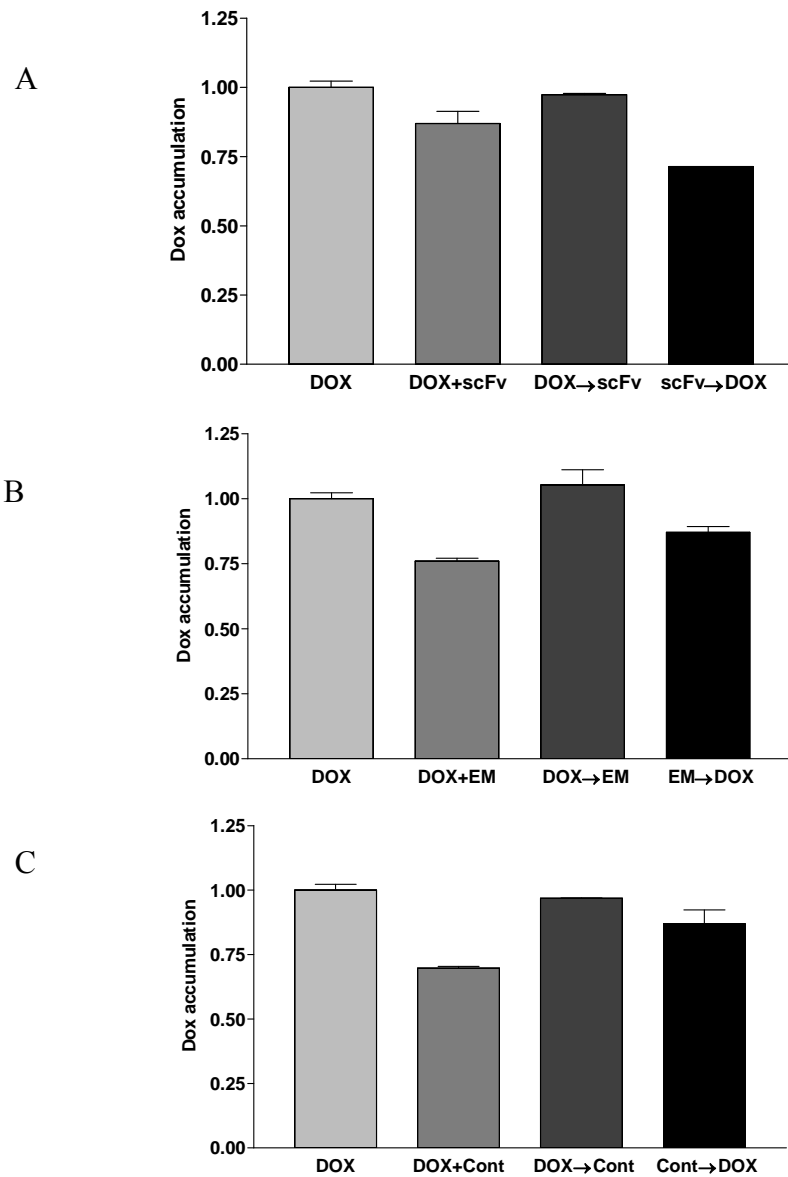
Figure 1.14
Sequencing of anti-IGF1R antibodies with doxorubicin did not affect doxorubicin accumulation in LCC6 cells.
Serum starved LCC6 cells were treated with doxorubicin (100ng/ml), scFv-Fc (30μg/ml)(A), EM164 (20μg/ml) (B) , anti-CD20 (20μg/ml, control antibody) (C) or DOX in combination with antibodies simultaneously or sequentially for 48hours. At end of incubation, cells were washed 3times with PBS and then observed by flow cytometry. Results are representative for 3 similar experiments.
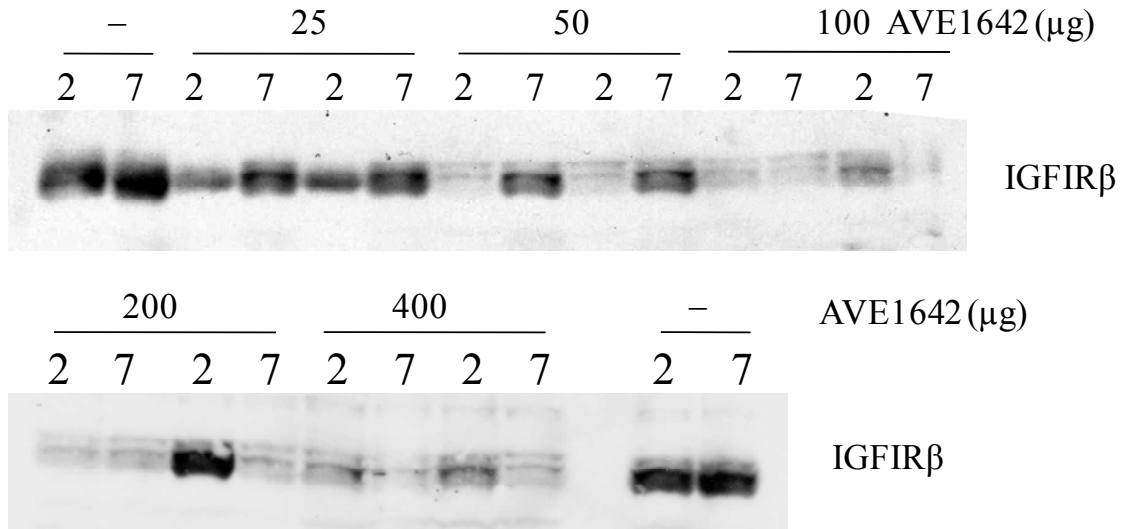
Figure 1.15
AVE1642 dose dependently decreased the IGF-IR levels *in vivo*.
Female athymic mice were injected with LCC6 cells in opposite mammary fat pads to
form xenograft tumors. When tumors had reached a volume of 80-100 mm^3, mice were
treated with AVE1642. Tumors were dissected 2 and 7 days after AVE1642 treatment.
A total of 100 µg of tumor extracts were immunoblotted for total IGF-1R$_ß$ levels.

Experiment Design *in vivo*
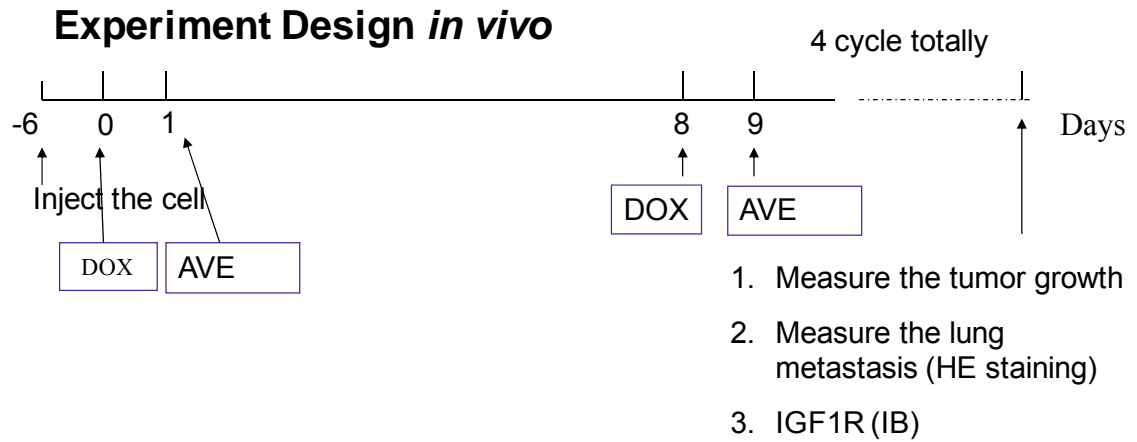
Figure 1.16
The schematic paradigm of sequencing of doxorubicin and AVE1642 in LCC6 mouse xenograft model.
The abbreviations used are: DOX, Doxorubicin (3mg/kg); AVE, AVE1642 (50, 800 µg/ml).
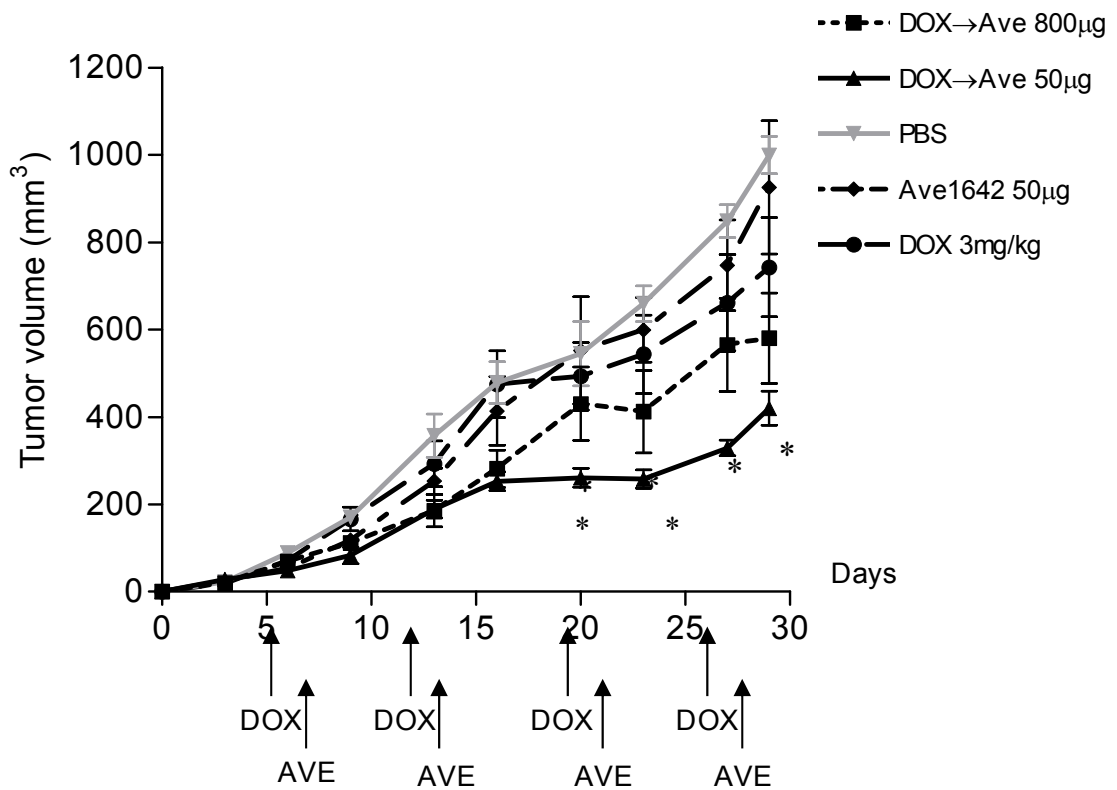
Figure 1.17
Sequential treatment of doxorubicin followed by a low dose of AVE1642 significantly inhibited mice xenografts growth.
Established LCC6 tumor xenografts (80 ~100 mm^3) were treated with PBS, DOX (3 mg/kg/week each, i.p.), AVE1642 (50 µg/mouse/week, i.p.), DOX 24 hours followed by AVE1642 50 µg or DOX 24 hours followed by AVE1642 800 µg four times at a 7 day interval. Tumors were measured every 3 days and shown as tumor volume calculated using the formula length ×width^2/2. Results are presented as mean ± SE.

66

A

PBS   AVE1642   DOX

DOX→AVE 50   DOX→AVE 800

B
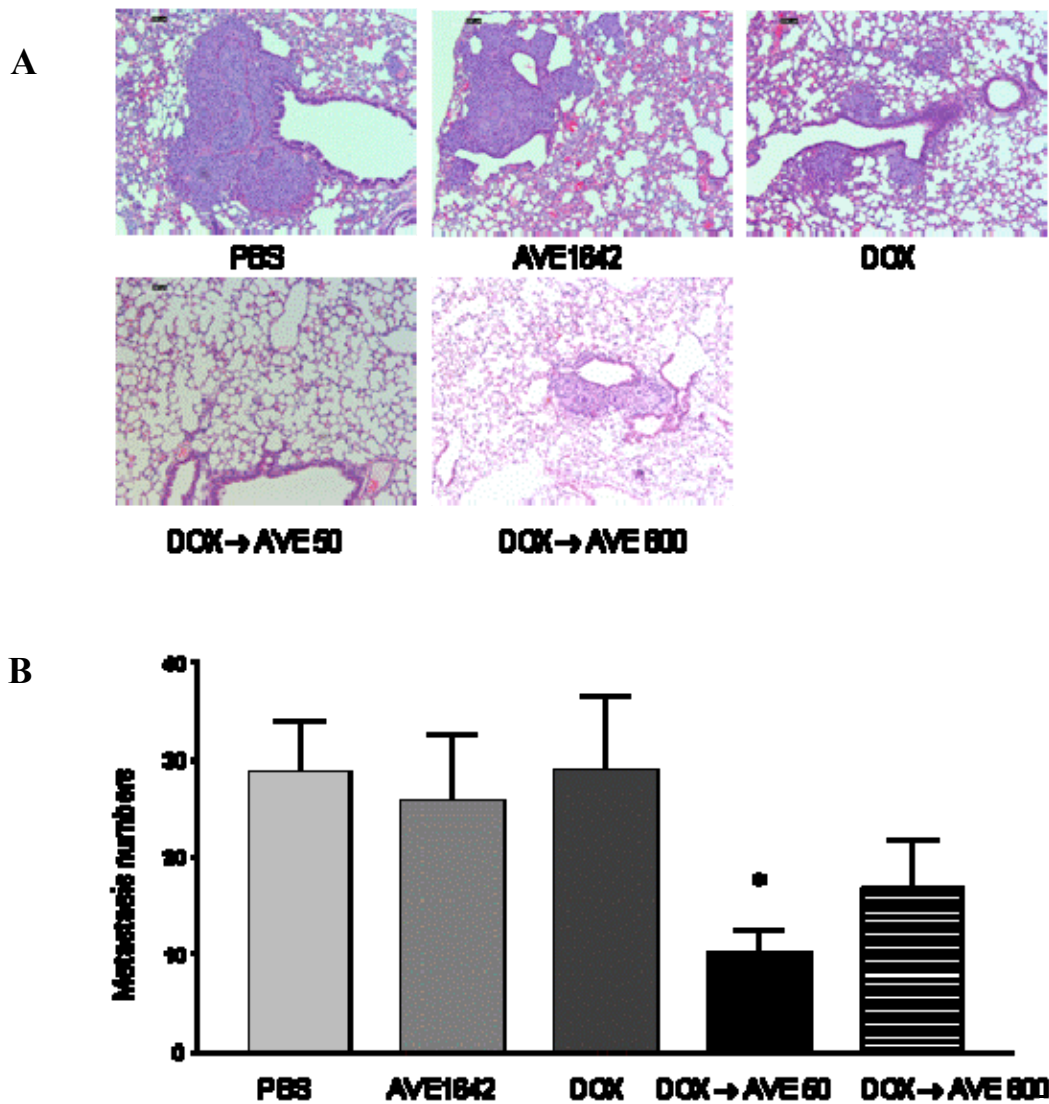
Figure 1.18
Sequential treatment of doxorubicin followed by a low dose of AVE1642
significantly inhibited lung metastases.
Established LCC6 xenografts (80 ~100 mm^3) were treated with PBS, DOX (3
mg/kg/week each, i.p.), AVE1642 (50 µg/mouse/week, i.p.), DOX 24 hours followed
by AVE1642 50 µg or DOX 24 hours followed by AVE1642 800 µg four times at a 7
day interval. 24 hours after the final treatment with AVE1642 and doxorubicin, mice
were sacrificed. The lung of each mouse was fixed, embedded, sectioned, and stained
with hematoxylin and eosin, and examined microscopically for metastatic deposits. A
representative view was shown for each treatment, ×200 magnification. D, Analysis of
lung micrometastatic deposits. Results are presented as mean ± S.E. (n=5) *P<0.05 *vs*
DOX alone.

67
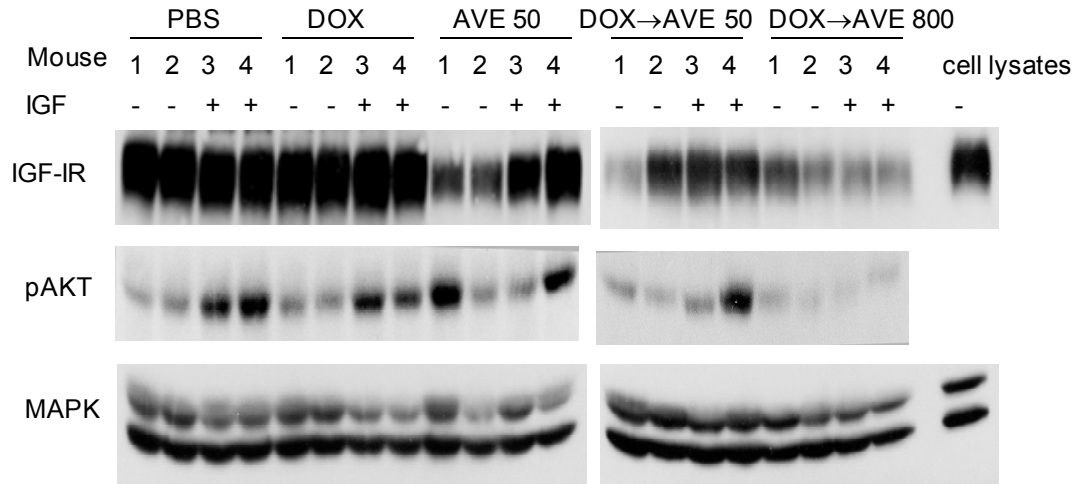
Figure 1.19
Doxorubicin followed by a low dose of AVE1642 did not affect the IGF-1R level and IGF-I signaling in LCC6 mouse xenograft model.
Seven days after the final treatment, mice were sacrificed, and tumor samples were frozen in liquid nitrogen and homogenized in TNESV buffer. A total of 100 μg of tumor extract and 40 μg of LCC6 cell lysates were immunoblotted for IGF-IRα, and MAPK.

CHAPTER 2

PQIP, a New IGF-IR Tyrosine Kinase Inhibitor Inhibits Cell Proliferation and Motility and Enhances the Cytotoxicity of Doxorubicin in Human Cancer Cells

INTRODUCTION

Signals from a variety of growth factors are required for tumorigenesis and cancer development in human malignancies, including breast cancers[158]. Receptor tyrosine kinases play key roles in cell proliferation, differentiation and cell apoptosis, and increased expression and dysregulation of receptor tyrosine kinases have been observed in various types of cancer. Therefore, receptor tyrosine kinases are attractive therapeutic targets, which can be blocked by small-molecule tyrosine kinase inhibitors or monoclonal antibodies. One of the potential targets is the type I insulin-like growth factor receptor (IGF-IR).

The insulin-like growth factor (IGF) system contains two receptor tyrosine kinases that are involved in mediating mitogenic signaling. IGF-IR is a heterotetrameric transmembrane receptor tyrosine kinase that is widely expressed in human tissues and is involved in embryonic development and postnatal growth[18]. The insulin receptor (IR) is also a key component of the IGF signaling pathway. Two IR isoforms exist, including IR-A and IR-B[159]. In addition to binding insulin, IR-A binds IGF-II, initiating mitogenic signaling [15]. Activation of IGF-IR and IR is also regulated by the IGF binding proteins (IGFBP) and the type II IGF receptor (IGF-IIR), which lacks tyrosine kinase activity. Both IGF-IR and IR consist of two α and two β subunits. Binding of the ligands IGF-I or IGF-II to IGF-IR or binding insulin or IGF-II to IR causes receptor auto-transphosphorylation and activates multiple signaling pathways, including the mitogen activated protein kinase (MAPK) and phosphatidylinositidie 3-kinase PI3K/AKT pathways[160, 161].

Activation of IGF-IR has been reported to stimulate proliferation, survival, transformation, metastasis, and angiogenesis[162-164]. IGF signaling also seems to play a vital role in the development of resistance to clinically useful anticancer therapy, including hormonal therapy, radiation therapy and chemotherapy[120, 165-168]. Thus, inhibition of IGF-IR by various strategies has been developed, including antisense oligodeoxynucleotides, anti-IGF-IR antibodies, dominant-negative IGF-IR, and small molecule inhibitors. These approaches have been shown to inhibit cell proliferation, induce cell apoptosis and reduce tumor xenograft growth in several types of cancer.

Tyrosine kinases include receptor protein kinase and nonreceptor protein kinase. They are crucial in mediating cell signaling pathways related to cell proliferation, differentiation, migration, angiogenesis, cell-cycle regulation and others. ([169-171]. Small-molecule inhibitors directly target the catalytic activity of the by interfering with the binding of ATP or substrates. Imatinib mesylate (Gleevec), which is the first successful small-molecule inhibitors, inactivates the kinase activity of the BCR-ABL fusion protein in chronic myeloid leukemia (CML). It has shown remarkable efficacy for the treatment of patients with Philadelphia chromosome-positive CML ([172]. EGFR is also a known target for small-molecule inhibitors [173]. Gefitinib[174] and erlotinib[175] selectively inhibit EGFR, and both are efficacious against EGFR-expressing cancers such as non-small-cell lung cancer and head and neck squamous-cell carcinoma. Unlike monoclonal antibodies (mAbs), small-molecule inhibitors can translocate through plasma membranes and interact with the cytoplasmic domain of cell-surface receptors. Compared to mAbs, small –molecule inhibitors are more convenient to administer than mAbs because of smaller molecular weight. In addition,

the half-lives of the small-molecule inhibitors are much shorter than mAbs[176]. This characteristic can be used for sequentially combining chemotherapy with targeted therapy studies and not need to address the long-lasting effects of the inhibitors. IGF-IR is a known receptor tyrosine kinase, and the kinase activity is vital for its biological functions, such as cell proliferation, migration and cell survival. Several small-molecule inhibitors of IGF-IR have been developed over the years. Since the IGF-IR is highly homologous to IR, it is a challenge for the development of specific small molecule inhibitors of IGF-IR. From the earlier described inhibitors such as AG1024 and AG1034 [102]to recently developed inhibitors BMS-536924[177] and BMS-554417[178], they all inhibited IR kinase activity with similar potency with IGF-IR.

cis-3-[3-(4-methyl-piperazin-l-yl)-cyclobutyl]-1-(2-phenyl-quinolin-7-yl)-imidazo[1,5-a]pyrazin-8-ylamine (PQIP), a novel small-molecule IGF-IR kinase inhibitor, was found to be ~14 fold more potent for inhibition of ligand-dependent autophosphorylation of human IGF-IR than human IR in NIH3T3/huIGF1R cells[179]. Moreover, PQIP inhibits the ligand-induced activation of Akt and mouse xenograft growth in GEO human colorectal cancer cell lines. Here, we assess the effects of PQIP on signaling blockade, growth, apoptosis induction, cell cycle and migration in MCF-7 breast cancer and LCC6 melanoma cell lines. We also analyze the effects of sequencing of PQIP with doxorubicin using MCF-7 and LCC6 in cell proliferation inhibition and cell apoptosis induction. Different from the report in NIH3T3/huIGF1R cells, we found PQIP inhibits both the IGF-IR and IR with similar potency by blocking Akt and MAPK activation in breast cancer cell lines. In addition, PQIP enhances the cytotoxicity of

doxorubicin in inhibiting cell proliferation. These observations provide the rationale for using PQIP alone or in combination with chemotherapy in breast cancer therapy.

Purpose of this study

Work discussed in chapter 1 demonstrated that sequencing of an anti-IGF-IR antibody with chemotherapy enhances the cytotoxicity of the DNA damaging agent doxorubicin. Doxorubicin followed by anti-IGF-IR antibody is the optimal sequence to inhibit cell proliferation and induce cell apoptosis. Further, the reverse sequence of antibody followed by doxorubicin protects cells from the cytotoxicity of doxorubicin. Since small-molecule tyrosine inhibitors are another big group of drugs for blocking of IGF-IR activation, we investigated the biological effects of PQIP and the effect of sequencing of PQIP with doxorubicin in inhibiting cell proliferation and inducing cell apoptosis in MCF-7 breast cancer and LCC6 melanoma cell lines.

Hypothesis

PQIP inhibits cell proliferation, induces cell apoptosis and reduces cell motility in breast cancer cell lines; sequencing of PQIP with doxorubicin affects the cytotoxicity of doxorubicin.

RESULTS

PQIP inhibited cell proliferation in a dose-dependent manner. To assess activity of PQIP in tumor cell lines, the IC50 concentrations were determined using MTT assays

(Figure 2.1). These assays were done with or without IGF-I (5 nM). MCF-7 cells treated

with PQIP exhibited a dose-dependent decrease in proliferation compared with

untreated cells (Figure 2.1A). The IC50 for this anti-proliferative effect was in the

submicromolar range in the presence of IGF-I.

LCC6 cells, which do not appear to rely on IGF-IR for intrinsic growth, also

exhibited a dose-dependent inhibition of proliferation but were 4.5- fold less sensitive to

PQIP compared with MCF-7 cells (Figure 2.1B). There was no significant difference in

the sensitivity of LCC6 cells to PQIP in the absence or presence of IGF-I.

PQIP inhibited IGF-I stimulated cell motility in LCC6 cells. Our lab previously

showed that IGF-I enhances cell motility in LCC6 cells[180]. To address whether PQIP

inhibits IGF- induced cell motility, LCC6 cells were pretreated with PQIP for 1 hour

and then placed in a modified Boyden chamber with or without IGF-I for 5 hours. As

shown in Figure 2.2, IGF-I significantly stimulated cell migration, and PQIP completely

and significantly inhibited the IGF-induced cell motility at its IC50 concentration in

LCC6 cells.

PQIP inhibited the phosphorylation of both IGF-IR and IR in a dose-dependent

manner in MCF-7 cells. To test the ability of PQIP to inhibit the activity of IGF-IR

and IR tyrosine kinase, receptor phosphorylation was examined in the presence or

absence of IGF-I (5 nM) or insulin (10 nM) (Figure 2.3). After PQIP pretreatment, IGF-

IR and IR were immunoprecipitated from treated cells and analyzed by Western blotting

using a phosphotyrosine-specific antibody. In MCF-7 cells, there was no detectable

phosphorylation of the immunoprecipitated IGF-IR and IR without the addition of growth factors. In the presence of IGF-I or insulin, there was detectable phosphorylation of the IGF-IR and IR, respectively. PQIP dose-dependently inhibited the phosphorylation of IGF-IR and IR by their cognate ligands. As shown in Figure 2.3, PQIP was found to be 3-fold more potent toward inhibiting the phosphorylation of IGF-IR than IR. There was no change in the amount of immunoprecipitated IGF-IR or IR between the various treatment groups as determined by Western blotting for total receptors.

PQIP dose-dependently inhibited IGF-IR and IR downstream signaling of ERK and Akt pathways. To elucidate the mechanism by which PQIP inhibiting cell proliferation, we investigated the pathways known to be important in IGF-I and insulin-induced growth and mitogenesis. Serum-starved MCF-7 and LCC6 cells were pretreated with various concentrations of PQIP for 1 hour and then stimulated with IGF-I (5 nM) or insulin (10 nM) in serum-free medium for 10 minutes. Cells were lysed and Western blotting for the phosphorylated forms of IRS1/2, MAPK and AKT was performed. In MCF-7 cells (Figure 2.4A), there was relatively low basal phosphorylation of ERK1/2 and AKT in the unstimulated state. In the presence of IGF-I or insulin, MAPK and AKT phosphorylation were dramatically increased. At concentration of 0.1 µM, PQIP completely inhibited both IGF-I and insulin stimulated phosphorylation of IRS1/2, MAPK and AKT. This shows the equal potency of PQIP to inhibit both the IGF-IR and IR signaling pathways. Since the MTT assay demonstrated that PQIP inhibited LCC6 cell proliferation as well, we assessed the ability of PQIP to

inhibit IGF-I and insulin stimulated signaling pathways in LCC6 cells. PQIP dose

dependently inhibited IRS1/2 and AKT phosphorylation in LCC6 cells. However, the

phosphorylation of ERK was constitutively active and was not affected by PQIP (Figure

2.4B).

PQIP dose-dependently inhibited IGF-I stimulated anchorage-independent growth of MCF-7 cells. To examine whether PQIP affects anchorage independent growth of

MCF-7 cells, we performed a soft agar colony formation assay. Cells were grown in 1%

FBS containing agar, in the presence of various concentration of PQIP, with and

without IGF-I. After 14 days, the number of colonies formed was determined. As

shown in Figure 2.5, PQIP dose-dependently inhibited anchorage-independent growth

with or without IGF-I stimulation. However, PQIP did not inhibit colony growth in

LCC6 cells (Figure 2.6).

PQIP inhibited IGF-I–mediated G1 to S phase transition in MCF-7 cells. IGF-I is

a well known mitogen that stimulates G1 to S phase progression through

phosphoinositide 3-kinase and ERK activation. To investigate the ability of PQIP to

interfere with IGF-I − mediated cell cycle progression, we analyzed MCF-7 cell cycle

distribution by flow cytometry. Serum-starved MCF-7 cells were treated with PQIP

(0.3 µM) in the absence or presence of IGF-I for 24 hours, and cell cycle progression

was analyzed. As shown in Figure 2.7, about 20% of serum-deprived cells were found

in the S phase and IGF-I induced a significant increase in S phase cells; PQIP at its

IC50 concentration completely inhibited the IGF-I stimulated S phase increase.

PQIP did not induce cell apoptosis in MCF-7 and LCC6 cells. Because PQIP treatment inhibited cell proliferation and IGF-I induced activation of the PI3K pathway, which is generally regarded as the predominant pathway used by the IGF-IR for protection against apoptotic cell death, we investigated whether PQIP treatment could initiate apoptosis. MCF-7 and LCC6 cells were incubated in serum-free medium or 1% FBS in the absence or presence of PQIP for 24 hours. After treatment, both adherent and nonadherent cells were collected and subjected to Western blotting with antibodies to Poly (ADP) ribose polymerase (PARP), a caspase substrate that is cleaved in a variety of cells undergoing apoptosis. At its IC50 dose, PQIP did not increase PARP cleavage in both MCF-7 and LCC6 cells (Figure 2.8). It was further confirmed by Annexin-V staining experiment (Figure 2.9).

PQIP did not increase the cytotoxicity of doxorubicin in monolayer growth of MCF-7 cells. Several studies have shown that activation of IGF-IR protects breast cancer cells from apoptosis induced by chemotherapy and radiation, and receptor activation mediates resistance to these therapies. Therefore, inhibiting IGF-IR signaling may enhance the sensitivity of cancer cells to chemotherapy. To test whether PQIP affects the cytotoxicity of doxorubicin in cell proliferation, in the presence of IGF-I, serum starved MCF-7 cells were treated with increasing concentrations of doxorubicin (DOX) and PQIP ($0.03-1\mu M$) for 72 hours, and MTT assay was performed. To our surprise, the IC50 of doxorubicin did not change when doxorubicin was given alone or in combination with PQIP (Figure 2.10). This result is consistent with our data in

chapter 1, where combining doxorubicin with an anti-IGF-IR antibody simultaneously does not benefit chemotherapy in mono layer growth assay. Sequencing needs to be considered for combining PQIP with doxorubicin. In addition, cell survival may also play a role in the effect of PQIP in combination with doxorubicin.

PQIP increased the cytotoxicity of doxorubicin in an anchorage-independent growth assay in MCF-7 cells. Anchorage-independent growth is the gold-standard for *in vitro* testing of potential chemotherapeutic agents. Moreover, cellular tumorigenicity in nude mice is correlated with anchorage-independent growth *in vitro*[181]. Thus, to further characterize the interaction among these drugs, we performed a soft agar assay. In contrast to MTT assay, both 0.01 and 0.03 µM PQIP decreased the IC50 of doxorubicin in an anchorage-independent growth assay in MCF-7 cells (Figure 2.11).

Combining doxorubicin with PQIP simultaneously or sequentially (DOX followed by PQIP) significantly inhibited anchorage-independent growth. As described in chapter 1, combining doxorubicin with anti-IGF-IR antibodies are sequence-dependent, and only doxorubicin followed by antibody significantly inhibited the colony formation. To determine whether combining the IGF-IR tyrosine kinase inhibitor PQIP with doxorubicin is sequence-dependent, MCF-7 cells were treated with PQIP in combination with doxorubicin as the following schedules: (1) IGF-I; (2) DOX (25 ng/ml); (3) PQIP (0.1 µM); (4) DOX in combination with PQIP simultaneously; (5) DOX 24 hours followed by PQIP; (6) Pretreated with PQIP for 24 hours and followed by DOX. In this experiment, we used a dose of doxorubicin (25ng/ml) that by itself, it

did not inhibit colony formation in MCF-7 cells. As shown in Figure 2.12, in comparison with the other groups, cells were either treated with PQIP and DOX simultaneously or DOX followed by PQIP significantly inhibit the colony growth. Consistent with the antibody data, PQIP followed by DOX did not enhance the cytotoxicity of doxorubicin.

To further confirm the effects of inhibition of IGF-IR on chemotherapy in cancer cells, LCC6 cell were used, which did not depend on IGF-IR for monolayer growth. As shown in Figure 2.13, similar results were obtained, as DOX in combination with PQIP simultaneously or DOX followed by PQIP significantly inhibited the anchorage-independent growth.

PQIP followed by doxorubicin decreased PARP cleavage in LCC6 cells. Our previous data showed that PQIP alone did not increase PARP cleavage in both MCF-7 and LCC6 cells, which is similar as the effects of anti-IGF-IR antibodies scFv-Fc and EM1642. IGF-I is a survival factor for a variety of cell type and several literature demonstrated that IGF-IR signaling protects cell from apoptosis induced by chemotherapy. To determine whether PQIP could increase the chemotherapy induced apoptosis, serum starved cells were treated with doxorubicin in combination with PQIP simultaneously or sequentially. Both adherent and nonadherent cells were collected and subjected to immunoblotting. In comparison with doxorubicin alone, PQIP did not increase PARP cleavage in MCF-7 cells in any combination (Figure 2.14). When we examined PARP cleavage in LCC6 cells, PQIP followed by doxorubicin significantly decreased PARP cleavage in LCC6 cells (Figure 2.15).

DISCUSSION

Searching for new therapeutic approaches to improve the efficacy of currently

available therapy in breast cancer is urgent needed. Multiple lines of evidence have

shown that IGFs are important for cell proliferation, survival, chemosensitivity, and

motility of breast cancer cells. Therefore, IGF-IR blockade is a promising approach for

cancer therapy. Several studies have shown that inhibition of IGF-IR using blocking

monoclonal antibodies or small-molecule inhibitors are effective. A main concern for

such therapy is trying to design drug which selectively inhibit IGF-IR, without

inhibiting IR at therapeutic dose in order to decrease side effects such as

hyperglycemia[96, 103, 182-184]. PQIP, a potent IGF-IR inhibitor, efficiently inhibited

IGF-I and insulin stimulated cell proliferation in a dose-dependent manner in several

types of cell lines. Moreover, PQIP strongly inhibited IGF-I mediated phosphorylation

of IGF-IR and insulin mediated phosphorylation of IR. Consistent with the known role

of the IGF system in cancer cells, treatment with PQIP resulted in inhibition of MAPK

and Akt phosphorylation stimulated by both IGF-I and insulin without causing receptor

down-regulation. Several epidemiological studies have shown that hyperinsulinaemia is

associated with an increased risk for a number of malignancies, including carcinomas of

the breast, prostate, colon and kidney[68, 70, 185-187]. In addition, IR, especially the

A isoform, is over-expressed in cancers. Moreover, IGF-II also binds to IR-A and IGF-

IR/IR-A hybrid receptors[15]. Due to the role of the IR in cancer, a strategy that target

only IGF-IR may not be sufficient to disrupt the malignant phenotype regulated by this

growth factor system and targeting of both IR and IGF-IR may be necessary to inhibit

IGF action. Our lab has reported that IGF-IR down-regulation by siRNA sensitized cells to insulin activation of downstream signaling pathways in several breast cancer cell lines[188]. Indeed, Haluska *et al.* have already shown that the small-molecule inhibitor BMS-55417 inhibits cell proliferation, induces cell apoptosis and inhibits the xenograft tumor growth *in vivo* by blocking both the IGF-IR and IR[178]. In this study, we showed that PQIP can inhibit both IGF-I and insulin mediated signaling activation with the same potency. This is different from Pachter's report that PQIP was 14-fold more potent against human IGF-IR than IR in NIH3T3/HuIGF-IR cell lines[179]. The possible reason is that different cell lines are used in our study. There are also many more IGF-IR receptors in the 3T3/IGF-IR cell line than in MCF-7 and LCC6 cell lines. Several studies have already shown that IGF-IR expression affects the sensitivity of anti-IGF-IR therapy and cell lines with high levels of IGF-IR are more sensitive to the drug[184].

The IGF-IR signaling has been described to be involved in metastasis in some tumor types [189]. Our lab has shown that LCC6 cell overexpressing a truncated IGF-IR has reduced cell motility *in vitro* and inhibited lung metastasis *in vivo*[55]. In our study, we found that PQIP inhibited the IGF-I mediated cell motility in LCC6 cell lines. Thus, PQIP may have the potential to inhibit tumor metastasis. This needs to be further confirmed using mouse xenograft growth model. In contrast to other reports, our study showed that PQIP did not increasePARP cleavage at its IC50 dose, a substrate of caspase activation. The reason may be that we used a different cell lines. Taken together, PQIP inhibited cell proliferation and cell motility but not inducing cell apoptosis in MCF7 and LCC6 cells.

Since IGF-I is able to attenuate the effect of conventional chemotherapeutic drugs[119, 128, 190]. We combined PQIP with doxorubicin, one of the most widely used drugs in breast cancer treatment, to further sensitize cancer cell lines to doxorubicin. We observed that PQIP enhanced the cytotoxicity of doxorubicin in anchorage-independent growth but not monolayer growth. These anchorage-independent growth inhibition effect, suggests the possible benefits of PQIP in combination with doxorubicin treatment *in vivo*. In chapter 1, we showed that anti-IGF-IR antibody in combination with doxorubicin is sequence-dependent and only doxorubicin followed by antibody enhances the cytotoxicity of doxorubicin. Since small-molecule tyrosine kinase inhibitors are another group of drugs used to block IGF-IR activation, we next investigated the effects of sequencing of PQIP with doxorubicin in inhibiting cell proliferation and inducing cell apoptosis. Interestingly, combining PQIP with doxorubicin simultaneously or doxorubicin followed by PQIP significantly inhibited the anchorage-independent growth, while the sequence PQIP followed by doxorubicin did not enhance the cytotoxicity of doxorubicin. Moreover, in contrast to antibody in combination with doxorubicin, both the sequence combining PQIP with doxorubicin simultaneously and doxorubicin followed by PQIP did not further increase PARP cleavage induced by doxorubicin in LCC6 cells. The sequence pretreatment with PQIP followed by doxorubicin attenuates PARP cleavage. In chapter 1, we found that pretreatment with an anti-IGF-IR antibody decreased the topoisomerase IIα mRNA, protein and activity. The underlying mechanism of pretreatment with tyrosine kinase inhibitor causing resistance to apoptosis needs to be explored further.

In summary, PQIP can be used alone or in combination with chemotherapy for breast cancer treatment. As anti-IGF-IR agents are being evaluated in phase I/II/III clinical trials, sequencing of anti-IGF-IR agents and chemotherapy need to be considered. Furthermore, since PQIP blocks both the IGF-IR and IR in some cancer cell lines, the hyperglycemia effects need to be evaluated.
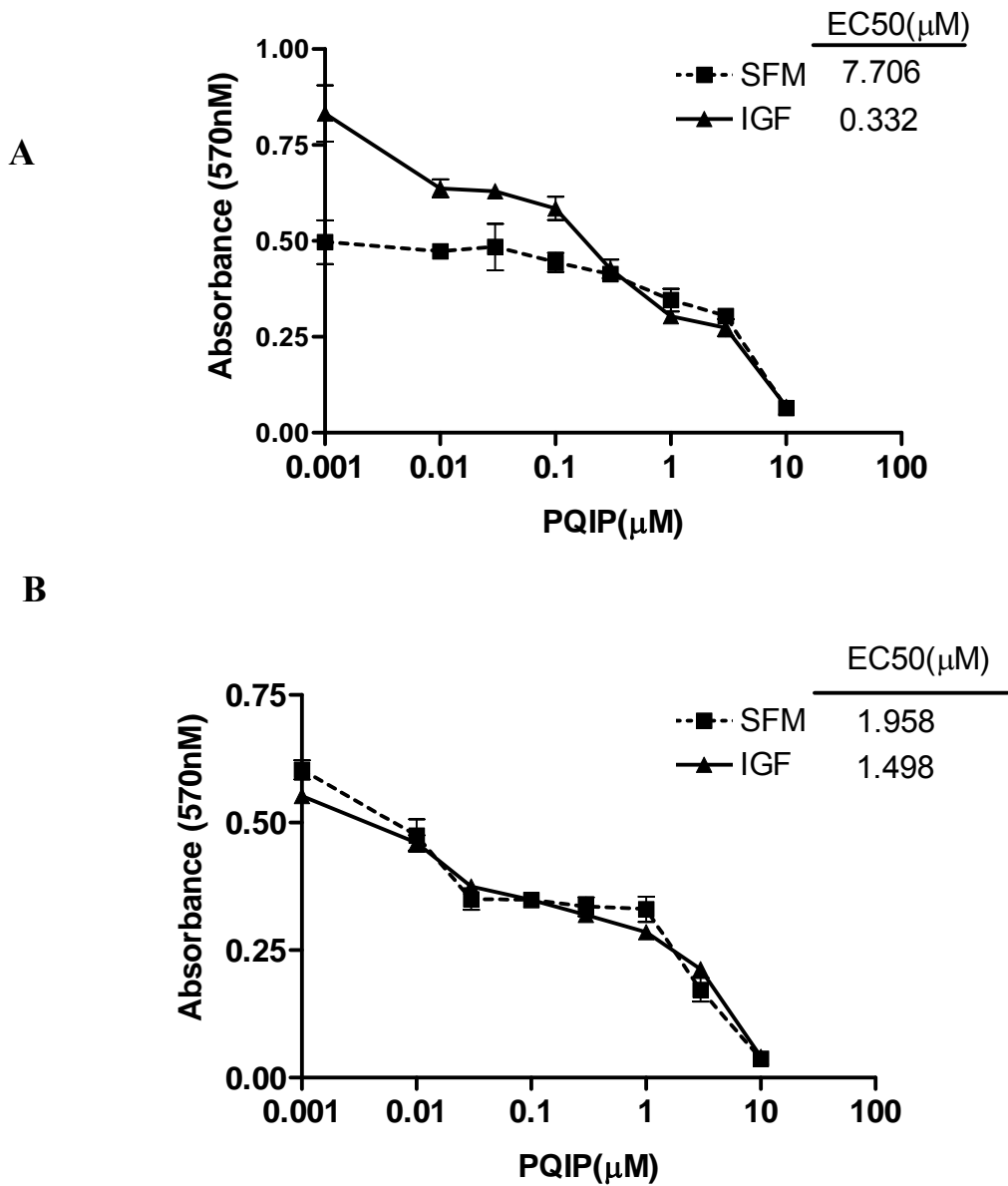
Figure 2.1
Anti-proliferative effects of PQIP.
MCF-7 cells (A) or LCC6 cells (B) were exposed to various concentration of PQIP in serum-free medium containing with or without 5 nM IGF-I for 72 hours. MTT assay was performed as an indicator of cell number.
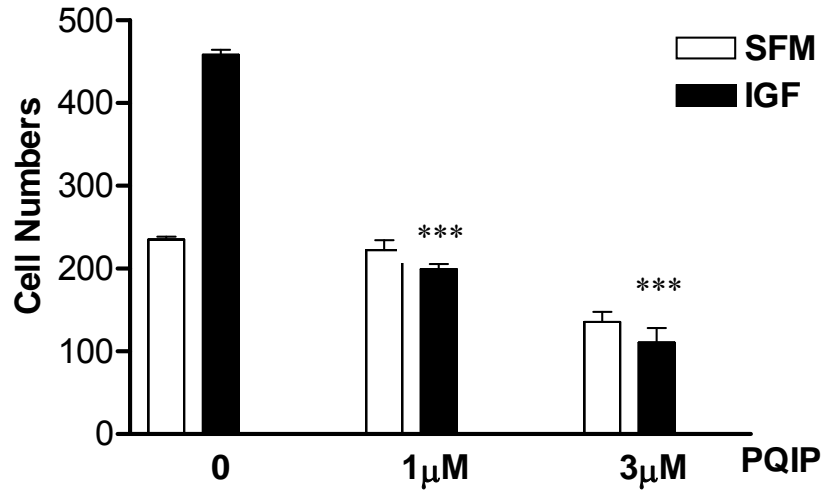
Figure 2.2
PQIP significantly inhibited IGF-I stimulated cell motility in LCC6 cells.
LCC6 cells were incubated in increasing concentrations of PQIP. Cell motility was
examined in both basal and 5 nM IGF-I containing medium using the modified Boyden
chamber assay. *** P<0.001vs IGF alone.
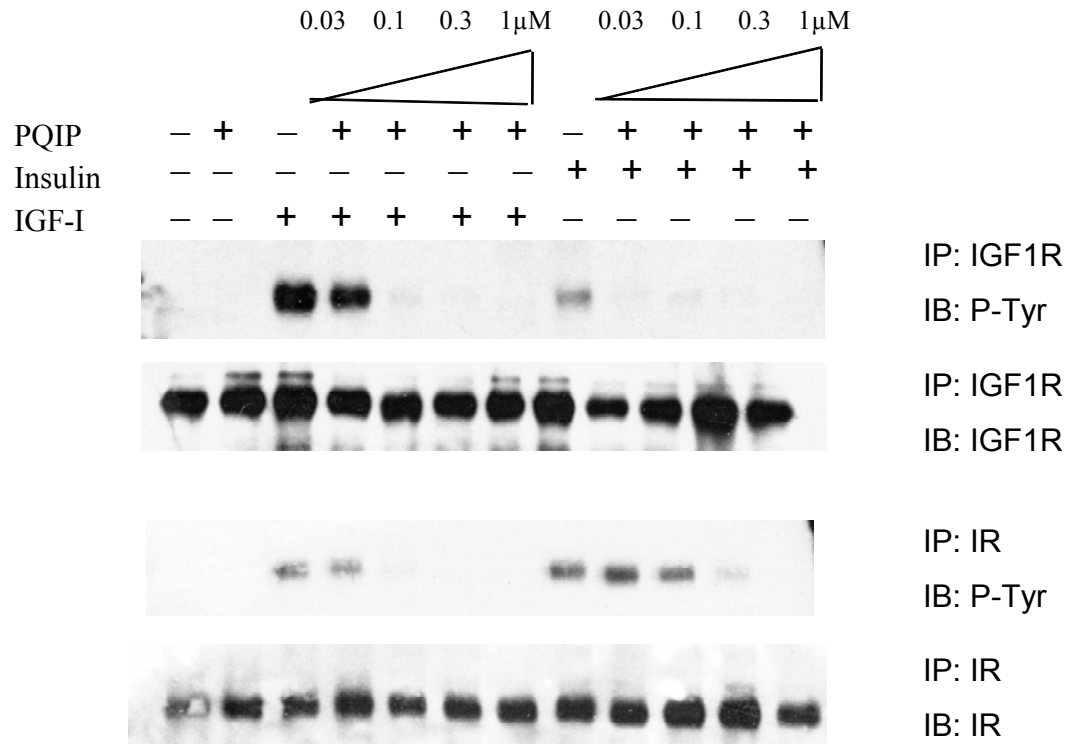
Figure 2.3
PQIP dose dependently inhibited IGF-IR and IR phosphorylations in MCF-7 cells.
Serum starved MCF-7 cells were incubated with PQIP at indicated concentrations for 1 hours at 37^oC, followed by stimulation with or without IGF-I (5 nM) or Insulin (20 nM) for 10 min. Cells were lysed and lysates were then analyzed by immunoprecipitation of IGF-IR and IR (IR), followed by immunoblotting for phosphotyrosine with a PY20 antibody.
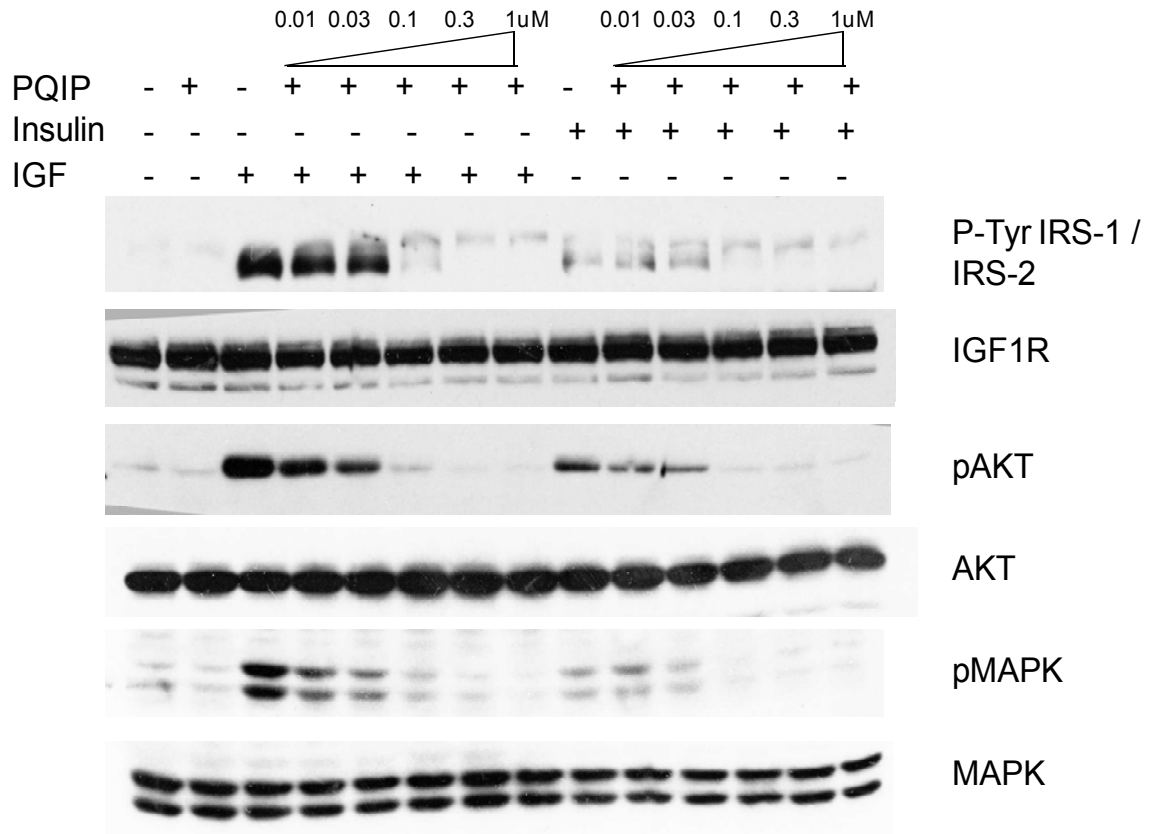
Figure 2.4A
PQIP dose dependently inhibited IGF-I and Insulin stimulated ERK and Akt activation in MCF-7 cells.
Serum starved MCF-7 cells were incubated with PQIP at indicated concentrations for 1 hours at 37°C, followed by stimulation with or without IGF-I (5 nM) or Insulin (20 nM) stimulation for 10 min. Cell lysates were then analyzed by immunoblotting for total and phosphorylated target protein content.
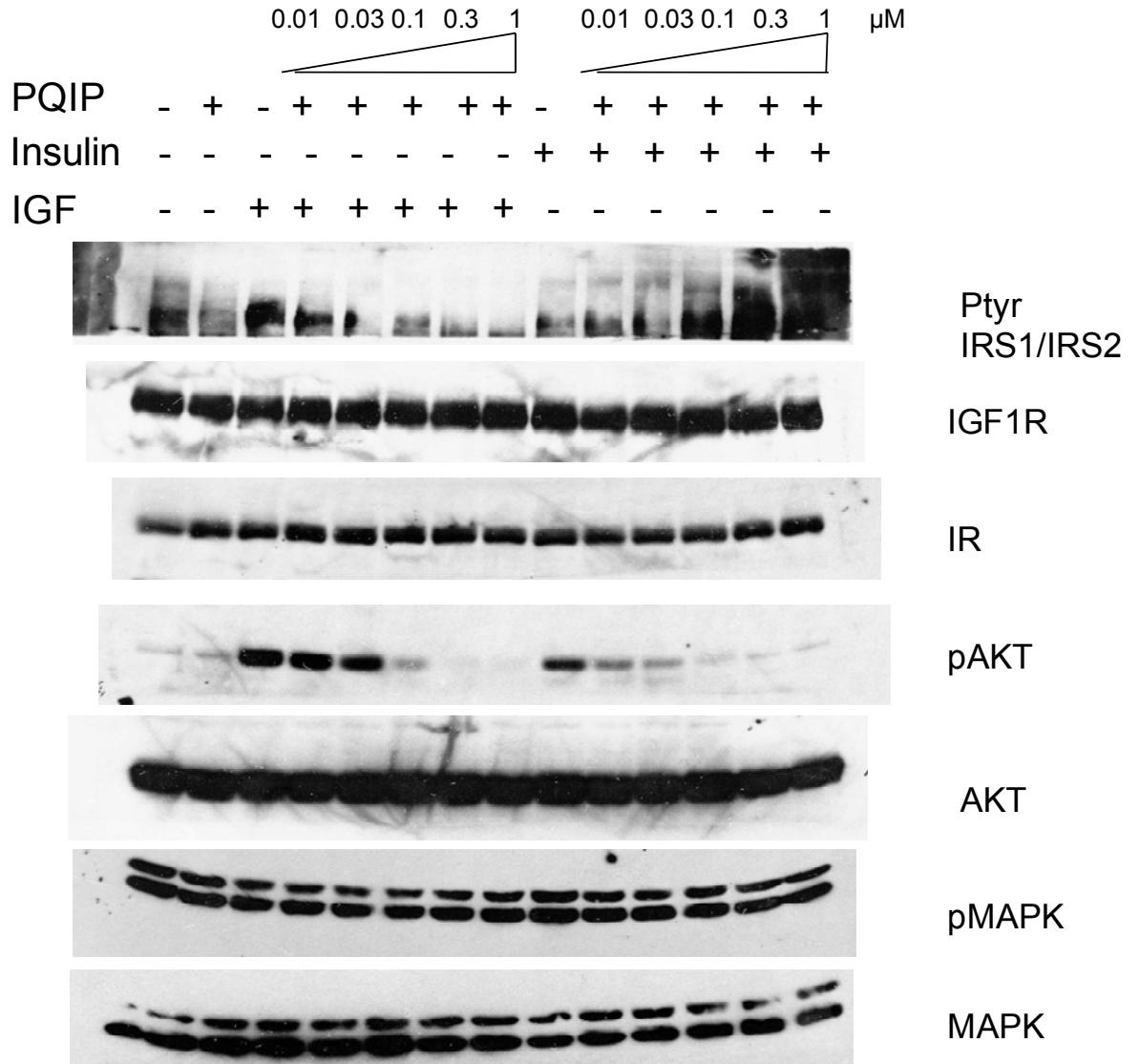
Figure 2.4B

PQIP dose dependently inhibited IGF-I and Insulin stimulated ERK and Akt activation in LCC6 cells.

Serum starved LCC6 cells were incubated with PQIP at indicated concentrations for 1 hours at 37°C, followed by stimulation with or without IGF-I (5 nM) or Insulin (20 nM) stimulation for 10 min. Cell lysates were then analyzed by immunoblotting for total and phosphorylated target protein content.
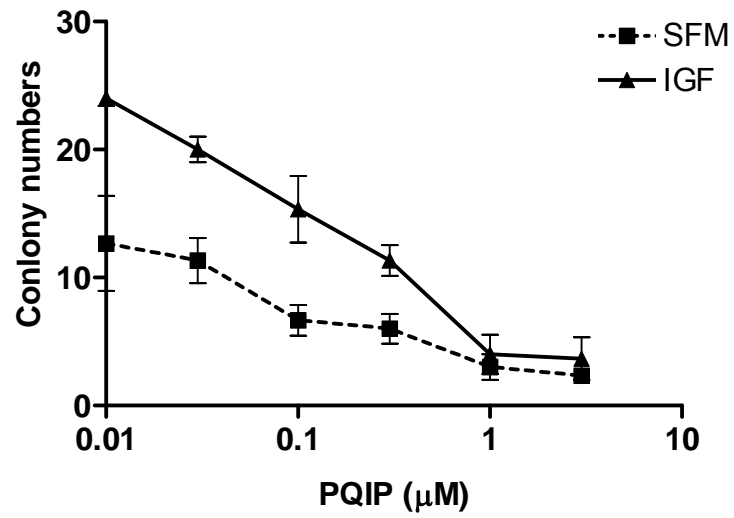
Figure 2.5
PQIP dose dependently inhibited IGF-I stimulated anchorage-independent growth in MCF-7 cells.
Cells were treated with increasing concentrations of PQIP in the presence of absence of IGF-I and the soft agar assay was performed. The number of colonies formed was counted after 14 days.
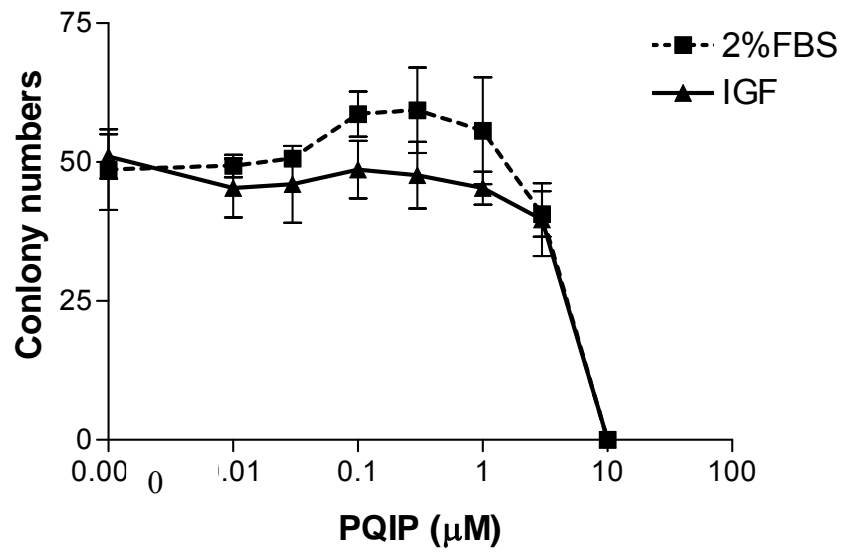
Figure 2.6
PQIP did not inhibit anchorage-independent growth in LCC6 cells.
Cells were treated with increasing concentrations of PQIP in the presence of absence of
IGF-I and the soft agar assay was performed. The number of colonies formed was
counted after 10 days.
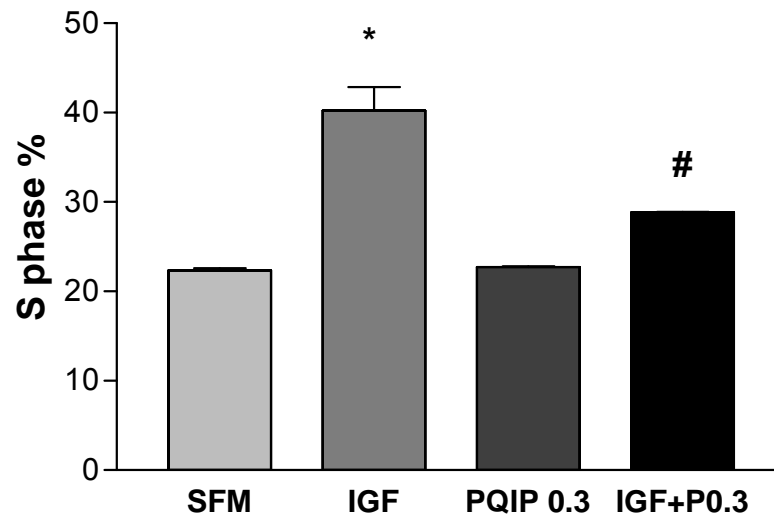
Figure 2.7
PQIP inhibited IGF-I stimulated cell cycle progression in MCF-7 cells.
Serum starved MCF-7 cells were exposed to either serum-free medium, 5 nM IGF-I, 0.3
µM PQIP or IGF-I plus 0.3 µM PQIP for 24 hours. Cells were harvested and analyzed
for cell cycle by flow cytometry. * $P<0.05$ vs SFM; # $P<0.05$ vs IGF.
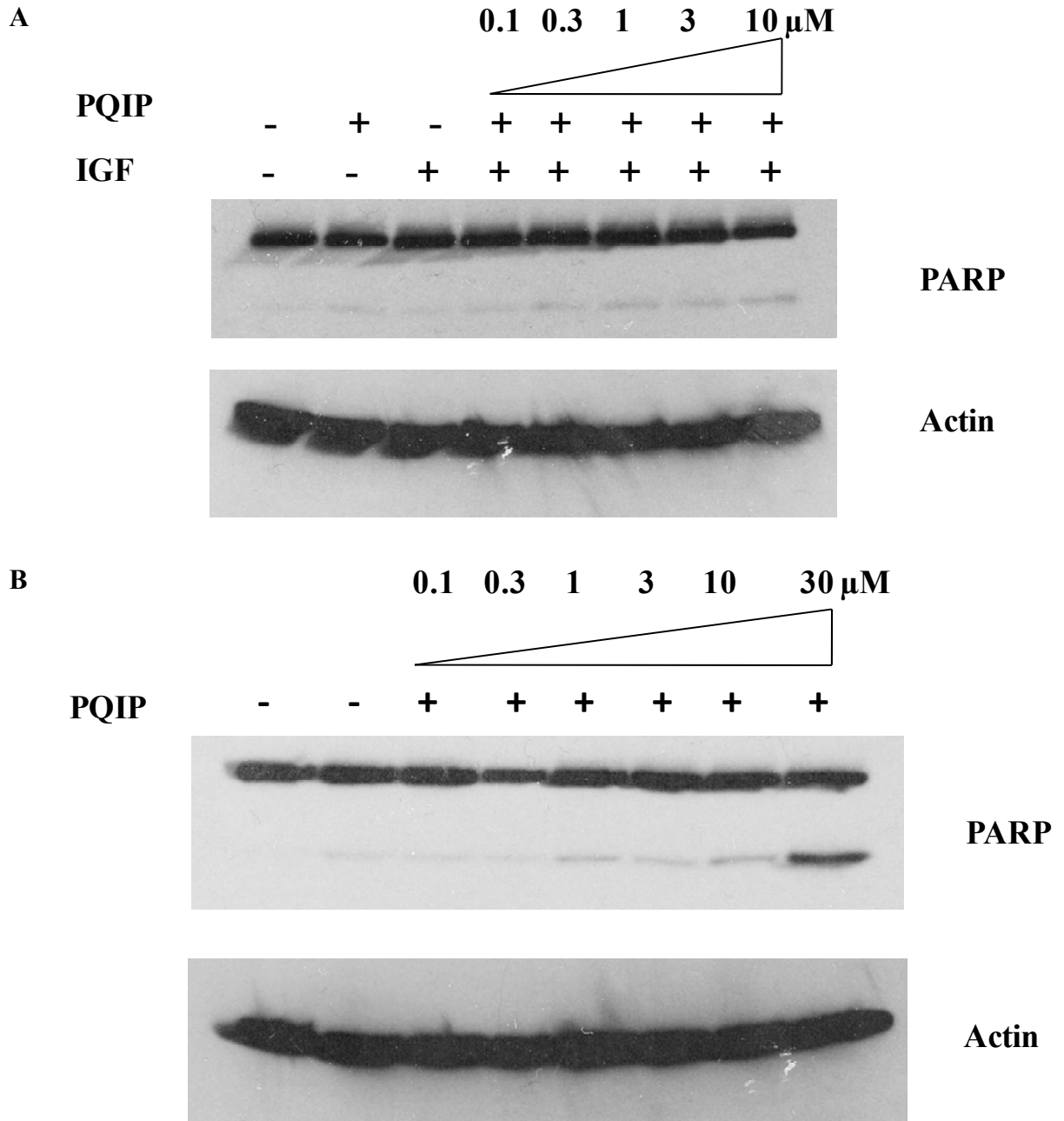
Figure 2.8
PQIP did not increase PARP cleavage in MCF-7 and LCC6 cells.
Cells were serum starved for 24 hours, and treated with or without PQIP at indicated
concentrations in the absence or presence 5 nM IGF-I in MCF-7 cells (A), or in the
presence of 1% FBS for 24 hours in LCC6 cells (B), and Poly(ADP) ribose polymerase
(PARP) cleavage was assessed by immunoblotting.

A
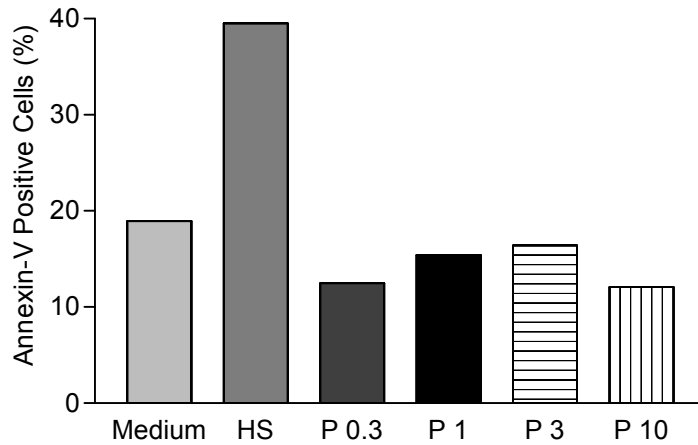
B
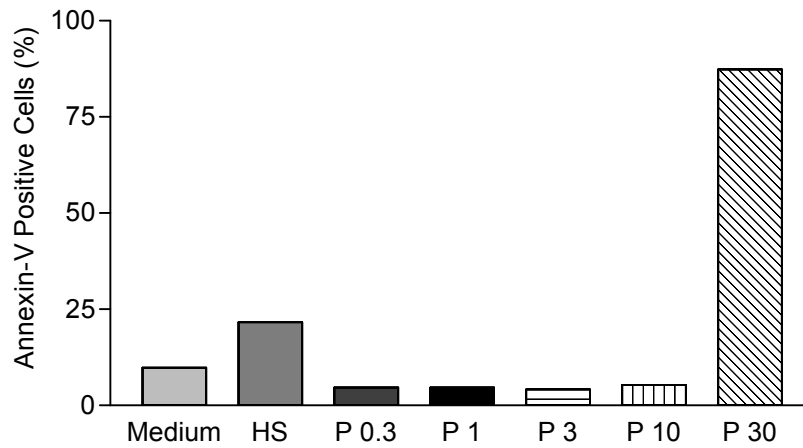
Figure 2.9
PQIP did not increase cell apoptosis in MCF-7 and LCC6 cells.
MCF-7 (A) and LCC6 (B) cells were treated with different concentration of PQIP for 24 hours, and then cell were collected and stained with APC–Annexin V and 7-AAD and analyzed by flow cytometry. The experiment was repeated three times with similar results; a representative experiment is shown for cells of apoptosis (percentage of all cells that were apoptotic). Heat shocked cell is the positive control. P 0.3, 1, 3, 10, 30 represents PQIP 0.3, 1, 3, 10, 30μM.
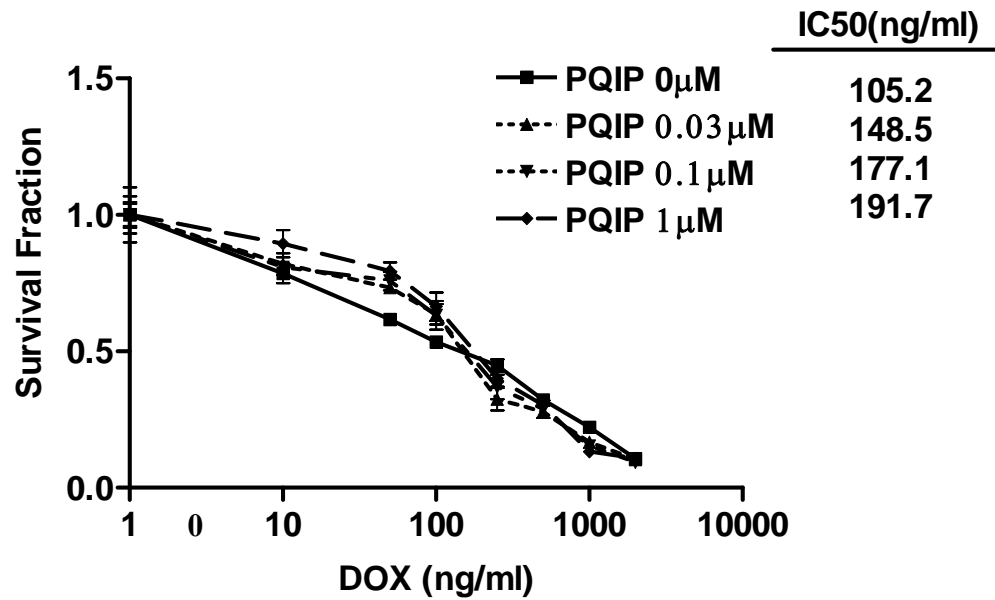
Figure 2.10
PQIP did not increase the cytotoxicity of doxorubicin in mono-layer growth in MCF-7 cells.
Serum starved cells were treated with increasing concentration of doxorubicin (DOX) in combination with PQIP (0.03-1μM) for 72 hours, and proliferation was measured by MTT assay. Cell survival is displayed as a percentage of growth over non-DOX treated control.
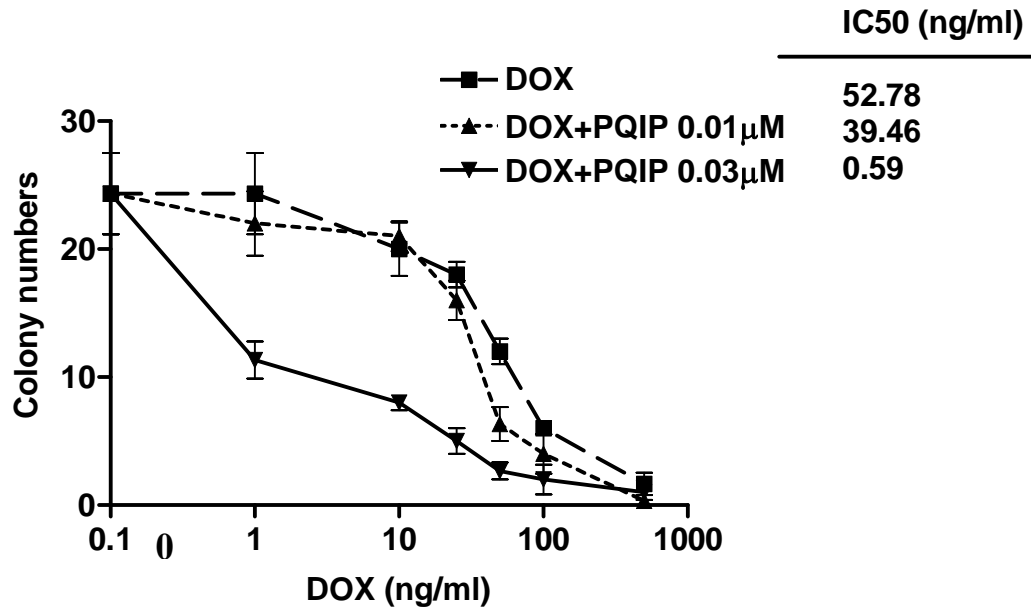
Figure 2.11
PQIP increased cytotoxicity of doxorubicin in anchorage-independent growth assay in MCF-7 cells.
70% confluent cells were treated with increasing concentrations of doxorubicin with or without PQIP (0.01, 0.03μM) and the soft agar assay performed. The number of colonies formed was counted after 14 days.
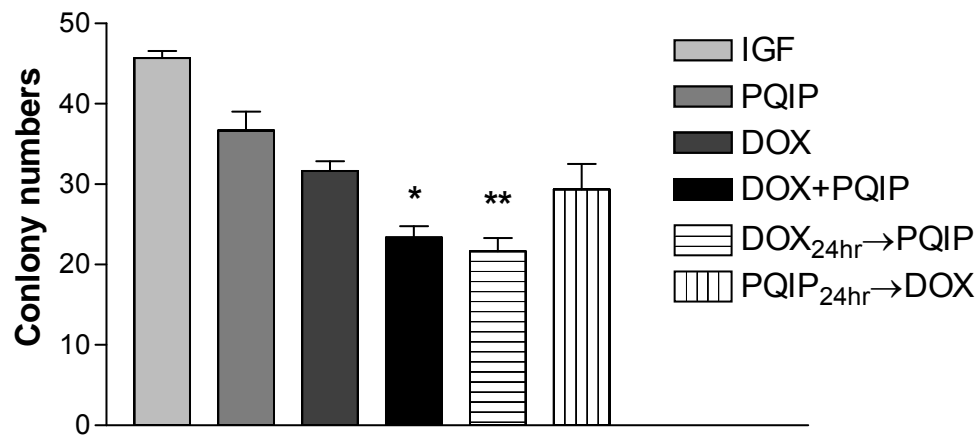
Figure 2.12
Combining doxorubicin with PQIP simultaneously or sequentially significantly inhibited anchorage-independent growth in MCF-7 cells.
Cells incubated with or without PQIP (0.1 µM) and/or doxorubicin (DOX, 25 ng/ml in the presence of 1% FBS were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates and incubate for 24 hours. If a second treatment was needed (shown by the *arrow*), it was added as a solution to the top agar with continued incubation for 14 days. Five randomly selected fields were counted for each well. Each treatment was done in triplicate, and the results are shown as the average number of colonies ± SE. * $P<0.05$ *vs* DOX alone; ** $P<0.01$ *vs* DOX alone.
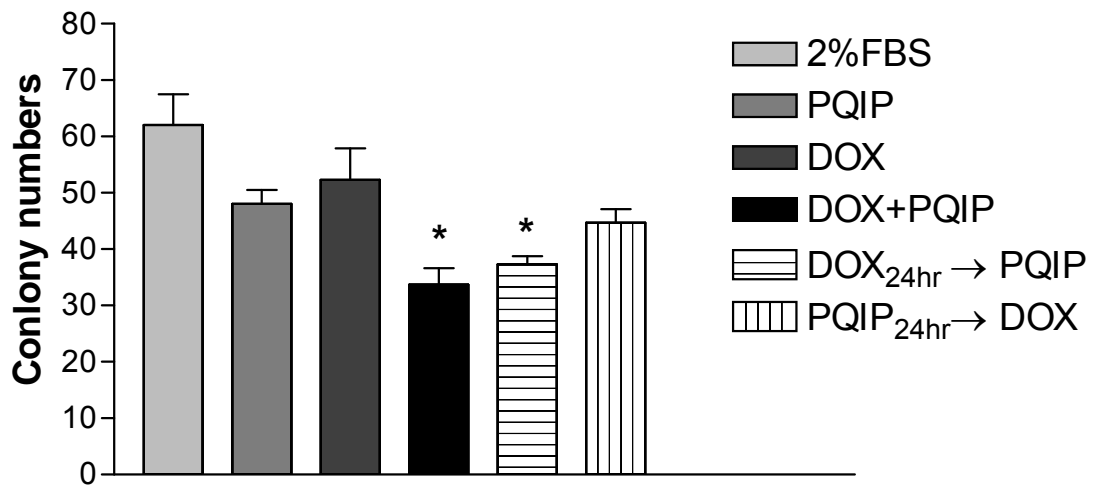
Figure 2.13

Combining doxorubicin with PQIP simultaneously or sequentially significantly inhibited anchorage-independent growth in LCC6 cells.

Cells incubated with or without PQIP (3μM) and/or doxorubicin (DOX, 100 ng/ml in the presence of 2% FBS were mixed with 0.45% agarose and overlaid over 0.8% bottom agar in 6-well plates and incubated for 24 hours. If a second treatment was needed (shown by the *arrow*), it was added as a solution to the top agar with continued incubation for 10 days. Five randomly selected fields were counted for each well. Each treatment was done in triplicate, and the results are shown as the average number of colonies ± SE. * P<0.05 *vs* DOX alone.
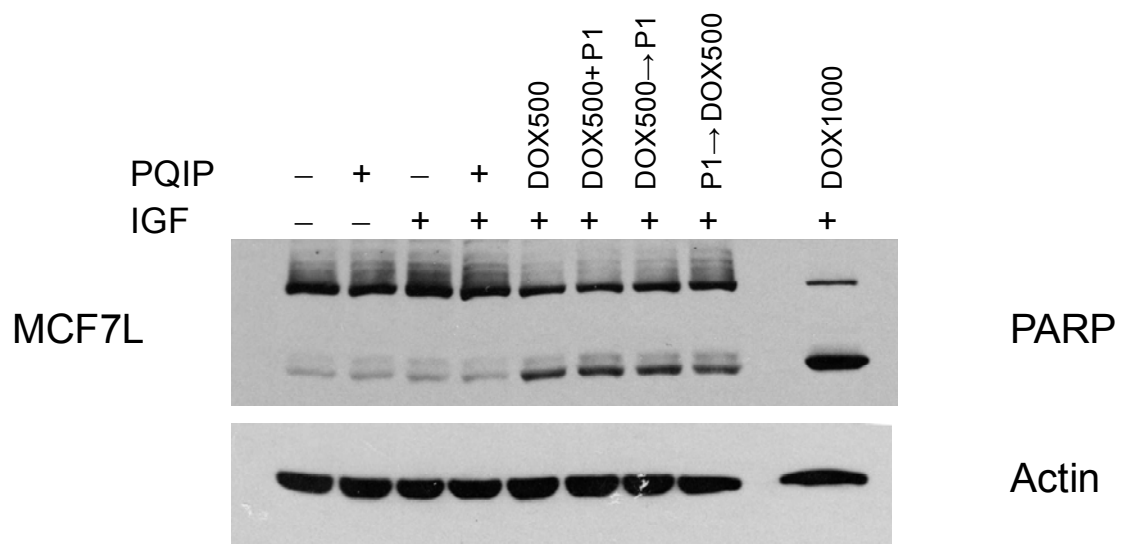
Figure 2.14
Combining PQIP with doxorubicin did not increase PARP cleavage in MCF-7 cells.
Serum starved MCF-7 cells were treated with 1% FBS (control), doxorubicin, PQIP or PQIP in combination with DOX simultaneously or sequentially for 48 hours totally. PARP cleavage was determined by Western blot analysis of whole cell lysates as described under "Materials and Methods." The abbreviations used are: P1, PQIP (1μM); DOX1000 (doxorubicin at 1000 ng/ml); DOX 500 (doxorubicin at 500 ng/ml).
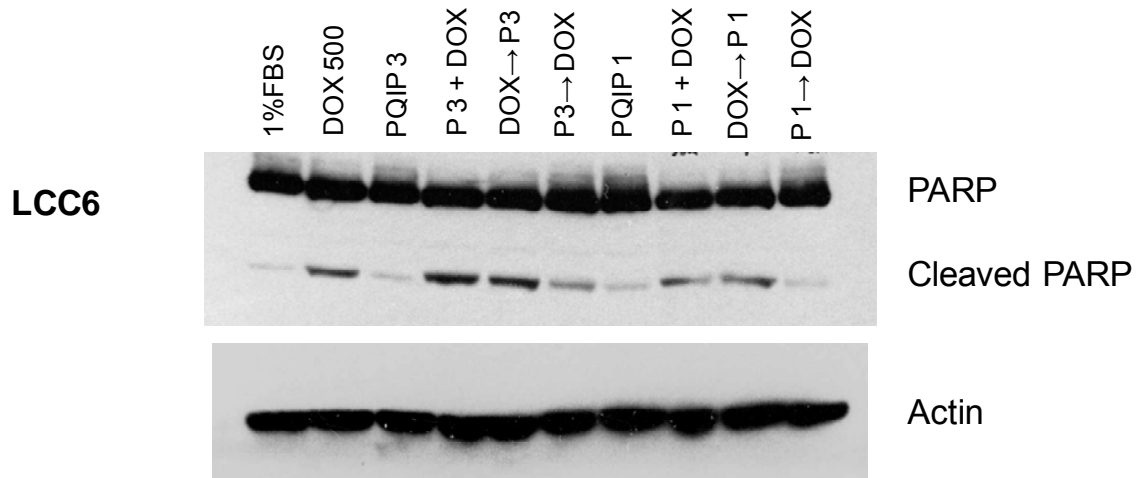
Figure 2.15
PQIP followed by doxorubicin decreased PARP cleavage in LCC6 cell lines.
Serum starved LCC6 cells were treated with 1% FBS (control), doxorubicin, PQIP or
PQIP in combination with DOX simultaneously or sequentially for 48 hours totally.
PARP cleavage was determined by Western blot analysis of whole cell lysates as
described under "Materials and Methods." The abbreviations used are: P1, PQIP (1µM);
P3, PQIP (3 µM); DOX1000 (doxorubicin at 1000 ng/ml); DOX 500 (doxorubicin at
500 ng/ml).

CONCLUDING REMARKS

This thesis described here provides the evidence that combining anti-IGF-IR antibody with chemotherapy is sequence-dependent. Chemotherapy given prior to exposure to an anti-IGF-IR monoclonal antibody is the optimal sequence for anti-cancer effects and the opposite sequence protects the cell from cytotoxicity of chemotherapy. Moreover, lower doses of antibody, which resulted in transient receptor suppression in *vivo*, were more effective than continuous receptor suppression caused by higher antibody doses. In addition, we show that a novel IGF-IR tyrosine kinase inhibitor, PQIP inhibits both the IGF-IR and IR with similar potency by blocking Akt and MAPK activation in human cancer cell lines. Furthermore, our sequencing study demonstrate that both combining PQIP with doxorubicin simultaneously and sequentially in the order of doxorubicin followed by PQIP significantly inhibits the anchorage-independent growth.

Our data in chapter 1 demonstrate that the sequencing of IGF-IR blockade plays a critical role in determining efficacy and the sequencing of IGF-IR inhibition with chemotherapy may distinguish between synergy and interference. These findings are important and novel, and will contribute significantly to the design of the anti-IGF-IR clinical trials. However, we only demonstrated the enhanced anti-tumor effects for combining a low dose of AVE1642 with doxorubicin in LCC6 xenograft model, which does not appear to rely on IGF-IR for intrinsic growth. Whether or not these *in vivo* observations are true in other cell lines such as MCF-7 and T-47D that depends on IGF-IR for growth and survival, is still unclear. As we show doxorubicin followed by scFv-Fc or EM164 inhibited the mono layer growth in MCF-7 cells, the sequence in MCF-7 cells needs to be further examined *in vivo*. In this study, mouse xenograft tumor growth

model is used to verify our *in vitro* sequence. It is known that the lymphocyte-mediated response to the tumor is lost in athymic nude mice, thus, this model may not fully mimic the situation in humans. The best way to validate our result is in human clinical trials. Our results argue that the human clinical trials against IGF-IR in combination with chemotherapy should be carefully designed to consider the potential sequence effects.

We demonstrated that PQIP inhibited both the IGF-IR and IR signaling *in vitro*. It is known that almost all anti-IGF-IR antibodies and small kinase inhibitors partially or completely inhibit both receptors. However, whether inhibiting both receptors is more beneficial than inhibiting a single receptor is still debated. This should be tested in the future using siRNA or shRNA technologies that can distinguish between the two receptors. Moreover, we also demonstrated that PQIP enhance the cytotoxicity of doxorubicin in anchorage-independent growth. These observations need to be confirmed in mouse xenograft tumor model. First, whether PQIP alone inhibits the tumor growth will be tested; second, combining of PQIP with doxorubicin simultaneously will be tested; third, sequencing of PQIP with doxorubicin will be examined in the future study. Since PQIP blocks the IR, it may affect the glucose metabolism to cause toxicity such as hyperglycemia. Thus, long-term *in vivo* treatment would be problematic. Short-term treatment or co-administration of PQIP with chemotherapeutic agents may be a better strategy for such kind of therapy.

In conclusion, IGF-IR is an attractive target for cancer therapy and both antibodies and small-molecule inhibitors are promising tools for anti-IGF-IR therapy. Disruption of IGF-IR signaling with anti-IGF-IR antibody or small kinase inhibitor in

combination with other cytotoxic agents emerges as an important and useful strategy in

cancer treatment. Dose and sequencing of cytotoxic chemotherapy with IGF-IR

blockade should be considered in the design of future clinical trials.

MATERIALS AND METHODS

Reagents

All reagents and chemicals were purchased from Sigma (St. Louis, MO), and cell culture reagents were from Invitrogen/Life Technologies, Inc. (Rockville, MD) unless otherwise noted. IGF-I was purchased from Novozyme (Adelaide, Australia). The anti-IGF1R antibody scFv-Fc was engineered and purified as described previously[93]. EM164 and AVE1642 (a humanized EM164) antibody were kindly provided by Sanofi-aventis[96]. PQIP were kindly provided by OSI pharmaceuticals, Inc (New York, NY). Horseradish peroxidase-conjugated PY-20 anti-phosphotyrosine antibody was purchased from Transduction Labs (Lexington, KY). Antibodies against ERK1/ERK2, phosphorylated ERK1 and 2, total Akt and phosphorylated Akt (Ser473), cleaved caspase 7 were purchased from Cell Signaling (Beverly, MA). The polyclonal antibodies against IGF1R α and ß subunits were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Poly (ADP-ribose) polymerase (PARP) and cleaved PARP was purchased from Roche Diagonstics (BV, Germany). HRP-linked anti-rabbit and anti-mouse antibodies were purchased from Amersham (Piscataway, NJ). The anti-ß-actin was from Sigma-Aldrich (St.Louis, MO). Protein A agarose was purchased from Santa Cruz Biothechnology, Inc. (Santa Cruz, CA). Anti-Topo IIα antibody was from TopoGEN (Columbus, OH). Anti-rabbit and anti-mouse secondary antibodies conjugated to HRP were from GE Biosciences (Piscataway, NJ). Sam68 antibodies were purchased from Cell Signaling (Beverly, MA). Doxorubicin was purchased from BMS (New York, NY).

Cell Culture

MCF-7 cells were originally obtained from Dr. C. Kent Osborne (Baylor College of Medicine, Houston, TX) and were routinely maintained in Iscove's modified essential medium (IMEM) with Zinc Option (Richter's modification) with 5% fetal bovine serum, 11.25 nM human insulin (Eli Lilly, Indianapolis, IN), 50 units/ml penicillin, and 50 µg/ml streptomycin. LCC6 cells were obtained from Dr. Robert Clarke (Georgetown University, Washington D. C.). LCC6 cells were routinely maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 11.25 nM human insulin, 50 units/ml penicillin and 50µg/ml streptomycin.

Lysate Preparation

Cells were plated in growth medium in a 60 mm dish and grown to 70-80% confluence, incubated in serum free medium (SFM) overnight, then treated with doxorubicin, scFv-Fc, EM1642 or doxorubicin in combination with anti-IGF1R antibody simultaneously or sequentially for 48 hours. Cells were washed twice with ice-cold PBS on ice and lysed with 500 µl TNESV lysis buffer [50 mM Tris-Cl (pH 7.4), 1% NP40, 2mM EDTA (pH 8.0), 100 mM NaCl, 10 mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin and 20 µg/ml aprotinin]. For apoptosis assay, adherent and non-adherent cells were collected, washed twice with ice-cold PBS on ice and lysed with 500 µl TNESV lysis buffer. Lysates were cleared by centrifugation at $12,000 \times g$ at $4^{\circ}C$ for 25 minutes. Protein concentrations of the lysates were determined using the bicinchoninic acid kit (Pierce, Rockford, IL).

Nuclear Extract Preparation

For studies on the effect of combining the anti-IGF-IR antibody with doxorubicin on topo IIα protein levels, nuclear extracts were prepared from LCC6 cells treated with AVE1642, doxorubicin, or AVE1642 in combination with doxorubicin concurrently or sequentially. Nuclear extracts were purified by lysis of cells with a high-salt buffer [100 mM NaCl, 20 mM KCl, 20 mM Tris, and 0.5 mM Na2HPO4 (pH 7.4)] containing 0.5% Triton X-100 and 1% NP40. Nuclei were pelleted, and the nuclear proteins were solubilized by sonication in 1% SDS. Lysates were cleared by centrifugation at 12,000 × g at 4°C for 25 minutes. Protein concentrations of the lysates were determined using the bicinchoninic acid kit (Pierce, Rockford, IL).

Western Blotting

40 μg or 50 of total cell lysates in 1× Laemmli sample buffer were separated by using 8% SDS-PAGE and transferred to nitrocellulose. Nitrocellulose membranes were blocked with 5% nonfat dry milk in TBST (0.05% Tween in Tris buffered saline w/v) for one hour at room temperature. Primary antibodies were used according to the manufacturer's direction. IGF-IR, Insulin Receptor (IR), PARP, Caspase 7, Actin, Topo IIα and Sam68 antibodies were used at 1:2000 in 5% milk overnight at 4°C. Total MAPK, phosphorylated MAPK and total Akt antibodies were used at 1:2000 in TBST overnight at 4°C. Phosphorylated Akt (Ser473) antibody were used at 1:2000 in 1% BSA overnight at 4°C. Following incubation with primary antibody, membranes were washed in TBST six times for five minutes each and incubated for one hour at room temperature with HRP-linked anti-rabbit (1:2000) or anti-mouse (1:5000) secondary

antibody in 5% milk. HRP-conjugated PY-20 antibody was used at 1:4000 in TBST for one hour at room temperature. Membranes were washed six times for five minutes each and chemiluminesence detected.

Immunoprecipitation

500 µg total cellular lysates were pre-cleared with 50 µl protein A agarose. Precleared lysates were incubated overnight with IGF-1R and IR antibody at 4°C followed by incubation with protein A agarose for 4 hours at 4°C. Immunoprecipitates were collected by centrifugation at 12,000 × g for 5 minutes at 4°C and washed three times in TNESV buffer. Samples were resuspended in sample buffer and analyzed by 8% SDS-PAGE followed by immunoblotting for phosphorylated tyrosine residues with HRP-conjugated PY-20 antibody.

Monolayer Growth Assay

Assay was performed as previously described. Briefly, MCF-7 or LCC6 cells were plated in triplicate in 24 well tissue culture plates at a density of 20,000 cells per well in growth media. After 24 hours, cells were washed twice with 1× PBS and switched to serum free medium (SFM) for 24 hours. Then cells were treated according to the following schedules: (1) doxorubicin (DOX) alone for 72 hours; (2) DOX and antibody simultaneously for 72 hours; (3) pretreatment with DOX for 24 hours followed by antibody treatment for 48 hours; (4) pretreatment with antibody for 24 hours followed by DOX treatment for another 48 hours. For PQIP experiment, serum-starved cells were treated with increasing concentrations of PQIP with or without 5nM IGF-I and

incubated for 72 hours. Cell number was estimated using the 3-[4,5-Dimethylthiazol 2-yl]2,5-diphenyltetrazolium bromide (MTT) assay as described previously[191]. 60 µl of 5 mg/ml MTT reagent in PBS was added to each well and plates were incubated for 3 hours at 37°C. Wells were aspirated and 0.5 ml of solubilizing solution (95% DMSO + 5% IMEM) was added to solubilize the formazan crystals. Absorbance was measured at 570nm using a 670nm differential filter. IC 50 of the different combination was caculated by Prism software, and data was fitted to the equation Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope)) .

Annexin V staining

The annexin V–APC-labeled Apoptosis Detection Kit I (BD Biosciences, San Diego, CA) was used to detect apoptosis by flow cytometry according to the manufacturer's instructions. LCC6 cells (4 x 10^5 cells/well) were seeded in 60-mm dishes and cultured overnight in serum-free DMEM medium. The next day, cells were treated according to the following schedule: (1) 1% FBS; (2) DOX (100 ng/ml) 48 hours; (3) scFv-Fc (30 µg/ml) or EM164 (15 µg/ml) 48 hours; (4) DOX in combination with scFv-Fc or EM164 simultaneously for 48 hours; (5) DOX 24 hours followed by scFv-Fc or EM164 for another 24 hours; (6) Pretreated with scFv-Fc or EM164 for 24 hours and followed by DOX for another 24 hours. Cells were harvested in cold PBS after treatment. Heat shocked cells (cells were incubated in 56°C water bath for 1 min) were used as positive control. Cells were then resuspended at a density of 1 x 106 cells/ml in 1x binding buffer (HEPES, 10 mM, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2) and stained simultaneously with APC-labeled annexin V (25 ng/mL) and 7-

AAD (50 ng/mL). 7-AAD was used as a cell viability marker. Cells were analyzed

using a fluorescence-activated cell sorter (FACS) flow cytometer (Becton Dickinson,

San Jose, CA), and the data were analyzed with CellQuest software.

Cell Cycle Analysis

70% confluent LCC6 cells were trypsinized and plated at a density of 5x 10^5 cells per

60 mm dish. After 24 hours, cells were washed twice with $1 \times$ PBS and switched to

SFM for 24 hours. Cells were then treated with scFv-Fc, EM164, doxorubicin or

doxorubicin in combination with scFv-Fc or EM164 concurrently or sequentially for 48

hours as previously described. Cells were collected on ice in 1 ml of 1x PBS, and

stained with propidium iodide (Sigma-Aldrich Corp. St. Louis, MO). Cells were

analyzed for phase of the cell cycle by flow cytometry. Results were analyzed using

Modfit software.

Anchorage-independent Growth

Anchorage-independent growth assays were performed as previously described[94].

Briefly, a bottom agar was prepared by solidifying 1 ml of 0.8% SeaPlaque agarose

(BioWhitaker, Rockland, ME) in 2% FBS-containing growth media in each well of a 6-

well plate. The bottom agar was overlaid with 800 µl of a 0.45% top agar mixture

containing 10,000 cells per well in the presence of DOX, scFv-Fc, EM164, or DOX and

anti-IGF1R antibody. The plates were incubated at 37°C for 24 hours, then growth

media with or without DOX and antibody was added to the top of agar. For the PQIP

experiment, cells were mixed with DOX, PQIP or DOX in combination with PQIP and the second treatment was added top of the agar. After 10-14 days, colonies were counted using a light microscope with an ocular grid. Only colonies larger than two-thirds of a grid square were counted. Five random fields were counted for each well and the average number of colonies per well is shown. Results are representative of one experiment performed in triplicate for each treatment.

Boyden Chamber Cell Migration Assay

Cell motility was measured by a modified Boyden chamber assay as described previously[44]. Cells were serum starved for 24 hours, then pretreated with PQIP (1, 3μM) for 1 hour. Cells were briefly detached by trypsin-EDTA, washed twice with SFM, and then resuspended in SFM, 150,000 cells were placed in the upper chamber of 1 10-well Boyden chamber apparatus.Upper and lower chamber were separated by a polycarbonate polyvinylpyrrolidone free filter with 12 μm pores. 0.4 ml SFM with or without IGF (5 nM) was placed in the bottom wells of the chamber. After 5-6 hours of incubation at 37°C in a humidified atmosphere containing 5% CO_2, cells remaining on the topside of the filter were removed with cotton swabs. The filter was then removed from the chamber, and the cells that had migrated to the underside of the filter were fixed and stained in HEMA3. The filter was mounted on to a glass microscope slide and cells were counted in five random areas using a light microscope with an ocular grid.

Topoisomerase II Decatenation Assay

The assay was performed according to manufacturer's instructions (TopoGen, Inc., Port Orange, FL). LCC6 cells were treated with doxorubicin, AVE1642, or doxorubicin with AVE1642 simultaneously or sequentially. Nuclear extracts were prepared as noted above. The total reaction volume was held at 20 μl. Assay buffer (120 mM KCL, 50 mM Tris-HCl, 10 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM ATP, and 30 μg/mL BSA) containing 100 ng of catenated kinetoplast DNA (kDNA) and 2 μg LCC6 nuclear extract was incubated for 30 min at 37°C. The reaction was stopped upon addition of 4 μl of stop buffer (4% glyceol, 1% sarkosyl, and 0.025% bromphenol blue), and then analyzed by 1% agarose gel electrophoresis.

Intracellular Doxorubicin Accumulation Assay

Intracellular doxorubicin accumulation was evaluated by flow cytometry[192]. Serum starved cells were treated with scFv-Fc, EM1642, doxorubicin or doxorubicin in combination with antibody concurrently or sequentially for 48 hours as previously described. After incubation, the cell medium was removed and tyrpsin-EDTA was used to detach the cells from the plates. Cells were harvested, washed twice in ice-cold PBS, and placed on ice (<1 hour) until analysis. The intracellular mean fluorescence intensity associated with doxorubicin was measured with FACScan flow cytometry (Becton Dickinson). Excitation was performed by an argon ion laser operating at 488 nm and the emitted fluorescence was collected through a 530 nm pass filter. Data analysis was performed using Cell Quest software.

RNA Extraction and Quantitative RT-PCR

Total cellular RNA was extracted using PureLinkTM Micro-to Midi total RNA purification kit (invitrogen), according to the manufacturer's procedure. Two micrograms of RNA were reverse transcribed with random hexamers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR amplification was performed in a 96-well plate. Primers were designed intron spanning based on the sequence data obtained from the NCBI database by the Primer3 software. The 5' and 3' primer pairs designed for Topo IIα were 5'-TGGCTGAAGTTTTGCCTTCT-3' and 5'-GGCCTTCTAGTTCCACACCA -3'. Each PCR contained 5 μl cDNA, and 0.5–1 μmol/liter of each primer. Real-time quantitative PCR analysis was performed with a LightCycler (Roche Diagnostics) according to the manufacturer's instructions as described previously (17 Carol and Emily paper). Melting-curve and agarose gel electrophoresis analysis confirmed product-specific amplification; the results were normalized to β-actin.

Tumor Growth in Athymic Mice and Tumor Extract Analyses

Four week-old female athymic mice were used for *in vivo* study. To study the effect of AVE1642 dose on IGF1R downregulation *in vivo*, 5×10^6 LCC6 cells were injected into the mammary fat pads on each side of each mouse. When tumor volume reached 100 mm^3, AVE1642 was injected intraperitoneally (i.p). Tumors from the left side of all mice were surgically resected and snap-frozen in liquid nitrogen two days after AVE1642 treatment. The tumors on the right side of the mouse were collected and frozen after another 7 days. Tumors were homogenized and blotted for IGF1Rβ as noted above.

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For the tumor growth study, 5 x 10^6 LCC6 cells in serum-free IMEM were injected into the mammary fat pad of the mice. Tumor growth was measured every 3 days and tumor volume was estimated from bidirectional measurements using the formula length x breadth^2 / 2. When the tumors reached volume of 80~100 mm^3, the mice were randomized into 5 groups of 5 animals each. The dose of AVE1642 was based on the findings from the previous experiment. Mice were treated with PBS, DOX (3 mg/kg body weight/week), AVE1642 (50 μg/week), DOX followed 24 hours later by AVE1642 (50 μg/week) or DOX followed 24 hours later by AVE1642 (800 μg/ week) by intraperitoneal injection. Treatment was given weekly for 4 weeks. Twenty four hours after the final treatment, mice were sacrificed. The lungs of the mice were harvested, fixed, embedded, sectioned and stained with hematoxylin and eosin, and examined microscopically for micro-metastatic deposits. In the second set of experiments, the mice were sacrificed one week after the final treatment. Tumors were harvested, nap-frozen and homogenized. Tissue homogenates were suspended in 500 μl TNESV lysis buffer. 100 μg of each tumor extract were subjected to 8% SDS-PAGE followed by immunoblotting for the level of IGF1R. Total levels of MAPK were used as loading control. All the animal protocols were approved by the University of Minnesota's Institutional Animal Case & Use Committee (IACUC).

Statistical Tests

Statistical significance between means of control and antibody in combination with doxorubicin was assayed using Student's t test; 95% confidence interval ($P < 0.05$) was considered significant. For comparisons between more than two treatments in the

xenograft experiments, One-way ANOVA analysis was performed. If not otherwise

indicated, error bars in all experiments represent the SD.

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