Aus der Klinik für Strahlentherapie und Radioonkologie

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# Targeted therapy against VEGFR and EGFR with ZD6474 enhances the therapeutic efficacy of irradiation in tumor-bearing nude mice

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**Abbreviations:**

| | |
|---|---|
| EGFR | Epidermal growth factor receptor |
| Flt-1 or VEGFR-1 | fms-like tyrosine kinase |
| IC50 | half inhibit concentration |
| KDR or VEGFR-2 | kinase-insert domain containing receptor |
| MAPK | Mitogen-activated protein kinase |
| TGF | Transforming growth factor |
| VEGF | Vascular endothelial growth factor |
| VEGFR | VEGF receptor |
| RT | Radiotherapy |

# 1. Abstract

## 1.1 Purpose

ZD6474 is a newly developed anti-angiogenic agent that selectively targets two key pathways in tumor growth by inhibiting vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) tyrosine kinase activity. While VEGFR signaling promotes an array of responses in the endothelium including endothelial cell proliferation and angiogenesis, EGFR signaling regulates principal cellular processes including proliferation, differentiation and apoptosis after cytotoxic stress. The purpose of the present study was to evaluate the antitumor efficacy of a combination of ZD6474 and radiotherapy in a human tumour xenograft model (Fadu, squamous cell carcinoma) and to investigate whether the effects of the treatments are related to changes in tumor perfusion, proliferation and apoptosis.

## 1.2 Methods and Materials

Tumor-bearing nude mice received either vehicle or ZD6474 (50mg/kg, by mouth, once daily for the duration of the experiment), with or without irradiation (5x2 Gy, days 1-5) (4 treatment-groups: control; ZD6474 alone; radiotherapy (RT) alone; ZD6474 + RT). The antitumor efficacy of the different treatment modalities was evaluated by the tumor growth delay. For the different treatment-groups the tumor vascularisation was evaluated by immunofluorescence analysis of CD34 positive vessel segments (tumor vascular density) and the proliferative capacity and apoptotic degree of the tumor tissue were analysed by the quantification of Ki67 positive nuclei and Capase-3 positive cell.

## 1.3 Results

The tumor growth delay induced by the combined treatment (ZD6474 + RT) was greater than that induced by ZD6474 or radiotherapy alone ($21.4 \pm 2.8$ versus $17.7 \pm 3.0$ versus $12.5 \pm 0.7$ days). The application of ZD6474 had significant effects on the tumor vasculature resulting in clearly reduced neoangiogenesis. Moreover, the proliferative capacity of the tumor tissue was significantly decreased by ZD6474 and apoptosis of tumor tissue was significantly increased by ZD6474.

## 1.4 Conclusion

When irradiation is combined with VEGFR and EGFR blockade, significant enhancement of antiangiogenic, antivascular, and antitumor effects were observed. These data provide support for clinical trials of biologically targeted and conventional therapies in the treatment of cancer.

## 2. Introduction

Ionizing radiation is an effective modality for the treatment of many tumors. It is a widely used treatment for cancer, with over half of all cancer patients receiving radiation therapy during their course of treatment (Owen et al., 1992; Hendry et al., 1992). Although widely used, a need remains to improve the cure rate by radiation therapy alone. The most frequent treatment is to combine cytotoxic chemotherapeutic agents with irradiation (McGinn et al., 1996; Stratford et al., 1996). The cytotoxicity of chemotherapeutic agents, however, is not limited to tumor cells because treatment of tumors with these agents can result in significant normal tissue toxicity (Archer et al., 2003).

There has been a recent rapid development of a new class of drugs termed antiangiogenesis or vascular targeting agents that target the relatively genetically stable tumor-associated vasculature (endothelial cells) rather than the genetically unstable tumor cell (Kerbel et al., 1991; Lin et al., 1998). The importance of targeting tumor vasculature development and function first became apparent in the 1970s through the seminal studies of Judah Folkman (Folkman, 1971), who demonstrated that angiogenesis is important for the growth and survival of tumor cells. The relationship between angiogenesis and tumor growth suggests that both tumor cells and their supporting endothelial cells are potential targets for cell killing and should be considered when planning cancer treatment (Camphausen et al., 2002). At least four theoretical advantages exist for considering tumor endothelial cells as targets for cancer therapy: (*a*) endothelial cells are more easily accessed by antiangiogenic/vascular targeting agents compared with drugs that act on tumor cells directly and have to penetrate large bulky masses; (*b*) antiangiogenic/vascular targeting agents may avoid tumor drug resistance mechanisms because they are directly cytotoxic to endothelial cells; (*c*) angiogenesis occurs in very limited circumstances in adults (wound healing and ovulation), thus antiangiogenic therapies targeting specific receptors on proliferating tumor endothelium potentially are safe and should avoid normal tissue toxicities; and (*d*) because each tumor capillary potentially supplies hundreds of tumor cells, targeting of the tumor vasculature should lead to a potentiation of the antitumorigenic effect (Scappaticci et al., 2002).

Investigations of antiangiogenic/vascular targeting agents that have been conducted in

preclinical and clinical trials indicate that tumor cures are limited when these agents are used as the sole method of treatment (Kerbel et al., 2002). Some recent preclinical studies suggest that the combination of radiotherapy and angiogenic blockade enhances the therapeutic ratio of ionizing radiation by targeting both tumor cells and tumor vessels (Gorski et al., 1998; Mauceri et al., 1996; Mauceri et al., 1998). At present, there is great interest in combining antiangiogenic/vascular targeting strategies with conventional cytotoxic therapies such as radiotherapy to improve therapeutic gain. The mechanisms by which tumor response to radiation is enhanced by these new agents, however, are not currently understood.

## 2.1 Role of angiogenesis in primary tumor growth and metastasis

A competent and expanding vascular supply is a necessary component of the progressive growth of solid tumors because cells in solid tumors, like normal tissue, must receive oxygen and other nutrients to survive and grow (Folkman, 1976; Jain, 1988; Jain, 1999). The connection between oxygen supply and tumor growth was first made in the 1950s, histological analyses of human and rodent tumors performed by Thomlinson and Gray (Thomlinson and Gray, 1955) were the first studies to suggest that regions of viable cells exist close to tumor blood vessels and that these walls or cords of viable tumor cells correspond in thickness to the distance that oxygen can diffuse (1–2 mm$^3$). The "tumor cord" model implied that hypoxic cells exist in a state of oxygen and nutrient starvation at the limits of the diffusion range of oxygen, and it was hypothesized that tumor cells could proliferate and grow only if they were close to a supply of oxygen from tumor stroma. In the 1970s, Folkman (Folkman, 1971) corroborated the earlier findings and proposed the importance of tumor vasculature as a viable target for anticancer therapy. He reported that a tumor without an adequate blood supply would grow only to a few thousand cells in size or around 1–2 mm$^3$, which is the distance that nutrients can enter tumor cells by passive diffusion (Folkman, 1971). To increase in size beyond this passive diffusion-limited state, the growing tumor mass must acquire new blood vessels. Thus angiogenesis is a necessary condition to maintain tumor growth and their subsequent metastasis (Fig. 1).
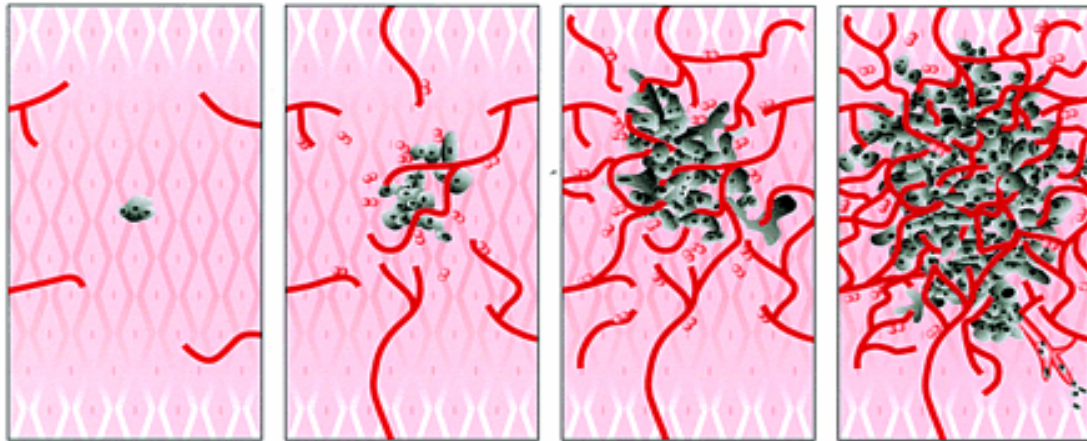
Fig. 1: Angiogenesis is a necessary condition for sustained tumor growth (Ferrara et al., 2004).

## 2.2 Actions of VEGF in angiogenesis

A switch to the angiogenic phenotype allows the tumor to expand rapidly. This so-called "angiogenic switch" (Hanahan et al., 1996) is regulated by environmental factors and by genetic alterations that act to either up-regulate proangiogenic factors (*i.e.,* VEGF and basic fibroblast growth factor) (Rak et al., 2001) and transforming growth factors (TGF-α and TGF-β) and/or downregulate inhibitors of angiogenesis [*i.e.*, angiostatin, endostatin, thrombospondin, and Alpha interferon (IFN-α) (Los et al., 2001)]. Tumor angiogenesis is a multistep process of degradation of the extracellular matrix, migration and proliferation of endothelial cells from postcapillary venules, and, finally, tube formation (Carmeliet et al., 2000). More than 20 endogenous activators and inhibitors have been identified in this process (Scappaticci et al., 2002). VEGF is the most potent and specific growth factor for endothelial cell activation (Ferrara et al., 2001). Evidence for the importance of VEGF-induced angiogenesis in tumor growth was demonstrated by use of neutralizing antibodies or a dominant-negative soluble receptor to inhibit VEGF action and growth of primary and metastatic experimental tumors (Gorski et al., 1999, Asano et al., 1995). VEGF also functions as a powerful antiapoptotic factor for endothelial cells in new blood vessels (Benjamin et al., 1999).

It has been described that angiogenesis is a multistep process, and VEGF acts at several stages to induce the angiogenesis (Fig. 2). *(1)* VEGF is a potent mitogen for vascular endothelial cells, but, with a few exceptions, is not mitogenic for other cell types (Ferrara et al., 1997). *(2)* VEGF mediates the secretion and activation of enzymes involved in degrading the extracellular matrix (Unemori et al., 1992; Lamoreaux et al., 1998). *(3)* VEGF acts as a survival factor for endothelial cells through the inhibition of apoptosis (Alon et al., 1995). This action is mediated through the induction of expression of the antiapoptotic proteins: B-cell lymphoma gene-2 and A1 protein, regulation of the phosphatidylinositol 3-kinase/Akt pathway, increased phosphorylation of focal adhesion kinase, and stimulation of endothelial cell production of prostaglandin I2 (Ferrara et al., 2001; Zachary et al., 2001). *(4)* VEGF is essential for the mobilization of bone marrow-derived endothelial cell precursors in the promotion of vascularization (Asahara et al., 1999; Bertolini et al., 2000). *(5)* In addition to promoting division of endothelial cells, VEGF also has an important role in modulating their migration to sites of angiogenesis (Rousseau et al., 2000). Other actions of VEGF include increasing vascular permeability (Senger et al., 1983), inhibition of dendritic cell differentiation (Gabrilovich et al., 1998), and induction of monocyte migration (Barleon et al., 1996).
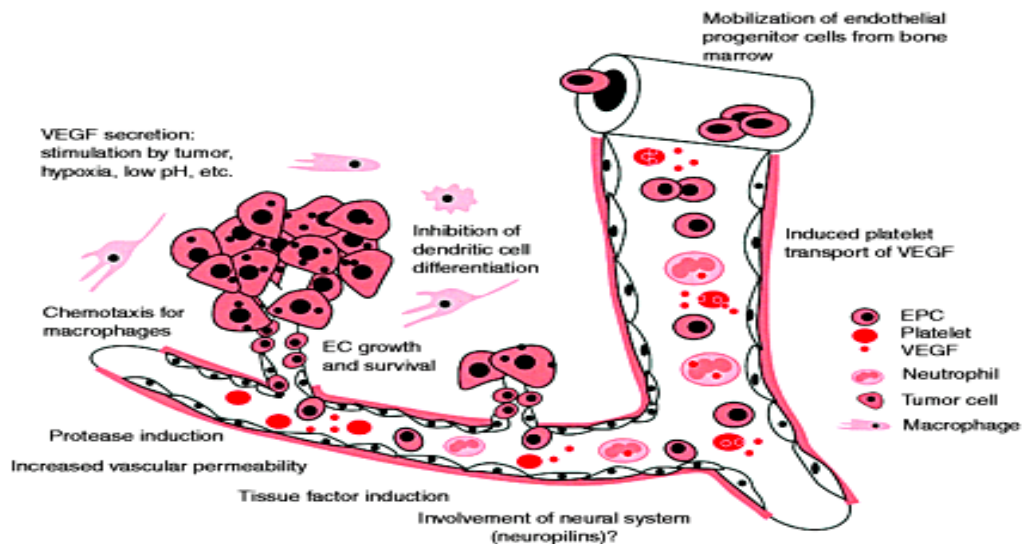


Fig. 2: Multiple functions of VEGF. Abbreviations: EC = endothelial cell; EPC = endothelial progenitor cell (Toi et al., 2001).

Many factor can induce the expression of VEGF, especially, VEGF is secreted by almost all solid tumors (Leung et al., 1989; Bellamy et al., 1999). Factors that lead to up-regulation of VEGF expression and secretion include external stresses such as ionizing radiation and tumor microenvironment factors such as hypoxia or a decrease in pH (Levy et al., 1995; Shweiki et al., 1995; Mukhopadhyay et al., 1995). Moreover, other growth factors stimulate VEGF production including insulin-like growth factor I and II, epidermal growth factor, and platelet-derived growth factor. Two specific signaling pathways are known to mediate the up-regulation of VEGF: (*a*) the phosphatidylinositol 3'-kinase/Akt (protein kinase B) signal transduction pathway, which leads to stabilization of hypoxia-inducible factor1α (Maxwell et al., 1997; Mazure et al., 1997); (*b*) The mitogen-activated protein kinase (MAPK) pathway, in which activation of extracellular signal-regulated kinase increases transcription of the VEGF gene (Fig. 3 summary of the VEGF signaling mechanisms).
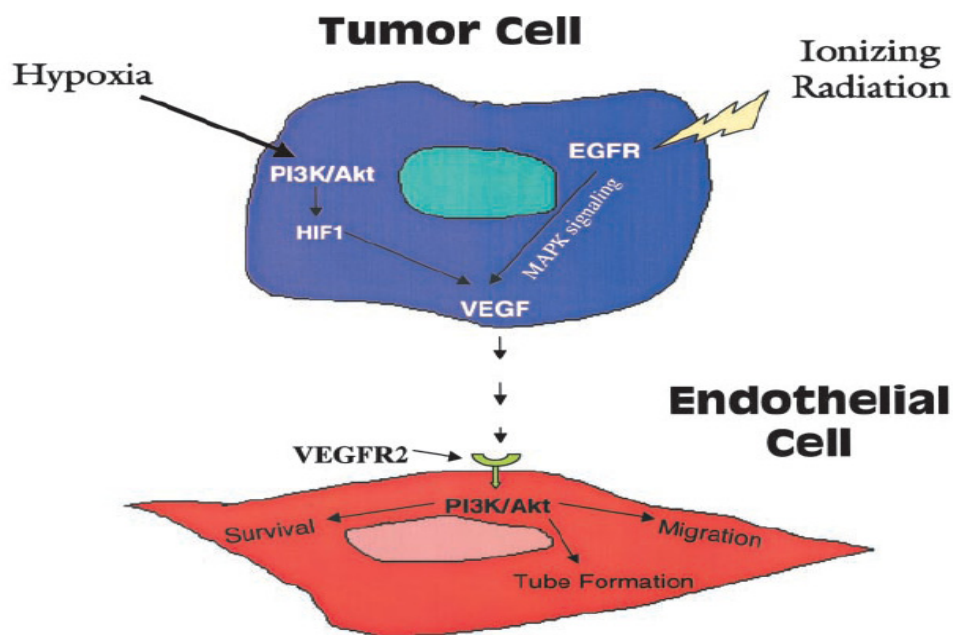


Fig. 3: Signaling mechanisms involved in VEGF induced tumor angiogenesis. Endothelial cell survival and neovascular processes are induced by VEGF/VEGFR2 signaling via PI3K/Akt signaling axis. VEGF is upregulated by external environmental stress (*i.e.*, ionizing radiation) and internal tumor microenvironmental stress (*i.e.*, hypoxia) (Jung et al., 1999).

Although the growth of solid tumors depends on angiogenesis from generation of a vascular network, the amount of newly formed vessels in the stroma of tumor does not necessarily lead to increased blood flow (Koukouraki et al., 2001). This inequality exists because newly formed microvessels in most solid tumors are abnormal when compared with the morphology of the host tissue vasculature (Vaupel et al., 1997). Endothelial cell lining of tumor blood vessels differ in many aspects with regard to gene expression from those of normal vasculature (St Croix et al., 2000). Due to the lack of adequate vascular maturation (Eberhard et al., 2000), vessels often have an incomplete or missing endothelial lining, and have interrupted basement membranes that result in an increased vascular permeability (Vaupel et al., 1997). Moreover, blood flow is often erratic, with even reversal of blood flow within individual vessels (Less et al., 1991). Consequently, oxygen availability to the tumor cells shows great variability. Many human tumors exhibit hypoxic regions that are heterogeneously distributed within the tumor mass (Movsas et al., 2002). Low perfusion rates and hypoxia may then coexist with high nonfunctional vascular density, creating hypoxic regions (Eberhard et al., 2000).  In these regions of hypoxia, endothelial cells may up-regulate survival factors to maintain their integrity and prevent apoptosis (Reinmuth et al., 2001). Thus, so-called "angiogenic hot spots" or localized regions of intense angiogenesis may be created and may be associated with failure of treatment (Koukourakis et al., 2001).

## 2.3 Radiation and angiogenesis: a vicious cycle

Tumor vasculature is abnormal, and the endothelial cell lining of tumor blood vessels have different phenotypic properties compared to those of normal vasculature (Eberhard et al., 2000). Consequently, increased tumor angiogenesis, as indicated by increased microvessel density or by increased VEGF expression, does not necessarily correlate with increased blood flow and oxygen availability. This situation, together with the existence of heterogeneous hypoxic regions within tumors, makes it difficult to predict how tumor angiogenesis will affect response to radiation therapy in a particular tumor.

Strong evidence exists that cytotoxic therapy alone, such as irradiation, can result in intensification of angiogenic processes (Koukourakis et al., 2001). Direct up-regulation of VEGF after irradiation of various cancer cell lines has been reported (Gorski et al.,

1998). This response is part of the overall cellular response to stress and is associated with the induction of a variety of transcription factors that can activate transcription of cytokines, growth factors, and cell cycle-related genes (Takahashi et al., 1998). The products of these genes regulate intracellular signaling pathways through tyrosine kinases, MAPKs, stress-activated protein kinases, and ras-associated kinases (Rak et al., 2002). These multiple pathways affect tumor cell survival or alter tumor cell proliferation. With regard to angiogenesis, radiation exposure can result in activation of the EGFR, which, in turn, can activate the MAPK pathway (Bowers et al., 2001). MAPK signaling is linked to increased expression of growth factors, such as TGF-$\alpha$ and VEGF. It is possible that radiation therapy itself contributes to radioresistance by upregulating and intensifying angiogenic pathways. The increased proliferation of tumor cells that is often seen after irradiation may be the result of up-regulated angiogenic pathways (Koukourakis et al., 2001). Although many tumors reoxygenate after irradiation, in tumors that are unresponsive to radiation therapy, up-regulated angiogenesis may lead to factors contributing to radiation resistance such as increased vascular permeability, decreased tumor perfusion, increased oxygen consumption, and up-regulated survival pathways (Hansen et al., 2000; Lee et al., 2000). Thus angiogenesis contributes to making radiation therapy less effective in some tumors (Kung et al., 2000; Teicher et al., 1994; Vaupel et al., 1989).

## 2.4 Radiation and antiangiogenic interactions

The existence of tumor microenvironmental factors, such as hypoxia, that can up-regulate angiogenic and survival pathways and hence induce resistance to radiation, therapy has prompted studies combining antiangiogenic agents with irradiation in an effort to overcome this resistance. Teicher *et al.* (Teicher et al., 1995) were the first to show an increased response to single-dose radiotherapy with antiangiogenic agents. Since that time a number of preclinical studies have indicated that antiangiogenic agents can enhance the tumor response to irradiation (Hess et al., 2001; Huang et al., 2002). The differences in tumor response to antiangiogenic agents may come from differences in angiogenic growth patterns arising from different angiogenic growth factors such as VEGF/VEGFR or EGFR. Radiotherapy plays a role in upregulating the expression of

VEGFR and EGFR, both of which are considered key targets for novel anticancer therapies. Data from various studies suggest that inhibitors of VEGFR- and EGFR-dependent signaling may enhance the cytotoxic effects of radiotherapy (Hess et al., 2001; Huang et al., 2002).

## 2.4.1 Radiation and VEGF/VEGFR signaling pathway inhibitors

Vascular endothelial growth factor has a critical role in pathological angiogenesis including sustained neovascularization that is required for all solid tumor growth. The signaling response is transmitted to the tyrosine kinase activity of the VEGF family of transmembrane receptors. Tyrosine kinase activation is stimulated after VEGF ligand binding and receptor dimerization. VEGF is a potent stimulator of endothelial cell proliferation, migration, and survival. In addition, VEGF acts as an important endothelial survival factor in newly formed vessels and stimulates vessel hyperpermeability, which may contribute to the high interstitial pressure commonly observed in solid tumors. The critical importance of VEGF in the growth of experimental tumors has been demonstrated when stasis or regression was observed after treatment with neutralizing antibodies (Kim et al., 1993; Asano et al., 1995; Kanai et al., 1998). Consequently, the development of clinically applicable inhibitors of VEGF signaling has been an area of avid research (Underiner et al., 2004), these can target either the VEGF protein directly or inhibit the activation of the cognate receptors. Of the three major receptors for VEGF family ligands [Flt-1 (VEGFR1), KDR (Flk-1; VEGFR2), and Flt-4 (VEGFR3)], VEGFR-2 is considered the most important receptor for mediating the angiogenic effects of VEGF. Therefore, inhibition of VEGFR-2 mediated tumor angiogenesis has become an important therapeutic goal. (Meyer et al., 1999; Zeng et al., 2001; Gille et al., 2001).

In addition to the effect of VEGF on tumor angiogenesis and growth, VEGF can also play an important role in the response of tumors to radiotherapy. This appears to be predominantly achieved through the ability of VEGF to enhance endothelial cell survival (Gorski et al., 1999; Geng et al., 2001; Hess et al., 2001), recently suggested to be the critical factor determining tumor radiation response (Garcia-Barros et al., 2003). These observations raise the possibility of combined therapeutic strategies, although there are complexities surrounding the application of antiangiogenic agents in a radiotherapy

context. In particular, inhibition of VEGF signaling has the potential to impact on tumor oxygenation and proliferation kinetics, which could have profound effects on the response to irradiation (Griffin et al.,.2002).

## 2.4.2 Radiation and EGFR signaling pathway inhibitors

Aberrant EGFR tyrosine kinase activity has been reported in a number of human tumors and may contribute to tumor growth and the development of metastases (Harari et al., 2004). Radiation exposure not only results in activation of EGFR, but also upregulates the downstream MAPK pathway. MAPK signaling in turn is associated with increased expression of other growth factors such as TGF-$\alpha$ and VEGF (Park et al., 2001).

Several preclinical studies have been conducted with inhibitors of EGFR signaling in combination with radiotherapy. Administration of cetuximab (C225), the humanized antibody against EGFR, improved the response to radiotherapy in xenograft models of non–small-cell lung cancer (Raben et al., 2005) and epidermoid carcinoma (Milas et al., 2000). Similarly, combining the EGFR tyrosine kinase inhibitor gefitinib with radiotherapy produced a cooperative antitumor effect in human colon and lung cancer xenografts (Bianco et al., 2002). Furthermore, clonogenic assays revealed a significant radiosensitizing action of gefitinib in bladder cancer cell lines (Maddineni et al., 2005). The potential mechanisms underlying the enhanced response to radiotherapy in the presence of EGFR signaling inhibition remain to be determined. However, possible explanations include enhanced tumor cell apoptosis and inhibition of EGFR-dependent production of endothelial cell survival factors such as VEGF. Of particular interest are the results from a randomized Phase III trial investigating high dose radiotherapy alone or in combination with cetuximab (humanized antibody against EGFR) in advanced head-and-neck cancer (Bonner et al., 2004). Patients who received combination therapy showed a significant prolongation of overall survival compared with those receiving radiotherapy alone. These highly promising data will encourage continued clinical evaluation of radiotherapy and inhibitors of EGFR as combination therapy.

## 2.5 ZD6474, an inhibitor of VEGFR and EGFR

ZD6474       [N-(4–bromo-2–fluorophenyl)–6–methoxy–7-[(1–methylpiperidin–4-yl) methoxy] quinazolin-4-amine], is a potent inhibitor of both VEGFR and EGFR tyrosine kinase activity (Wedge et al., 2002). ZD6474 inhibits both VEGFR- and EGFR-mediated intracellular signaling, and its use in combination with radiotherapy may provide significant clinical efficacy (Sandström et al., 2008; Shibuya et al., 2007). Simultaneous inhibition of both VEGFR and EGFR signaling may present a novel and exciting opportunity to augment the antitumor effects of radiotherapy (Fig. 4).
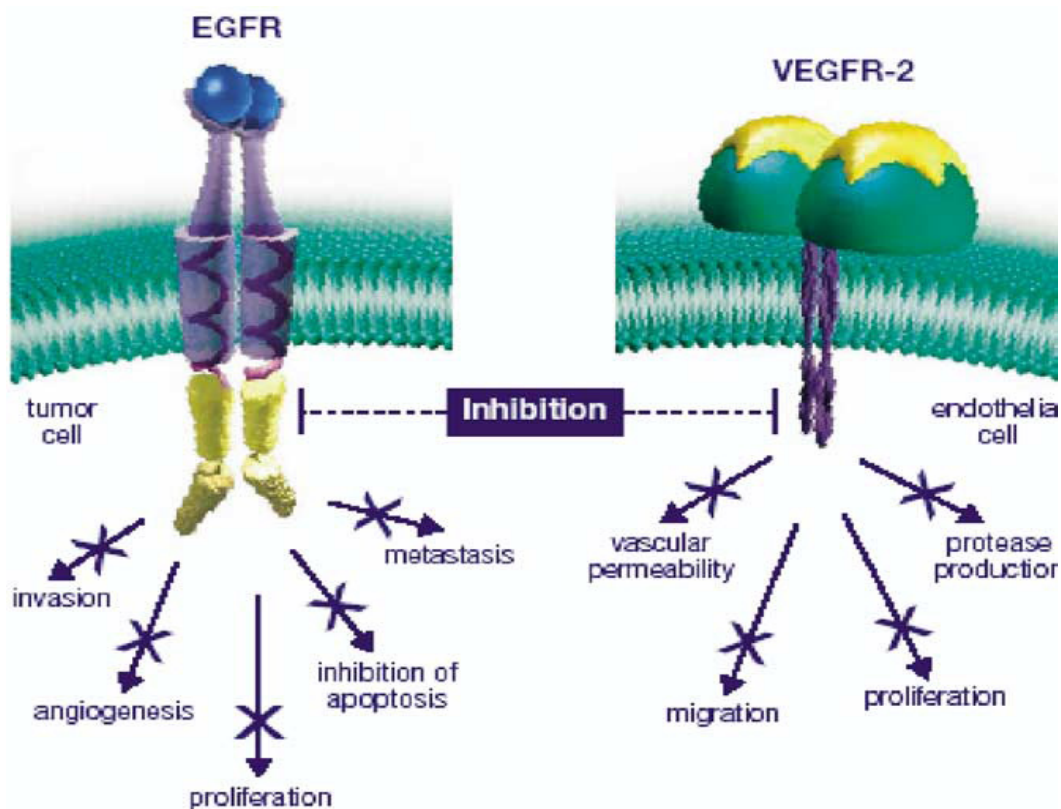


Fig. 4: Mechanism of action of ZD6474, which inhibits both VEGFR and EGFR tyrosine kinase activity (Frederick et al., 2006).

## 2.5.1 ZD6474 enhances the therapeutic efficacy of radiotherapy

Preclinical evidence supporting the combined use of ZD6474 and radiotherapy has recently been reported. One study investigated the effects of ZD6474 and radiotherapy on human umbilical vein endothelial cell proliferation, as well as on tumor growth in a human non–small-cell lung cancer xenograft model (H226 and A549). *In vitro,* ZD6474 inhibited human umbilical vein endothelial cell proliferation and induced accumulation of cells in G1, inhibited the formation of capillary-like networks, and enhanced the radiosensitivity of human umbilical vein endothelial cell in combination with radiotherapy. *In vivo,* the combination of daily oral ZD6474 plus radiotherapy produced significantly greater antitumor effects in H226 and A549 tumor xenografts compared with single-agent therapy alone (Hoang et al., 2006).

## 2.5.2  ZD6474 — overcoming resistance to EGFR inhibition

ZD6474 may also be a useful treatment option in the setting of acquired EGFR resistance. Overcoming resistance to EGFR signaling inhibition has been examined in a recent study from Ciardiello *et al.* (Ciardiello et al., 2004). Human colon cancer xenografts that had developed resistance to inhibitors of EGFR activity (gefitinib or cetuximab) were subsequently exposed to daily dosing of ZD6474. Significant tumor growth inhibition for the entire duration of dosing (up to 150 days) was observed with administration of ZD6474; in contrast, animals bearing gefitinib- or cetuximab-resistant tumors failed to respond when treatment with either EGFR inhibitor was reinitiated after a treatment break. Western blot analysis revealed increases in proangiogenic factors in the resistant GEO colon tumor cell lines, suggesting a potential mechanism underlying the strong antitumor effects of ZD6474 in these experiments.

## 2.5.3 Pharmacokinetic dosing of ZD6474 in vivo

Initial preclinical studies with ZD6474 in xenograft models have shown activity against a variety of tumor types using doses ranging from 12.5 to 100 mg/kg/d in mouse models (Wedge et al., 2002). In these initial studies, most human tumor xenografts continued to grow, albeit slowly at doses < 50 mg/kg/d. The most marked antitumor effects, including regression, usually required dosing at > 50 mg/kg/d (Gustafson et al.,

2004). Therefore, preclinical studies may provide more predictive information on treatment application of ZD6474.

The pharmacokinetics of ZD6474 in humans is linear, with a long terminal half-life (at least 100 hours) in both Japanese (Minami et al., 2003) and Western populations (Holden et al., 2005). At doses of 50–600 mg/d, the plasma levels at steady-state range of 100–1,100 ng/mL. Steady-state levels are attained only after 20–30 days of daily drug dosing owing to the long half-life. The terminal half-life of ZD6474 in mice is significantly shorter (30 h) than that seen in humans (Zirrolli et al., 2005). Overall, the pharmacokinetics of ZD6474 are consistent with the maintenance of therapeutic levels after once-daily treatment, and dosing in animal models can be used according to approximate human exposure.

## 2.5.4 Schedule optimization of radiotherapy and ZD6474

Schedule optimization of radiotherapy and antiangiogenic combination therapy may differ among a variety of tumor types. Williams *et al.* (Williams et al., 2004) assessed the *in vivo* effect of chronically administered ZD6474 (25 or 50 mg/kg/d), with or without radiotherapy (three fractions of 2 Gy on days 1–3) in a Calu-6 human lung cancer xenograft model. Two schedules were examined: *(1)* ZD6474 dosing initiated 2 h before the first radiation dose (concurrent schedule), and *(2)* ZD6474 dosing initiated 30 min after the last radiation dose (sequential schedule). The growth delay induced using the concurrent schedule was significantly greater than that induced by either ZD6474 or radiotherapy alone. However, when administered sequentially, the tumor growth delay was markedly enhanced. Recent studies by Gustafson *et al.* (Gustafson et al., 2004) of a head-and-neck squamous cell carcinoma xenograft model indicated that concurrent administration of ZD6474 and radiotherapy significantly delayed tumor growth and was superior to single modalities or sequential combination therapies. The antitumor effects of ZD6474 doses was also examined in a human colorectal cancer xenograft model (HT29), either alone or combined with radiotherapy (Siemann et al., 2004). Three different schedules of combination therapy were assessed: ZD6474 before radiotherapy, concurrent administration, and ZD6474 after radiotherapy. In that model, irrespective of sequencing, concurrent therapy resulted in a significantly greater growth delay than either

radiotherapy or ZD6474 treatment alone. The *in vivo* effects of combination therapy correlated with a reduction in VEGF expression.

The rationale for the development of VEGF signaling inhibitors in combination with radiotherapy was originally based on the observation that VEGF could enhance endothelial cell survival (Gorski et al., 1999). Consequently, a number of studies have shown that VEGF-targeting approaches can induce endothelial cell radiosensitization in vitro (Gorski et al., 1999; Geng et al., 2001). Some studies have suggested that VEGFR-blockade can lead to a transient normalization of the vasculature, leading to a window of opportunity when perfusion, oxygenation, and consequently, radiation response may be improved (Tong et al., 2004; Winkler et al., 2004). It is proposed that optimal treatment scheduling corresponds to administration of irradiation during a period of vascular normalization that is induced by the anti-VEGFR-2 therapy (Jain, 2001). Because tumors induce high levels of VEGF and angiogenesis, the antiangiogenic drug or VEGFR-blockade administration serves to balance this pathologic angiogenesis. Therefore, it is suggested that this period of normalization represents a balance between proangiogenic and antiangiogenic factors that transiently improves tumor blood flow and oxygenation (Jain, 2005). Because hypoxia is known to decrease the efficacy of radiotherapy (Wachsberger et al., 2003), giving radiation during this normalization window could serve to enhance treatment before the tumor vessels are irreversibly damaged and tumor blood flow is diminished by irradiation.

Overall, ZD6474, is a potent (half inhibit concentration, IC50, 40 nmol/L), orally active, low molecular weight inhibitor of VEGF, and has additional activity *versus* the EGFR tyrosine kinase (IC50, 500nmol/L) (Ciardiello et al; 2003). Chronic oral dosing of mice bearing human tumor xenografts with ZD6474 has been previously shown to induce a dose-dependent inhibition of tumor growth (Wedge et al., 2002). And some results have suggested that ZD6474 may be a successful agent combined with clinical radiotherapy in a variety of tumor types (Gustafson et al., 2008; Bianco et al., 2006; Damiano et al., 2005). However the optimal scheduling of treatments when ZD6474 combined with radiotherapy will continue to be an important issue to be assessed.

## 2.6 Aim of the project

ZD6474 is a newly developed anti-angiogenic agent that selectively targets two key pathways in tumor growth by inhibiting VEGFR and EGFR tyrosine kinase activity. While VEGFR signaling promotes an array of responses in the endothelium including endothelial cell proliferation and angiogenesis, EGFR signaling regulates principal cellular processes including proliferation, differentiation and apoptosis after cytotoxic stress. The purpose of the present study was to evaluate the antitumor efficacy of a combination of ZD6474 and radiotherapy in a human tumour xenograft model (Fadu, squamous cell carcinoma) and to investigate whether the effects of the treatments are related to changes in tumor perfusion and tumor proliferation and apoptosis.

# 3. Materials and Methods

## 3.1 Tumor cell line

Fadu tumor cells (squamous cell carcinoma of head and neck) were maintained as a monolayer in RPMI 1640 supplemented with 10% fetal bovine serum and 0.01% (v/v) L-glutamine and penicillin/streptomycin. All cells were maintained at 37°C in a 5% $CO_2$ incubator.

## 3.2 Animal cares

Female athymic nude mice (*nu/nu*, body weight, 20–25 g, 8–12 weeks of age), were purchased from Charles River Laboratories, Inc (Wilmington, MA). All mice were provided sterilized food and water and housed in a barrier facility with 12-h light and dark cycles and in laminar flow hoods with constant temperature and humidity for the whole course of the experiments and supplied with standard laboratory diet and water (Sano et al., 2007; Brazelle et al., 2006). The mice were allowed to acclimatize from shipping for 1 week before use. The care and treatment of all experimental mice was in accordance with institutional guidelines.

## 3.3 Tumors xenografts

2 days before the tumor xenografts were established, all the mice were whole-body irradiated with 4 Gy (6-MV linear accelerator, MDX, Siemens) to suppress the immunoreactions. Fadu cells were prepared at a concentration of 2 x $10^7$cells/mL in a 1:1 mix of serum-free RPMI1640, and were intradermal injected to the right hind leg of the mice with.

## 3.4 Drug and/or radiation treatment schedules

After 7 days, when established tumor size reached at about 6 mm in diameter, all the mice bearing Fadu tumors were randomly assigned to 4 study groups (*n* = 6–8 mice per group):

**Control group:**

No treatment was given to the mice.

**ZD6474 alone group:**

ZD6474 was administered daily by oral gavage (50 mg/kg, at volume of 0.1 mL/10g of body weight) for a period of 4 weeks (Monday to Friday).

**Radiotherapy alone group:**

Tumor irradiations were performed using a 6-MV linear accelerator, and localized radiotherapy was administered on anesthetized mice confined in plastic jigs. The tumor bearing limb was extended through an opening of the jig, allowing local tumor irradiation. The total dose was 10 Gy ($5 \times 2$ Gy, days 1–5).

**Combination treatment with ZD6474 and radiotherapy group:**

ZD6474 (50 mg/kg, by mouth, once daily) was given 2 hours before each dose of radiation ($5 \times 2$ Gy, days 1–5).

## 3.4 Tumor response assessment

Tumor size was measured using callipers every two days from the time of initial treatment to the time tumor size reached 2.5 cm$^3$. Tumor volumes (V) were determined by the two axes of the tumor (L, longest axis; W, shortest axis). Volume was calculated according to the following formula as:

Tumor volume (cm$^3$) = (L x W$^2$) x $\pi$/6, where L and W are the shortest and the longest diameter (Hessel et al., 2004).

The tumor size and days of tumor growth were recorded until tumor volume reached 2.5 cm$^3$.

## 3.5 Immunohistochemical evaluation of the xenograft tumors

Mice were implanted with Fadu cells, after 6 to 8 days, mice were divided into 4 groups to receive different treatment as described above in the tumor volume studies. In the drug treatment group, the mice were treated with 50 mg/kg ZD6474 p.o. daily for 5 days. Mice in the radiation treatment group were treated with 2 Gy daily fractions for 5 days, given by use of a 6 MV linear accelerator as described above in the tumor volume studies, and in combination treatment group, drug was delivered 2h before daily

irradiation. One week after 5 days treatments, mice were killed and the xenograft tumors sections were separated, tumor tissues from four mice from each treatment group were fixed and embedded in paraffin, then sectioned in 5 μm.

## 3.5.1 Evaluation of vessel density

To evaluate angiogenesis and vessel density, the antibody against CD34 was used. CD34 is a myeloid progenitor cell antigen and also present in endothelial cells, it can be detectable in all types of endothelium (Folkman, 1995). The monoclonal antibody against CD34 reacts with endothelium of arteries and venules, and has been found to stain more intensely capillary endothelium (Fina et al., 1990). It has been used for the diagnosis of vascular tumors and detection of small vessel proliferation representing angiogenesis (Schaerer et al., 2000).

To evaluate vessel density, an immunofluorescence technique and antibody against CD34 were utilized. Briefly After deparaffinization and rehydration using the following series of washes: two xylene washes (10 min each), followed by 100% ethanol, 96% ethanol, 90% ethanol, 80% ethanol, 70% ethanol rinses (2 min each), the sections were washed by $H_2O$ for 1 min. Antigen retrieval was performed by heating the sections for 60 min with citrate buffer (DAKO Retrieval puffer, #S-2031, Glostrup, Denmark pH 6.0) at 96°C for unmasking the antigenic sites. After washing the sections with PBS for 5 min on a shaker, samples were blocked with normal goat serum (cat. #642921 ICN, Irvine, CA, USA) at room temperature for 60 min in order to diminish non-specific binding sites. Afterwards the tissue sections were incubated with primary monoclonal antibody of rat anti-mouse CD34 (United States Biological Inc, Massachusetts, Cat #C2386-02C) at a dilution of 1:100 in PBS in the humidified chamber overnight at 4°C, then the sections were incubated with biotinylated Alexa Fluor 488-conjugated goat anti-rat secondary antibody IgG (Invitrogen, Karlsruhe, Germany, cat. #A11006) diluted 1:200 in PBS in a humidified chamber for 60 min at room temperature in dark. Finally the sections were counterstained with DAPI and mounted in Entellan (Merck, Darmstadt, Germany) at 4°C overnight protected in the dark. Between each step the sections were washed by PBS (3x10 min).

Sections were examined on a Nikon fluorescent microscope. Blood vessel density

was quantified that each slide was scanned at low power (x10-100) and the area with the higher number of new vessels was identified (hotspot). This region was then scanned at x600 microscope magnification. Three fields (0.15 mm$^2$ per microscopic field) were analyzed and, for each of them, the number of stained blood vessels was counted. For individual tumors, microvessel count was scored by averaging the three field counts (Sano et al., 2007).

## 3.5.2 Evaluation of tumor proliferation and apoptosis

To evaluate the prolifreration of tumor tissue, the proliferation antigen Ki67 was used to assess the proliferating rate of tumor in different treatment group. The proliferation antigen Ki67 is detectable in cells at all phases of the cell cycle except G0 (Gerdes et al., 1983), and the Ki67 labeling index (the percentage of cells with Ki67 positive nuclear immunostaining) is a measure of tumor proliferation (Lehr et al., 1999; Thor et al., 1999). Other studies (Chang et al., 2000; Archer et al., 2003) have suggested that a high Ki67 labeling index can be as a predictor of responsiveness to adjuvant therapy. To evaluate the prolifreration of tumor tissue, the antibody against Capase-3 was used. Capases are family of cytosolic aspartate specific cysteine proteases involved in the initiation and execution of apoptosis. Capase-3 is a member of apoptosis execution group of capases, it can be activated when cells undergo apoptosis (Nicholson et al., 1995).

The immunofluorescence technique and antibody against Ki67 and Capase-3 were utilized. After deparaffinization and rehydration using the following series of washes: two xylene washes (10 min each), followed by 100% ethanol, 96% ethanol, 90% ethanol, 80% ethanol, 70% ethanol rinses (2 min each), the sections were washed by H$_2$O for 1 min, antigen retrieval was performed by heating the sections for 60 min with citrate buffer (DAKO Retrieval puffer, #S-2031, Glostrup, Denmark pH 6.0) at 96°C for unmasking the antigenic sites. After washing the sections with PBS for 5 min on a shaker, samples were blocked with normal goat serum (cat. #642921 ICN, Irvine, CA, USA) at room temperature for 60 min in order to diminish non-specific binding sites. Afterwards the tissue sections were incubated with primary monoclonal antibody of rat anti-mouse Ki67 (at a dilution of 1:200, Dako Gmbh, Hamburg, Germany) or rabbit anti-Capase3 (1:100, Epitomic Inc, Burlingam CA, USA) in the humidified chamber overnight at 4°C,

then the sections were incubated with biotinylated Alexa Fluor 488-conjugated goat anti-rat (Invitrogen, Karlsruhe, Germany, cat. #A11006) or goat anti-rabbit (Invitrogen, Karlsruhe, Germany, cat. #A11008) secondary antibody IgG diluted 1:200 in PBS in a humidified chamber for 60 min at room temperature in dark. Finally the sections were counterstained with DAPI and mounted in Entellan (Merck, Darmstadt, Germany) at 4°C overnight protected in the dark. Between each step the sections were washed by PBS (3x10 min).

Sections were examined on a Nikon fluorescent microscope. To determine the percentage of positive cells with Ki67, at least 500-1000 tumor cells per slide were counted, the number of Ki67-positive cell was scored and the positive rate was counted. To quantify the analysis of apoptosis, the number of apoptotic cells or Capase-3 stained positive cells was counted in 5 fields (0.15 mm$^2$ per microscopic field) under x600 microscope magnification in each tumor section per treatment group; results were expressed as positive cells per field.

# 4. Results

## 4.1 Assessment of tumor growth delay

### 4.1.1 Inhibition of xenograft growth in nude mice

We evaluated the effects induced by radiotherapy and ZD6474 treatment in an *in vivo* model. 1 week after tumor cell injection, tumor size reached about 6 mm in diameter; all the mice were treated differently in 4 groups according to the plan. When tumor volume reached 2.5 cm$^3$, mice were sacrificed. The progress of tumor growth was recorded every other day. The tumors in the control group (untreated mice) needed about 28 days to reach a volume of 2.5 cm$^3$. At the same time point, the tumor growth in mice treated with either radiotherapy alone or ZD6474 alone or combination treatment with ZD6474 and radiotherapy was markedly inhibited. Respectively, it needed about 40 days, 45 days and 49 days for the tumor volume to reach 2.5 cm$^3$. So the inhibition effect in combination treatment with ZD6474 and radiotherapy group was better than ZD6474 alone group, and better than radiotherapy alone group (Fig. 5; Table 1).

### 4.1.2 ZD6474-mediated tumor growth inhibition

In the present study, ZD6474 has shown to produce a significant inhibition of Fadu cell xenograft growth. Fadu cell is an aggressive tumor cell line with high proliferating rate, the time taken for control tumors to achieve 2.5 cm$^3$ was about 28 days. However, at the same time the mean tumor volume in ZD6474 alone group was 1.0 cm$^3$, far smaller than in control group, it was about 45 days for ZD6474 alone group to reach end point, the difference between ZD6474 alone group and control group is obvious. (Fig. 5; Table 1). Thus ZD6474 treatment induced a highly significant inhibition of tumor growth in the present study.

## 4.1.3 ZD6474 combined with radiotherapy produces an enhancement of antitumor activity

The potential of ZD6474 to enhance the outcome of radiotherapy in Fadu cell xenografts was investigated in a concurrent schedule whereby 50 mg/kg ZD6474 was given 2 hours before radiation (2 Gy per day) every day for a period of 5 days. When tumor in radiotherapy alone group reached 2.5 cm$^3$, the mean tumor volume was 0.7 cm$^3$ in ZD6474 combined with radiotherapy treatment group. The time to achieve the end point in the combined treatment group was about 49 days, obviously greater than that seen in tumors treated only with radiotherapy (40 days) or only with ZD6474 (45 days), that there was an apparent difference between radiotherapy and ZD6474 combined with radiotherapy treatment (Fig. 5; Table 1).

Table.1: Chronic daily dosing of ZD6474 (50 mg/kg) enhances the response of Fadu xenografts to fractionated radiotherapy

| Treatment | Mean days (1) when Tumor volume (2.5cm$^3$) | Mean days (2) Growth delay |
|---|---|---|
| Control | 27.5 ± 1.2 | NA |
| Radiotherapy alone (RT) | 40.0 ± 0.7 | 12.5 ± 0.7 |
| ZD6474 alone | 45.2 ± 3.0 | 17.7 ± 3.0 |
| ZD6474+RT | 48.9 ± 2.8 | 21.4 ± 2.8 |

NOTE:

(1) Tumors were treated daily in different groups until the tumor volume reached 2.5 cm$^3$ was. The days of tumor growth were recorded and compared.

(2) Tumor Growth delay is a comparison of the days of tumor growth between different treatment group and control (days of TV 2.5 cm$^3$ treated – days of TV 2.5 cm$^3$ control).

Values shown are the mean ± SE.

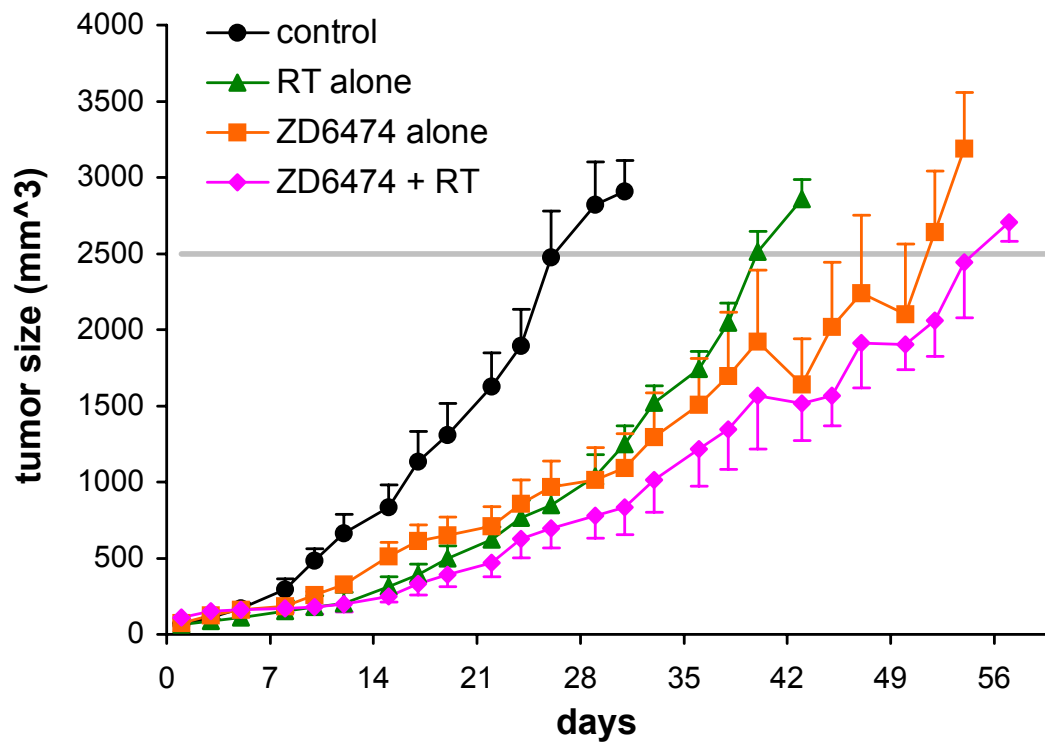Abbreviations: NA, not applicable; TV, tumor volume

Fig. 5: Inhibition of *in-vivo* growth of Fadu tumors in nude mice treated with radiotherapy alone (RT: 5 x 2 Gy, days 1-5), ZD6474 alone (ZD6474: 50 mg/kg/d) and their combination (ZD6474 + RT) compared with control without treatment. Data points represent mean values obtained from six to eight animals. Error bars represent the SE of 6-8 experiments.

## 4.2 ZD6474 with radiotherapy reduces vascular density in Fadu xenograft tumors

To determine the effects of ZD6474, radiation, and combination treatment with ZD6474 and radiotherapy on endothelial cells in vivo, histological assessments of vessel density were done on tumors excised one week after 5 days of continuous treatment. To relate changes in the endothelial compartment to the pathology of the tumor as a whole, the extent of tumor proliferation and apoptosis were also assessed. An antibody for CD34 was used to stain for blood vessels. A representative image is shown in Fig. 6 A. The number of vessels per microscopic field was determined for each treatment group. Sections were examined on a Nikon fluorescent microscope. Blood vessel density was quantified that each slide was scanned at low power (x10-100) at first and the area with the higher number of new vessels was identified (hotspot). This region was then scanned at x600 microscope magnification. Three fields (0.15 mm$^2$ per microscopic field) were analyzed and, for each of them, the number of stained blood vessels was counted. For individual tumors, microvessel count was scored by averaging the three field counts (Sano et al., 2007). Our study has shown control tumors had an average of 30.9 ± 1.4 vessels per microscopic field, radiotherapy alone had 21.6 ± 1.7 vessels, ZD6474 alone had 17.5 ± 2.3 and combination treatment with ZD6474 and radiotherapy had 12.5 ± 0.6, the number of vessels was lowest in the combination treatment sections. Both ZD6474 (50 mg/kg) daily for 5 days and radiotherapy alone (5 × 2 Gy) reduced vessel density in Fadu tumors compared with control. However, it was when both agents were applied concomitantly that this change achieved significantly enhanced compared with radiotherapy alone (Fig. 6 A and B).
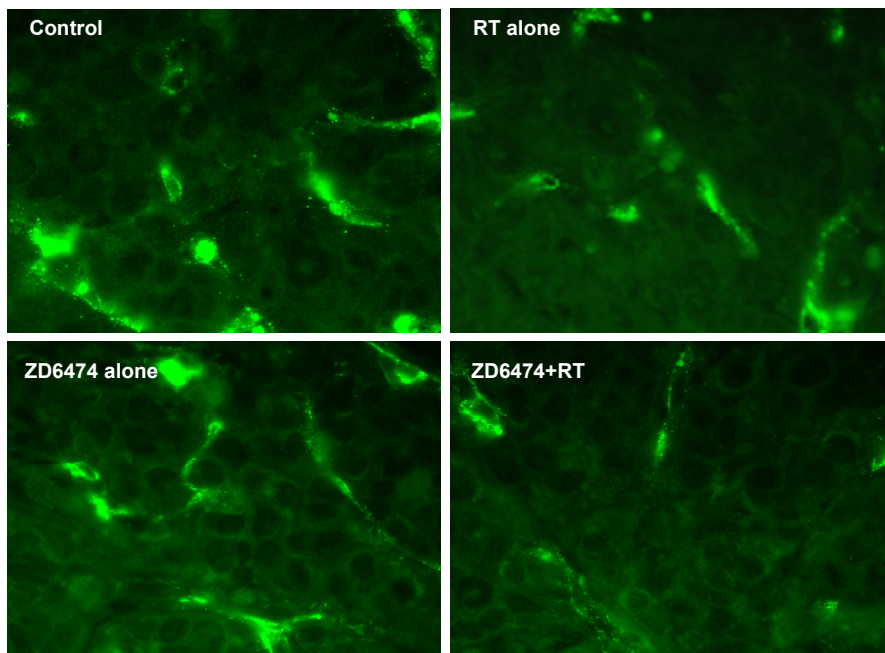
Fig. 6 A: ZD6474 in combination with irradiation decreases vascular density in Fadu xenograft tumors. Histological sections were obtained from the tumors of the mice in each treatment group after one week treatment with ZD6474 and with or without irradiation; total vessel density was assessed using immunostaining for CD34 as a marker of endothelial cells. Images were shown at ×600 magnification.
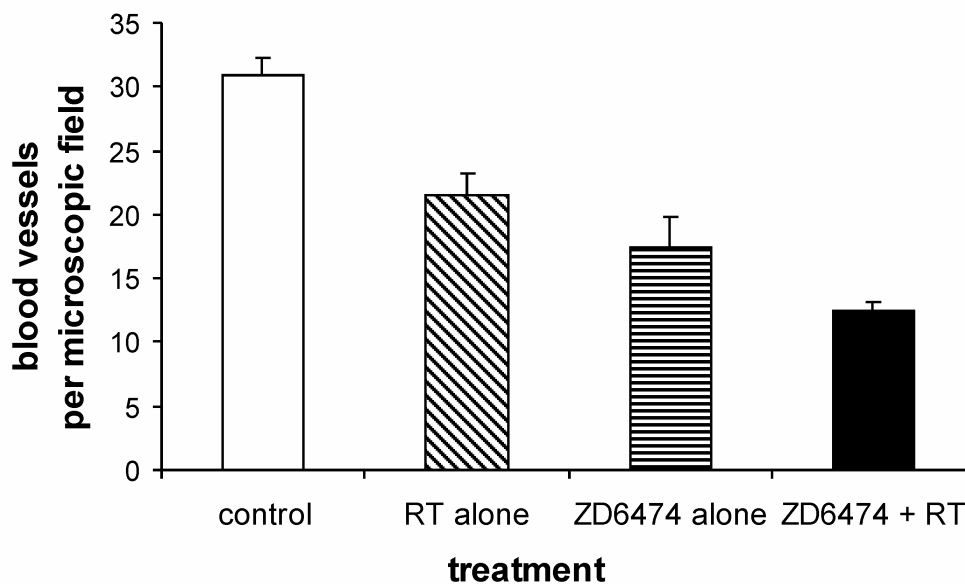


Fig. 6 B: Quantification analysis of blood vessel in tumor. Average blood vessel density of each treatment group was determined by counting the number of blood vessels at an area of 0.15 mm$^2$ per microscopic field. Data presented are average values. Error bars represent the SE of 3-4 experiments.

## 4.3 ZD6474 with radiotherapy reduces proliferation in Fadu xenograft tumors

To determine whether the tumor growth delay from the combined therapy results from decreased tumor proliferation, immunofluorescence technique and antibody against Ki67 were utilized to evaluate tumor proliferation. Ki67 staining was done using tissue sections from Fadu tumors in all treatment groups. Sections were examined on a Nikon fluorescent microscope and the proliferating rate was then determined in each treatment group. To determine the percentage of positive cells with Ki67, at least 500 - 1000 cancer cells per slide were counted and the number of positive cell were scored. Results were shown in Fig. 7 A and B.

Our results have shown tumor in mice of control group had a very high proliferating rate with an average positive rate of 50% ± 1%; radiotherapy alone had an average positive rate of 21% ± 2%; ZD6474 alone had an average positive rate of 15% ± 1%, and combination treatment with ZD6474 and radiotherapy had an average positive rate of 9% ± 1%. Compared with control, ZD6474 alone or radiotherapy alone inhibited tumor proliferation; the difference was obvious. However, when ZD6474 was combined radiotherapy, the positive rate of Ki67 was lowest in all treatment sections, and compared with ZD6474 alone or radiotherapy alone, the inhibited effect was increased. The difference between ZD6474 combined with radiotherapy and ZD6474 or radiotherapy alone was significantly increased.

Fig. 7 A: ZD6474 in combination with irradiation reduces Ki67 expression in Fadu xenograft tumors. Histological sections were obtained from the tumors of the mice in each treatment group after one week treatment with ZD6474 and with or without irradiation; standard Ki67 stainings were done. Immunofluorescence staining was shown at × 600 magnification in 4 groups.
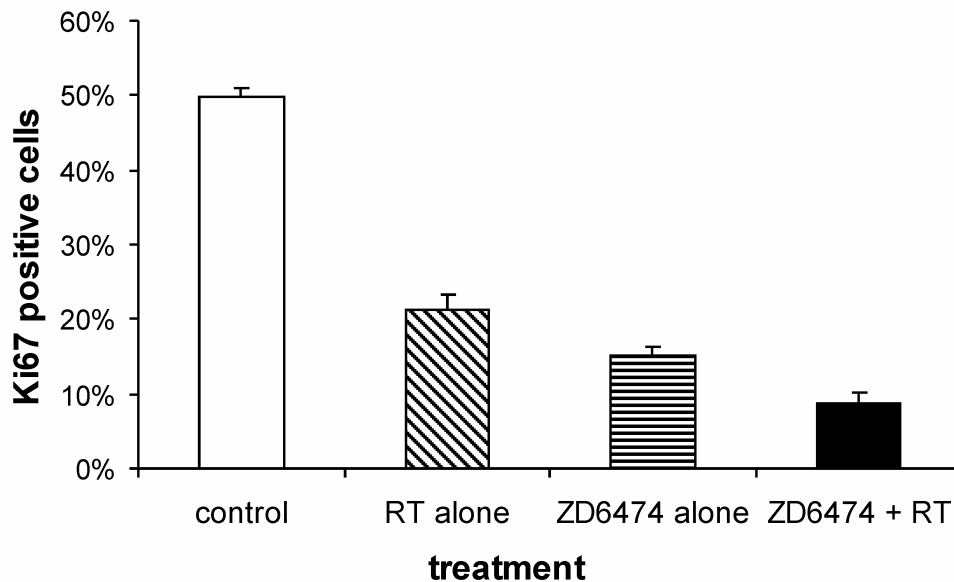


Fig. 7 B: Quantification of proliferative rate in tumor. Average proliferative rate of each treatment group was determined by counting Ki67 positive cells in 500-1000 tumor cell under microscopic field at ×600 magnifications. Data presented are average values. Error bars represent the SE of 3-4 experiments.

## 4.4 ZD6474 with radiotherapy increases apoptosis in Fadu xenograft tumors

To determine whether the tumor growth delay from the combined therapy results from increased tumor apoptosis, Immunofluorescence technique and antibody against Capase-3 were utilized to evaluate tumor apoptosis. Capase-3 staining was done using tissue sections from Fadu tumors in all treatment groups. Sections were examined on an Nikon fluorescent microscope and the apoptosis index was then determined in each treatment group. To quantificate analysis of apoptosis in tumor, 5 fields (0.15 mm$^2$ per microscopic field) were selected in each tumor section per treatment group under x600 microscope magnification, cells with positive expression of Capase-3 were counted, and results were expressed as positive cells per field. Results were shown in Fig. 8 A and B.

Our results have shown that tumors in the control group had a very low number of apoptotic cells per microscopic field (3.7 ± 0.3). The tumors in the radiotherapy alone group had an average number of 5.8 ± 0.3 and in the ZD6474 alone group had an average number of 5.2 ± 0.3 apoptotic cells per microscopic field. Combination treatment with ZD6474 and radiotherapy lead to a significantly higher number of apoptotic cells (11.5 ± 0.5) per microscopic field.

Compared with control, ZD6474 alone or radiotherapy alone increase apoptosis in tumor; the difference was obvious; however, when ZD6474 was combined radiotherapy, the difference was increased further. Compared with ZD6474 alone or radiotherapy alone, ZD6474 combined with radiotherapy enhanced the apoptosis in tumor. The difference between ZD6474 combined with radiotherapy and ZD6474 or radiotherapy alone was significantly increased.
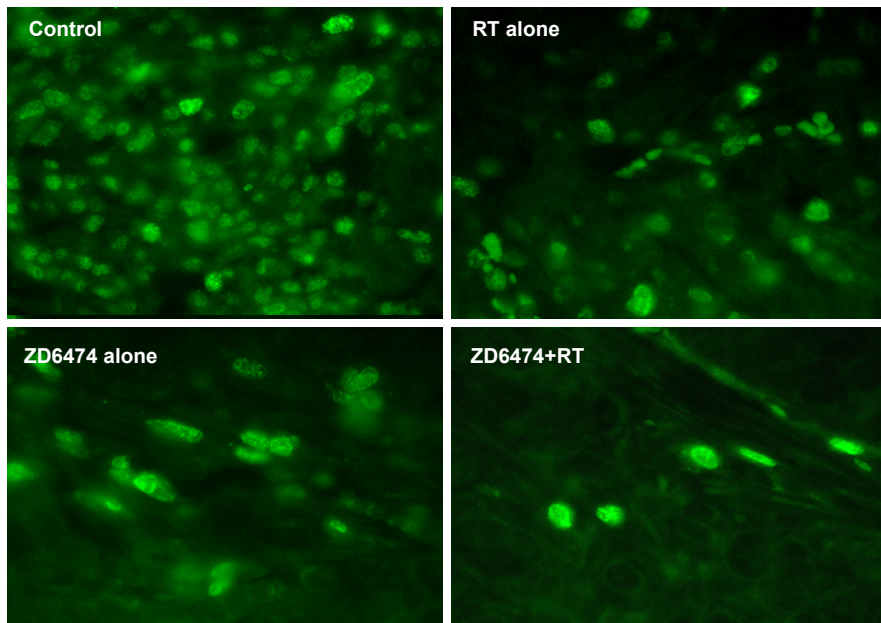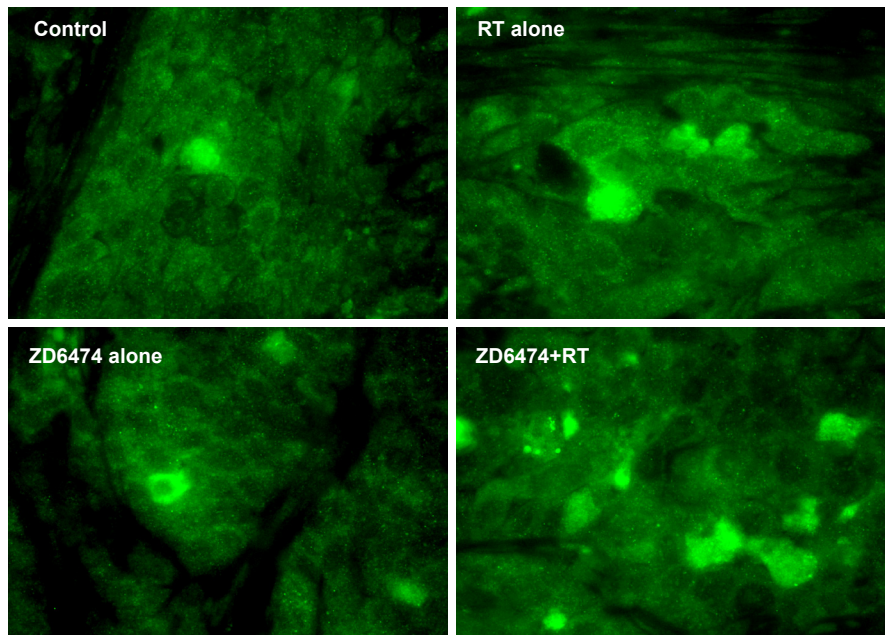
Fig. 8 A: ZD6474 in combination with irradiation increases Capase-3 expression in Fadu xenograft tumors. Histological sections were obtained from the tumors of the mice in each treatment group after one week treatment with ZD6474 and with or without irradiation; standard Capase-3 stainings were done. Immunofluorescence staining were shown at ×600 magnification in 4 groups.
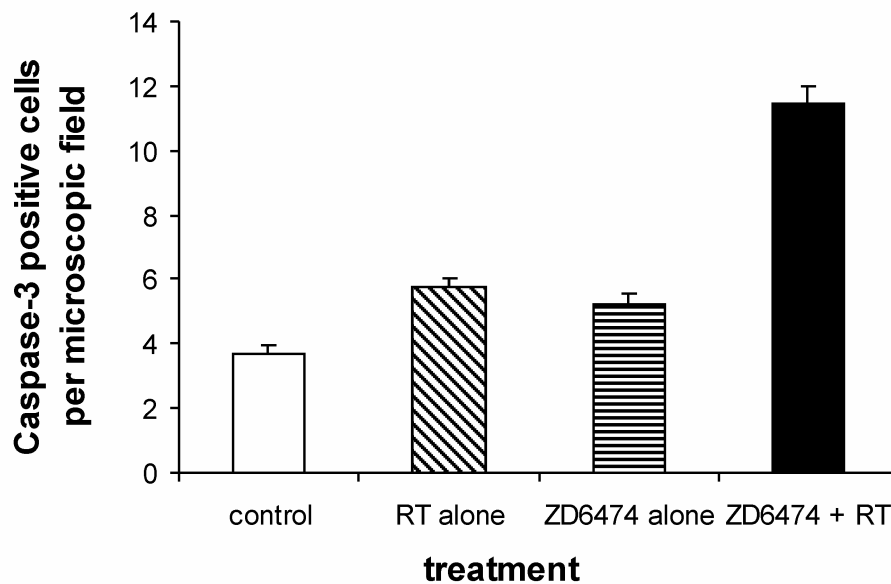


Fig. 8 B: Quantification analysis of apoptosis index in tumor. Average apoptosis index of each treatment group was determined by counting the number of Capase-3 positive cell at an area of 0.15 mm$^2$ per microscopic field. Data presented are average values. Error bars represent the SE of 3-4 experiments.

# 5. Discussion

## 5.1 ZD6474 inhibits VEGF signaling, angiogenesis, and tumor growth following oral administration

Tumor growth requires access to oxygen and nutrients carried by blood vessels. Tumor cells meet this nutritional need by influencing the surrounding host stroma to induce formation of new blood vessels, which subsequently grow within the tumor (angiogenesis). Angiogenesis plays a central role in the exponential growth of primary tumors and metastases. Strategies directed toward inhibition of tumor angiogenesis hold promise in cancer therapeutics with the first antiangiogenic agent achieving approval from Food and Drug Administration in 2004 (Tortora et al., 2004; Kerbel et al., 2004). Activated vascular endothelium represents an attractive target because of its rich accessibility to drugs, genetic stability (less likely to develop resistance), tissue homogeneity, and presence of a proliferating state primarily within tumor tissue.

Many factors have been implicated in promoting angiogenesis; VEGF plays a key role in tumor angiogenesis including induction of endothelial cell proliferation, migration, survival and capillary tube formation (Kanno et al., 2000). Enhanced production of VEGF is generally correlated with increased neovascularization within tumors (Kerbel et al., 2000; Fontanini et al., 1997). Different mechanisms, notably hypoxia can increase VEGF expression in cancer cells (Kerbel et al., 2000; Ferrara et al., 1992). VEGF binds to two distinct receptors on endothelial cells, i.e. flt-1 (VEGFR-1) and flk/KDR receptor (VEGFR2) (Ferrara et al., 1992). VEGFR-2 is considered to be the dominant signaling receptor for endothelial cell permeability, proliferation, and differentiation (Ferrara et al., 2003). VEGF and its receptors are good targets for cancer therapy because VEGF receptors are highly specific for VEGF and are expressed in increased numbers primarily during periods of tumor growth (Cherrington et al., 2000). The integral role of the VEGF/VEGFR2 pathway in tumor angiogenesis is supported by numerous studies, including those that show inhibition of tumor growth in nude mice by anti-VEGF antibodies (Willett et al., 2004), anti-Flk1 antibodies (Kozin et al., 2001), inhibitors of VEGFR2 tyrosine kinase (Drevs et al., 2000; Abdollahi et al., 2003), anti-VEGF antisense RNA (Saleh et al., 1996; Ellis et al.,1998 ), and VEGF toxin (Ramakrishnan et

al., 1996).

ZD6474 is a potent, low molecular weight inhibitor of VEGFR2 tyrosine kinase activity (Hennequin et al., 2002), which significantly inhibits tumor growth in a broad range of established human cancer xenografts in nude mice and is currently undergoing clinical evaluation (Miller et al., 2005; Heymach et al., 2005). ZD6474 also has additional activity against EGFR tyrosine kinase (Ciardiello et al., 2003; Ryan et al., 2005). ZD6474 has potent antitumor activity by a direct antiangiogenic mechanism via inhibition of VEGFR2 signaling in endothelial cells, and can also directly inhibit cancer cell growth by interfering with the EGFR autocrine pathway which is central to cancer growth and progression. Overexpression of EGFR and/or its ligands, transforming growth factor TGF-α and EGF has been reported in many human tumor types (Ciardiello et al., 2001). Furthermore, ZD6474 could block neoangiogenesis more efficiently than more selective anti-VEGFR agents, because in addition to a direct inhibitory effect on VEGFR2 signaling, it also has an indirect effect on angiogenesis via blockade of EGFR induced paracrine production of angiogenic growth factors (Wedge et al., 2002; Ciardiello et al., 2003).

In this study, ZD6474 proved to be an effective agent for suppressing growth of Fadu cells in vivo. Chronic once-daily oral administration of ZD6474 (50 mg/kg/day) produced an obvious inhibition of tumor growth in Fadu tumor xenograft models. To evaluate vessel density *in vivo*, histological analysis of immunohistochemical staining was utilized. Vessels were detected with an anti-CD34 antibody. After treatment with ZD6474, the number of CD34-positive endothelial cells decreased and the vessel number was significantly lower in the tumors treated with ZD6474 alone compared with control. To evaluate proliferating rate and apoptotic events *in vivo*, the marker of Ki67 and Capase-3 were utilized. After treatment with ZD6474, the number of Ki67-positive tumor cells decreased and the number of Capase-3 positive tumor cells was significantly higher in the tumors treated with ZD6474 than that in the control. These data are compatible with inhibition of VEGF signaling because a significant reduction in tumor neovascularization and VEGF-induced vascular permeability could be expected to result in greater tumor ischemia and the induction of tumor cell necrosis.

In conclusion, based on the present results, we show that in vivo ZD6474 was a

highly active antitumor agent by inhibition of VEGF-dependent angiogenesis, proliferation and induction of tumor cell apoptosis. These results suggest that ZD6474 may have dual antitumor effects by i) inhibiting VEGF dependent tumor angiogenesis and VEGF-dependent endothelial cell survival, and ii) inhibiting tumor cell proliferation and promoting tumor cell apoptosis. ZD6474 has the potential to inhibit two key pathways in tumor growth via inhibition of VEGF-dependent tumor angiogenesis and via inhibition of EGFR-dependent tumor cell proliferation. In further, the efficacy of ZD6474 combined with radiotherapy will be evaluated.

## 5.2 ZD6474 increases the tumor response to radiotherapy

### 5.2.1 ZD6474 produces an enhancement of radiotherapy through antiangiogenesis

Although antiangiogenic agents have been shown to reduce the growth rate of tumors, treatment with these agents alone is unlikely to be curative (Siemann et al., 2004). However, combining antiangiogenic agents with other modalities may lead to an overall increase in antitumor efficacy. When considering treatment options for combination studies, radiation therapy appears to be a good candidate given its extensive use and application in a wide variety of tumor types and clinical settings (DeVita et al., 1997).

Radiation represents a central modality of treatment for many human cancers. Approximately one-half of all cancer patients receive radiation at some point during the course of their disease. The effectiveness of radiation is often limited by normal tissue tolerance or by tumor cell resistance to therapy. Molecular targeting of the tumor vasculature may influence the efficacy of radiotherapy in achieving locoregional tumor control (Gong et al., 2003; Fenton et al., 2004; Raben et al., 2004). Indeed, approaches using angiostatin (Mauceri et al. 1998), endostatin (Hanna et al., 2000), vascular microtubule formation inhibition (Siemann et al., 2002), and anti-VEGF treatment (Gorski et al., 1999; Lee et al., 2000) have shown enhanced antitumor effect of radiation in several preclinical studies with human tumor xenografts. With specific regard to VEGFR2, a report by Kozin *et al.* (Kozin et al., 2001) demonstrates that VEGFR2 blockade by mAb DC101 reduces the dose of radiation required to control 50% of tumors

in two human xenograft model systems. Studies using an orally available inhibitor of VEGFR2 tyrosine kinase activity (ZD6474) also demonstrate antitumor activity (Wedge et al., 2002), and preliminary data in lung and head-and-neck tumor models suggest favorable interaction of ZD6474 with radiation (Shibuya et al., 2007; Hoang et al., 2006; Gustafaon et al., 2004).

In the present study, we examined in vivo antitumor and antiangiogenic effects of VEGFR2 blockade by ZD6474, we have confirmed chronic once-daily oral administration of ZD6474 (50 mg/kg/day) produced an inhibition of tumor growth in Fadu tumor xenograft models. The growth-inhibitory effect of ZD6474 (VEGFR2 blockade) on tumor xenografts seems to reflect antiangiogenic influence as revealed through an in vivo angiogenesis assay. We further investigated the effect of combined treatment with radiotherapy and ZD6474 on tumor xenograft response and angiogenesis. To explore the clinical viability of combining these two treatment modalities, we established a Fadu cell tumor xenograft model in nude mice. Mice were placed into four treatment groups: control, ZD6474 alone, radiotherapy alone, or ZD6474 combined with radiotherapy. To maximize clinical applicability, we chose to use five fractions dose (2 Gy per day) irradiation on our tumor model. We found that chronic administration of ZD6474 caused a reduction of tumor growth, consistent with previous studies (Wedge et al., 2002). And when combined with fractionated radiotherapy, a significant increase in tumor growth delay was observed compared with either radiotherapy or ZD6474 alone. When tumor size reached 2.5 cm$^3$, Mice in the control group needed about 28 days, 4 weeks after tumor implantation. Respectively, it needed about 49 days, 45 days, 40 days for mice in combination treatment with ZD6474 and radiotherapy, ZD6474 alone, radiotherapy alone group. The growth delay was $17.7 \pm 3.0$ day for ZD6474 alone, $12.5 \pm 0.7$ days for radiotherapy alone, and $21.4 \pm 2.8$ days for combination treatment (Fig. 5 and table 1). The difference in tumor growth between treatment groups of ZD6474 combined with radiotherapy and radiotherapy or ZD6474 alone is obvious, but compared with ZD6474 alone, the difference between combination treatment group and radiotherapy alone is significantly more obvious compared with ZD6474 alone (49 days versus 40 days versus 45 days for the tumor growth). In our project, the total dose of irradiation was only 10 Gy, but ZD6474 (50 mg/kg/day) was delivered for 4 weeks in

drug alone or combination treatment group. It can be the reason for the little difference between ZD6474 alone group and combination treatment group because of insufficient total dose of irradiation. In addition, because tumor regrowth after radiotherapy was slowed and the extent of tumor regression postradiotherapy was enhanced obviously in combination treatment group, it also can be explained as constantly excellent antitumor effect with the continuous application of ZD6474 after radiotherapy ended.

To determine the effects of ZD6474 treatment on tumor neovascularization, proliferation and apoptosis in vivo, 4 mice per group were sacrificed one week after 5 days treatment. Tumor tissues were then collected and analyzed, using antibodies against CD34; microvessel number was assessed in the most intense areas of neovascularization. And the marker of Ki67 and Capase-3 were utilized to evaluate proliferating rate and apoptotic events in vivo. Our results showed that tumor vascular density was significantly decreased by combination therapy with ZD6474 and radiotherapy (Fig. 6 A and B). This decrease in vascular density was observed 7 days after initiation of ZD6474 treatment. This abolition of tumor vasculature not only shows the value of ZD6474 in enhancing radiation-induced vascular damage but also underscores the previously discussed importance of optimal treatment scheduling when using anti-VEGFR2 agents. Consistent with the tumor growth delay, under the chosen treatment schedule, we observed decreased proliferation through Ki67 staining of histologic sections from the Fadu cell xenograft tumors. There was a notable decrease in Ki67 staining in the tumor sections that received treatment of ZD6474 combined with radiotherapy, compared with control or either treatment alone (Fig. 7 A and B). Similarly, apoptosis was increased in the combination treatment group compared with controls (Fig. 8 A and B). These data suggest that ZD6474 increases tumor response to radiotherapy through inhibition of tumor angiogenesis, and the mechanism of tumor growth delay also included both decreased proliferation and increased apoptosis of tumor cells.

## 5.2.2 The possible radiosensitive mechanisms of antiangiogenesis from ZD6474

ZD6474 treatment resulted in significant in vivo tumor growth retardation. This growth inhibition was significantly enhanced when ZD6474 was combined with fractionated dose radiotherapy. Regarding potential mechanisms whereby VEGFR2 blockade and radiation induce cell death, ZD6474 is thought to exert antitumor effects through inhibition of angiogenesis and induction of endothelial cell apoptosis (Wedge et al., 2002). Our present studies are compatible with this conclusion. Although one might speculate that VEGFR2 blockade could reduce the efficacy of radiation by reducing tumor oxygenation due to diminished vascularization, our results suggest the converse. In other words, VEGFR2 blockade in our model system showed enhanced efficacy of radiation on tumor response. The positive interaction observed when ZD6474 is used as an adjuvant to radiotherapy supports an important role for VEGF-mediated signaling in the tumor response to radiotherapy in vivo. Several mechanisms have been postulated by which inhibitors of VEGF signaling could improve the therapeutic effects of radiation. These include a normalization of tumor vasculature (Winkler et al., 2004) and a direct enhancement of the radiosensitivity of tumor endothelial cells (Gorski et al., 1999).

## 5.2.2.1 Vascular normalization and radiosensitization

The study by Kozin *et al.* similarly reports that DC101, a VEGFR2 blockade, can potentiate radiation response among a series of human tumor xenografts (Kozin et al., 2001). Furthermore, several recent studies (Gong et al., 2003; Winkle et al., 2004; Tong et al., 2004) suggest that VEGFR2 blockade may create a "normalization window", a period during which radiation treatment can induce maximal tumor impact. This window is characterized by an increase in tumor oxygenation, which is known to enhance radiation response. Because there is a critical window during which the interaction between anti-VEGFR2 therapy and radiotherapy is maximized, it is proposed that optimal treatment scheduling corresponds to administration of radiation during a period of vascular normalization that is induced by the anti-VEGFR2 therapy (Jain, 2001). Because tumors induce high levels of angiogenesis, the antiangiogenic drug administration serves to balance this pathologic angiogenesis. Therefore, it is suggested that this period of

normalization represents a balance between proangiogenic and antiangiogenic factors that transiently improves tumor blood flow and oxygenation (Jain, 2005). Because hypoxia is known to decrease the efficacy of radiotherapy (Wachsberger et al., 2003), giving irradiation during this normalization window could serve to enhance treatment before the tumor vessels are irreversibly damaged and tumor blood flow is diminished by radiation. Alternatively, a crucial role for VEGF in tumor remodeling and growth postradiotherapy has been suggested from studies demonstrating a positive interaction between radiotherapy and VEGF-targeting when the agent is given as an adjuvant to radiation (Zips et al., 2003; Williams et al., 2004; Zips et al., 2005). These data have lead to the suggestion that preirradiated tumor vasculature is more sensitive to inhibition of VEGF signaling. In our study, ZD6474 (50 mg/kg, by mouth, once daily) was given 2 hours before each dose of radiation (5 × 2 Gy, days 1–5) in combination treatment schedule, it did have produced a greater control of tumor growth during the course of radiotherapy and supported above ideas.

## 5.2.2.2 Endothelial radiosensitivity and radiosensitization

However, Fenton et al. (Fenton et al., 2004) also provide evidence to suggest that VEGFR2 blockade can enhance radioresponse by specifically sensitizing endothelial cells (possibly via enhancement of endothelial cell apoptosis) despite the induction of hypoxia in the tumor. This concept of the endothelial cell as primary target governing ultimate radiation response has been recently postulated using a mouse intestinal model system (Paris et al., 2001). Damage to the endothelial cell compartment would thereby facilitate subsequent damage to tumor cells, counter to the prevailing hypothesis that radiation damage targets primarily the malignant cell (Garcia-Barros et al., 2003). It has also been speculated that antiangiogenic therapies may block the endothelial cell VEGF production induced by radiotherapy, thus inhibiting a survival advantage for tumor endothelium (Kermani et al., 2001) and resulting in impairment of tumor cell growth and survival in a more indirect manner. Blockage of VEGF, it is hypothesized, would lead to an increase in endothelial cell apoptosis and thus lead to greater tumor cell death than radiation alone.

Our present results have presented combination of ZD6474 with radiotherapy induced

a notable decrease in tumor proliferation and increase of apoptosis compared with either treatment alone. If this observation is further generalized, increased endothelial radiosensitivity after VEGFR2 blockade might explain the potent interactive killing of tumor xenografts observed with the combination of ZD6474 and radiotherapy. Our improved understanding of the molecular interplay between radiation and these angiogenic factors should facilitate the design and testing of new clinical trial strategies.

## 5.3 Conclusion

In summary, the present studies indicate that VEGFR2-associated tyrosine kinase inhibition by the small molecule ZD6474 improves the response of Fadu tumor xenografts to fractionated radiotherapy through:

*(a)* ZD6474 appears to target tumor cells and tumor vasculature by both indirect and direct mechanisms (inhibition of EGFR/VEGF pathways).

*(b)* ZD6474 can decrease overall tumor resistance to radiation by targeting both tumor cells and tumor vasculature.

*(c)* Tumor growth and angiogenesis are part of a codependent cycle. ZD6474 can break the cycle and prevent revascularization after radiation.

*(d)* The enhancement effects observed with combined radiation and ZD6474 treatments on inhibition of tumor growth may arise from antiangiogenesis and the intrinsic radiosensitivities of endothelial cells.

These results provide further impetus for future evaluation of the combination of ZD6474 with radiotherapy in the clinical setting. As antiangiogenic agents experience increased applications in cancer patients and their potential for use in conjunction with radiotherapy grows, uncovering the complex interactions involved between the two therapies will become even more vital to successful therapies for solid tumors.
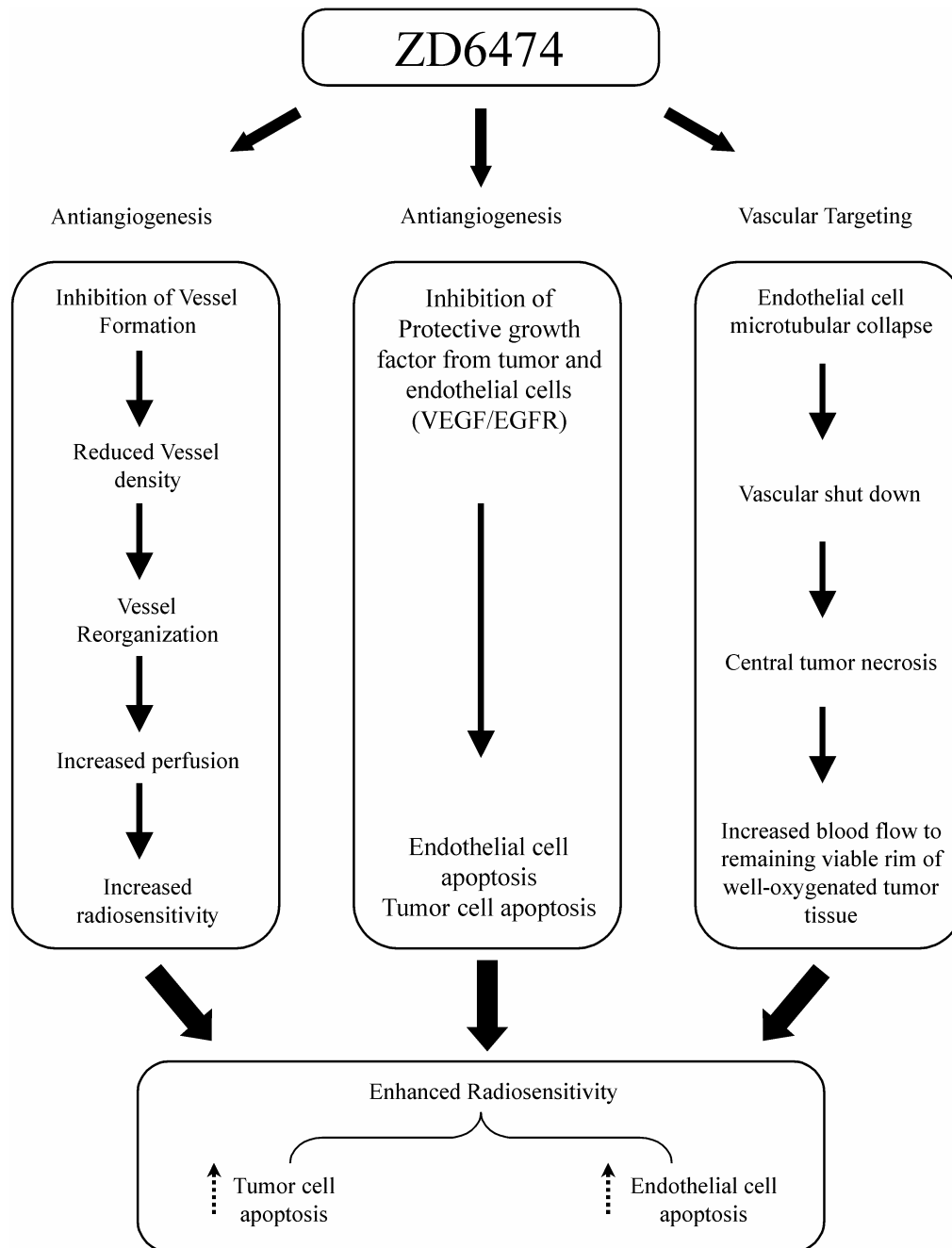
Fig. 9: Possisble mechanisms for enhanced tumor response to radiation with ZD6474. ZD6474 may work synergistically with radiation through inhibition of neovascularization processes. ZD6474 may also improve radiosensitivity by directly and/or indirectly inhibiting protective cell survival signaling pathways in both endothelial and tumor cells, resulting in increased apoptosis in both the tumor cell compartment and the tumor stromal (*i.e.*, endothelial cell) compartment.

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