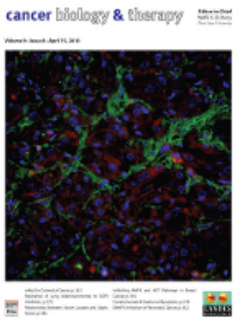

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ZD6474, a dual tyrosine kinase inhibitor of EGFR and VEGFR-2, inhibits MAPK/ERK and AKT/PI3-K and induces apoptosis in breast cancer cells

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Key words: epidermal growth factor receptor, vascular endothelial growth factor receptor, tyrosine kinase inhibitor, angiogenesis, apoptosis

Abnormalities in gene expression and signaling pathways downstream of the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) contribute to the progression, invasion, and maintenance of the malignant phenotype in human cancers, including breast. Consequently, the dual kinase inhibitor of EGFR and VEGFR ZD6474 represents a promising biologically-based treatment that is currently undergoing clinical trials for non-small cell lung cancer. Patients suffering from breast cancers have a poor prognosis because of the lack of effective agents and treatment strategies. We hypothesized that inhibition of phosphorylation of the EGFR and VEGFR by ZD6474 would inhibit breast cancer cell proliferation and induce apoptosis. This hypothesis was tested using human breast cancer cell lines. ZD6474 inhibited cell proliferation in a dose-dependent manner, by blocking cell progression at the G_0-G_1 stage, through downregulation of expression of cyclin D1 and cyclin E. In vitro, ZD6474 inhibited growth factor-induced phosphorylation of EGFR, VEGFR-2, MAPK and Akt. ZD6474 also downregulated anti-apoptotic markers including Bcl-2, upregulated pro-apoptotic signaling events involving expression of bax, activation of caspase-3, and induction of poly (ADP-ribose) polymerase during apoptosis. ZD6474 inhibited anchorage independent colony formation using soft agar assays, and invasion of breast cancer cells in vitro using Boyden chamber assays. In a xenograft model using human MDA-MB-231 breast cancer cells, ZD6474 inhibited tumor growth and induced cancer-specific apoptosis. Collectively, these data imply that ZD6474 a dual kinase inhibitor has potential for the targeted therapy of breast cancer.

Introduction

The epidermal growth factor (EGF) receptor (EGFR) is a member of the ErbB family, consisting of four closely-related tyrosine kinase receptors, which affects cell proliferation. It includes EGFR family members HER-1 (Erb-1), EGFR-2/HER-2 (Erb-2), HER-3 (Erb-3) and HER-4 (Erb-4). EGFR-1 and HER-2 are overexpressed in several carcinomas with high levels of expression being a prominent feature of the malignant phenotype in many solid human tumors,[1] including breast cancer. EGFR-1 activation occurs through four different ligands, most commonly EGF and transforming growth factor-α (TGFα), which form homodimeric complexes or heterodimeric complexes, preferably with HER-2, as well as with other members of the ErbB family of receptors.

Ligand binding and dimerization cause autophosphorylation of the intracytoplasmic domains and activation of the intracellular tyrosine kinase. Activated EGFR (phosphorylated EGFR [pEGFR]) stimulates several different signal transduction pathways, such as the MAPK/ERK and the phosphoinositide-3 kinase (PI3K)/Akt pathways that lead to uncontrolled cell proliferation and inhibition of apoptosis. Although EGFR has not provided a useful prognostic/predictive marker of clinical response to EGFR-targeted therapies,[2,3] other prognostic/predictive markers have been proposed, including the activated form of EGFR (pEGFR).[4] Previously, EGFR phosphorylation was shown to be associated with poor prognosis in non-small-cell lung cancer and has been proposed as an important predictor of clinical outcome.[5] Thus, therapies targeting EGFR signaling by blocking phosphorylation of EGFR and its downstream target molecules may be required to control the pathogenesis and progression of EGFR overexpressed breast cancers.

Vascular endothelial growth factor receptor (VEGFR) is also a member of the receptor tyrosine kinase (RTK) family, which includes VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1) and VEGFR-3 (flt-4) and a soluble form of VEGFR-1 (sVEGFR-1), a negative regulator of VEGF.[6] VEGFR-2 is proposed to function as a dominant receptor of VEGF/VEGFR signaling in the angiogenesis pathway. It plays a central role in both local tumor growth and distant metastasis in breast cancer.[7] Enhanced expression of

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VEGF generally correlates with increased neovascularization as measured by microvessel counts within the tumor. Expression of VEGF and other pro-angiogenesis factors and their receptors correlates with less favorable clinical outcomes in various cancers, including breast.[8-11]

Clinical trials with angiogenesis inhibitors targeting only the VEGF/VEGFR signaling pathway have been disappointing suggesting that additional inhibitors may be necessary to inhibit tumor growth and proliferation. There are common downstream signaling molecules involved in both EGFR- and VEGFR-mediated pathways. Specific cancer cells acquire resistance to anti-EGF/EGFR therapy by increased tumor-induced angiogenesis due to the constitutive overexpression of VEGF.[12] Additionally, activation of the EGFR signaling pathway also upregulates the production of VEGF. Therefore, both pathways represent viable targets for therapy, and because of their parallel and reciprocal activation, dual inhibition of the EGF(R) and VEGF(R) signaling may result in significant anti-tumor activity. Several preclinical and in vitro studies demonstrate the additive effect of combining EGFR and VEGFR inhibitors in reducing tumor growth.[13-15] Clinically, bevacizumab, a humanized antibody against VEGF, has shown considerable promise and improved survival for patients with colorectal, breast or lung cancer when used in combination with EGFR inhibitors or other chemotherapeutic drugs.[15,16]

The potential of dual targeting inhibitors as anti-cancer drugs has led to the clinical testing of ZD6474, a dual tyrosine kinase inhibitor (TKI) of EGFR and VEGFR-2, in various tumors. ZD6474 inhibits both the development of the tumor's blood supply through inhibition of VEGFR (anti-angiogenesis) and tumor cell proliferation and survival through inhibition of EGFR. It also inhibits oncogenic RET kinases in thyroid cancers. Apart from inhibiting the phosphorylation of tyrosine kinases, it also inhibits serine-threonine kinases.[17,18] ZD6474 is currently in phase III clinical trials for the treatment of follicular, medullary, anaplastic and locally advanced and metastatic papillary thyroid cancer, as well as for non-small cell lung cancer. Breast cancers display aggressive properties and are characterized by overexpression of EGFR and VEGFR. Despite improvements in chemotherapy, the long-term survival of patients with advanced and higher stage breast cancer has remained very low. The present study has tested the hypothesis that simultaneous inhibition of phosphorylation of both EGFR and VEGFR pathways by ZD6474 would improve breast cancer outcome, particularly in EGFR and/or VEGFR expressing advanced breast cancers. Studies have now been conducted to test this possibility using breast cancer cell lines in vitro, MCF-7, T-47D, ZR-75-1 and MDA-MB-231, and in a MDA-MB-231 orthothopic nude mouse model. These studies confirm significant activity of ZD6474 in inhibiting growth and inducing apoptosis both in vitro and in vivo providing support for the use of this agent for the therapy of breast cancer.

Results

ZD6474 suppresses the in vitro cell proliferation of breast cancer cells in a dose-dependent manner. ZD6474 inhibited cell proliferation of breast cancer cells MCF-7, MDA-MB-231, T-47D and ZR-75-1 in a dose-dependent manner and the cell inhibition was prominent mainly between concentrations of 1–10 μM (**Fig. 1A**). The IC_{50} values of ZD6474 in MCF-7, MDA-MB-231, T-47D and ZR-75-1 cells were 12.667 ± 0.667, 6.128 ± 0.446, 6.199 ± 0.356, 6.841 ± 0.237 and 5.6777 ± 0.487 μM respectively.

ZD6474 induces cell cycle arrest at the G_0-G_1 phase. ZD6474 induced cell cycle arrest at the G_0-G_1 phase, as shown in **Figure 1B**. There was 20% relative increase in the G_0-G_1 phase in all breast cancer cells treated with ZD6474 for 24 h as compared with control cultures. This increase in G_0-G_1 phase was time-dependent as shown in **Table 1**. There was a temporal decrease in cyclin D1 and cyclin E (**Fig. 1C**) with time, further confirming the role of ZD6474 in cell cycle arrest at the G_0-G_1 phase.

ZD6474 induces apoptosis in breast cancer cells. Apoptosis induction occurred in a time-dependent manner following ZD6474 treatment (**Fig. 2A**). ZD6474 induced more than 50% apoptosis after 72 h of treatment as quantified by flow-cytometry. This induction of programmed cell death was corroborated by DNA ladder formation in agarose gel electrophoresis (**Fig. 2B**). Oligonucleosomes were also observed under fluorescent microscopy after PI staining (**Fig. 2C**). Cleavage of poly(ADP-ribose) Polymerase (PARP) was also observed over time. Additionally, there was an increase in the pro-apoptotic Bax protein and a decrease in the anti-apoptotic Bcl-2 protein in a time-dependent manner in MCF-7, MDA-MB-231, ZR-75-1 and T-47D cells (**Fig. 2D**). A decrease in the inactive pro-form (32 Kd.) of caspase 3 was also observed with time, indicating the role of effector caspase 3 in DNA fragmentation and apoptosis induction in MDA-MB-231, T-47D and ZR-75-1 cells. Caspase-3 is absent in MCF-7, indicating the contribution of other caspases in DNA fragmentation. There was no change in expression of p53 (**Fig. 3A**), indicating that ZD6474 executed its apoptotic mechanism independent of the p53 pathway.

ZD6474 inhibits epidermal growth factor receptor and vascular endothelial growth factor receptor signaling in breast cancer cells. The expressions of EGFR and VEGFR-2 did not change in a temporal manner in MCF-7 and MDA-MB-231 cells (**Fig. 3A**). EGFR and VEGFR-2 remain unaltered even after 72 h of ZD6474 treatment. Next, we determined whether ZD6474 could inhibit the phosphorylation of EGF-mediated growth and survival signaling pathways in breast cancer cells growing in culture. Western blots revealed inhibition by ZD6474 of EGFR and VEGFR-2 phosphorylation, which was most pronounced in MCF-7, T-47D and MDA-MB-231 cells (**Fig. 3B**). The phosphorylated forms of both Akt and MAPK were also down-modulated in cells treated with ZD6474. Total levels of EGFR, VEGFR-2, Akt and MAPK protein remained unaltered by ZD6474 treatment (**Fig. 3B**).

ZD6474 inhibits colony formation, chemoinvasion and angiogenesis. The effect of ZD6474 on the capacity of MCF-7, T-47D and MDA-MB-231 to grow in a semisolid medium was determined. MCF-7, T-47D and MDA-MB-231 formed colonies which were further stimulated by EGF and ZD6474 inhibited EGF stimulated colony formation in soft agar (**Fig. 4A and B**). Tumor cell migration is a critical factor in the formation of

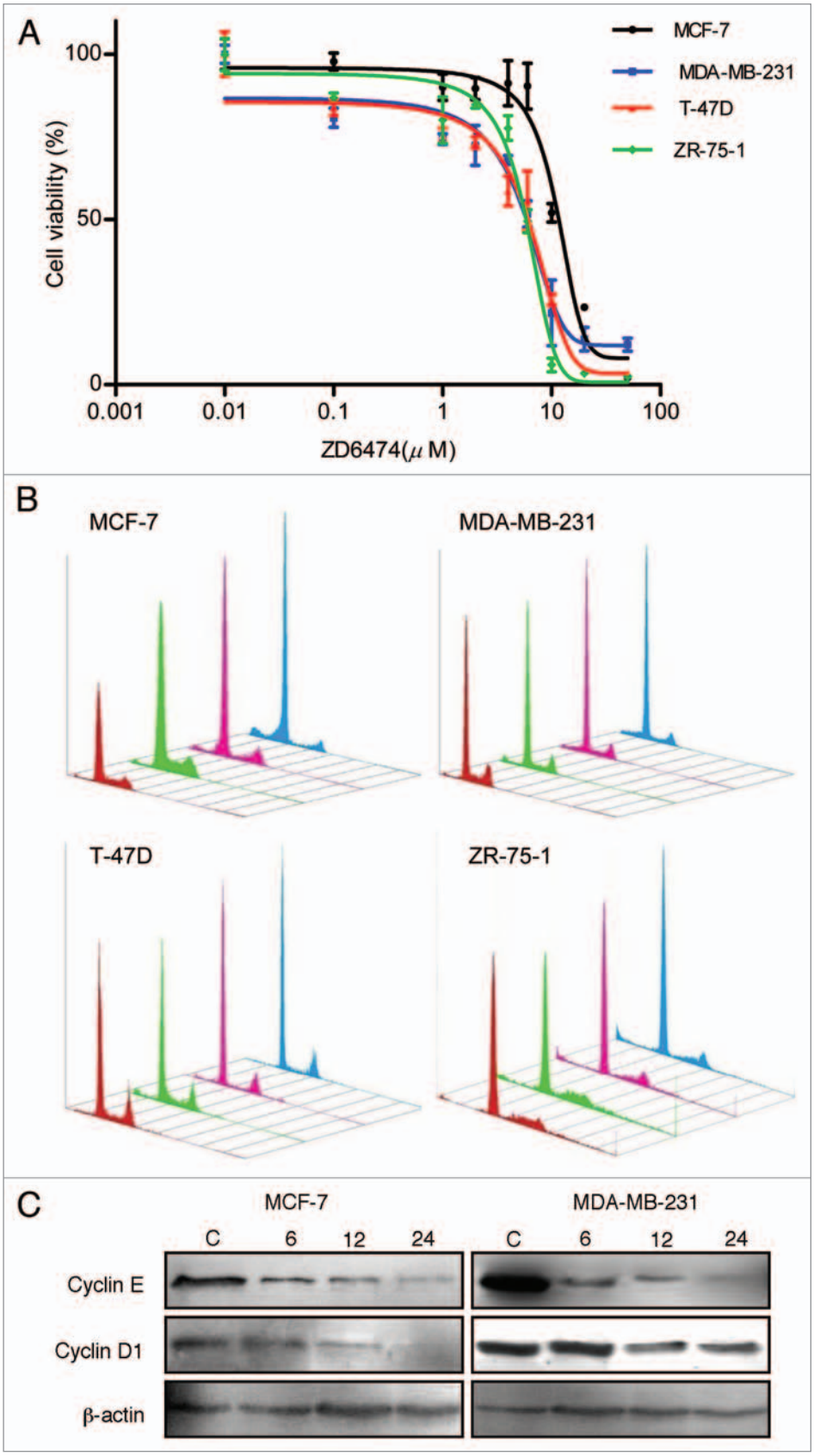

Figure 1. Dose-dependent growth inhibitory effects of ZD6474 on breast cancer cell lines. MCF-7, MDA-MB-231, T-47D and ZR-75-1 cells were treated with ZD6474 and incubated for 3 d and MTT assays were performed. (A) Points, average ± SD of three different experiments each performed in triplicate. Cell cycle analysis was performed by treating cells with an IC50 dose of ZD6474. (B) Representative histogram plot of breast cancer cells treated with ZD647 for 6 (second histogram from the left), 12 and 24 (extreme right) h along with control (extreme left) (0.1% DMSO). (C) Western blotting of MCF-7 and MDA-MB-231 treated with ZD6474 for the indicated time periods along with control (C). β-actin was used as an internal probe for equal loading. Representative data of three independent experiments.

solid tumors and is necessary for their spread to distant organs. The process of metastasis requires changes in cell adhesion, increased cell migration, and angiogenesis. To determine the effect of ZD6474 on cell invasion and migration, cells were treated and Boyden chamber assays were performed. These results show that ZD6474 effectively blocked cell invasion and migration of MCF-7, T-47D and MDA-MB-231 cells (**Fig. 4C and D**). Thus, the EGFR and VEGFR-2-TKI ZD6474 may be an effective tool in inhibiting tumor formation as well as blocking breast cancer invasion and potentially metastasis. Additionally, there was a decrease in CD-31 staining in MDA-MB-231 xenografts after ZD6474 treatment (**Fig. 6A**), suggesting its inhibitory effect on angiogenesis.

Antitumor effect of ZD6474 in nude mice. MDA-MB-231 xenografts were developed in nude mice to study the antitumor effect of ZD6474 on tumor growth. There was a significant reduction in tumor size and tumor mass ($p = 0.0002$, $n = 5$, unpaired t-test) (**Fig. 5A and B**) after ZD647 treatment compared to control. Tumor volume was significantly reduced (**Fig. 5C**). There was significant growth delay in attending tumor volume, RT_4 in the treatment groups as compared to control groups (**Fig. 5D**). The growth delay was almost 20 d (**Table 2**) between two groups. Using survival curve analysis by log-rank

Table 1. Cell cycle study of breast cancer cells

Cell	Time (h)	G_0/G_1 (%)	S (%)	G_2/M (%)
MCF-7	0	66.555 ± 3.359	25.450 ± 2.800	8.055 ± 0.488
	6	68.375 ± 2.397	24.435 ± 3.712	7.185 ± 1.038
	12	84.675 ± 0.615	9.620 ± 5.119	5.805 ± 4.377
	24	90.245 ± 0.474	7.040 ± 1.557	2.715 ± 2.029
MDA-MB-231	0	64.730 ± 2.998	19.525 ± 2.185	15.645 ± 5.042
	6	69.605 ± 2.807	17.950 ± 0.396	12.450 ± 2.418
	12	75.805 ± 1.068	13.280 ± 1.414	10.915 ± 2.482
	24	82.095 ± 5.084	10.685 ± 2.581	7.220 ± 2.517
T-47D	0	60.755 ± 3.274	21.755 ± 1.563	17.040 ± 1.075
	6	65.730 ± 3.917	16.910 ± 2.744	17.730 ± 0.651
	12	75.215 ± 0.615	8.235 ± 2.086	16.555 ± 2.694
	24	75.365 ± 1.718	8.880 ± 0.834	15.750 ± 2.546
ZR-75-1	0	62.123 ± 1.327	21.500 ± 2.121	15.439 ± 3.449
	6	67.245 ± 1.326	17.210 ± 0.071	14.608 ± 1.255
	12	72.124 ± 0.619	15.060 ± 0.085	12.378 ± 0.535
	24	81.182 ± 0.843	13.060 ± 0.085	7.354 ± 0.656

Cells were treated with ZD6474 for indicated time (hours) and analyzed by flow cytometry. Representative mean (±SD) of three independent experiments.

(Mentel-Cox) test, there was a significant difference ($p = 0.0019$) between control and ZD6474 treatment in attending tumor volume, RT_4. IHC analysis of Ki-67 confirmed the antiproliferative function and TUNEL assays confirmed the apoptosis promoting effects of ZD6474 (**Fig. 6A**).

ZD6474 blocks epidermal growth factor receptor and vascular endothelial growth factor receptor signaling in breast carcinoma cells growing subcutaneously in nude mice. Immunohistochemical studies revealed that the level of expression of EGFR and VEGFR did not vary significantly among tumors from control and ZD6474 treatment groups (**Fig. 6B**). In contrast, EGFR and VEGFR activation differed markedly in tumors from the mice treated with ZD6474 in comparison with the control animals. When antibodies specific to tyrosine-phosphorylated (activated) EGFR and VEGFR were used, both receptors showed high levels of phosphorylation in the absence of ZD6474 treatment. Levels of phosphorylation of EGFR and VEGFR-2VEGFR-2 were markedly reduced in tumors treated with ZD6474. The status of two of the major downstream targets of EGFR and VEGFR pathways, Akt and MAPK, was also assessed by immunohistochemical analysis. There were only negligible changes in the levels of expression of total Akt and MAPK proteins in the treatment vs. control groups. However, the phosphorylation status of both kinases, although high in the control, was distinctly downregulated in the mice treated with ZD6474 (**Fig. 6B**). These results correlate directly with those of in the in vitro experiments.

Discussion

In our study, ZD6474 effectively inhibited cellular proliferation and induced apoptosis of breast carcinoma cells. Previous reports indicated that ZD6474 inhibits angiogenesis and proliferation in vitro as well as in vivo in lung, ovarian, thyroid and breast carcinoma cells. We presently studied the detailed mechanisms of growth inhibition along with its additional apoptotic activity in breast cancer. ZD6474 suppressed cellular proliferation by inhibiting phosphorylation of EGFR, VEGFR-2, Akt and MAPK in vitro. In addition, it induced apoptosis by increasing the pro-apoptotic Bax, inhibiting anti-apoptotic Bcl-2, thus shifting the balance from survival to apoptosis. This dual TKI also inhibited anchorage-independent colony formation and chemoinvasion in vitro. The oral administration of ZD6474 in alternative days significantly reduced tumor size and volume in nude mice containing human MDA-MB-231 xenografts. There was a decrease in Ki-67 positivity and an increase in DNA fragmentation as shown by antibody staining and TUNEL assay, respectively, in ZD6474-treated compared to control mice bearing tumor, confirming a role as an antiproliferative and apoptotic promoting agent. IHC analyses further showed the in vivo inhibition of activated EGFR and VEGFR signaling pathways and downstream Akt and MAPK activities by ZD6474 in mice bearing MDA-MB-231 tumors.

In vitro, ZD6474 induced significant growth inhibition of breast cancer cells, including MCF-7, MDA-MB-231, T-47D and ZR-75-1. These cell lines express both EGFR and VEGFR with ZR-75-1 expressing VEGFR with low or no expression of EGFR,[23,24] indicating the role of VEGFR in tumor growth that is distinct from angiogenesis. In addition, VEGFR-1 and VEGFR-2 expression has been detected on various tumor cells, such as leukemias, melanomas, non-small cell lung carcinomas, prostate carcinomas and breast carcinomas.[25-28] The significance of this expression has not been studied in detail in breast cancer. Therefore, it is conceivable that angiogenic factors support tumor growth not only by inducing angiogenesis, but also by acting directly through VEGFR-1 and VEGFR-2 expressed on breast carcinomas. Moreover, VEGF overexpression was observed and shown to be regulated by hypoxia, which is a common feature of most solid tumors, including those of breast and this protein is highly expressed in metastastic cells.[29] Thus, VEGFR inhibitors may inhibit autocrine VEGFR-2 signaling in addition to inhibiting paracrine signaling with stromal and endothelial cells surrounding the tumor. Consistent with this hypothesis, recent studies have shown that for certain leukemias, VEGFR-1 may be essential for tumor cell growth by promoting a VEGF-A/VEGFR-1 autocrine loop, that when disrupted induces tumor growth arrest and apoptosis.[26,27] In breast cancer cell lines, increased survival and mitogenic signals are promoted by VEGF-A, but the receptor and mechanisms responsible for these observed activities have not been fully characterized.[29,30]

In this study, we also report that ZD6474 is an inhibitor of EGFR tyrosine kinase. This inhibition translated into dose- and time-dependent antiproliferative effects of ZD6474 in breast carcinoma cells. We demonstrate that ZD6474 blocks EGF-mediated proliferation of cells in vitro, as well as in vivo. Furthermore, our studies agree with studies showing ZD6474, a potent inhibitor of VEGFR-2, can inhibit EGFR phosphorylation in cells devoid of VEGFRs and which overexpress EGFR.[21]

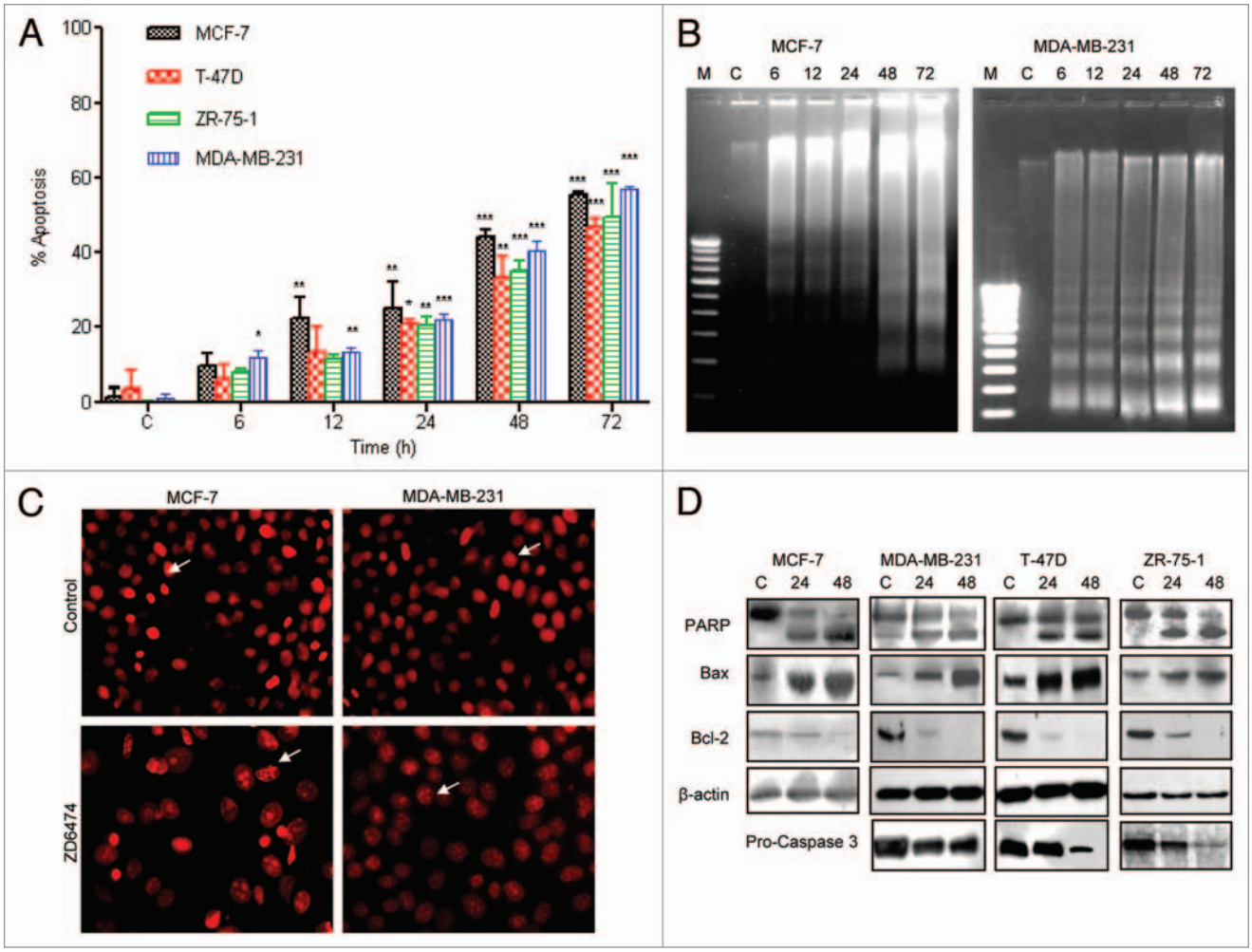

Figure 2. ZD6474 induces apoptosis in breast cancer cells. MCF-7, MDA-MB-231, T-47D and ZR-75-1 cells were treated with ZD6474 for the indicated time in hours. Flow-cytometry-based assays were done to measure the content of hypo-diploid cells (apoptosis). (A) Data represents values, each performed in triplicate; Bars, SD. *, ** and *** represent level of significance with p (<0.05) with respect to control. (B) DNA laddering in MCF-7 and MDA-MB-231 cells treated with ZD6474. (C) Fluorescent microscopy of MCF-7 and MDA-MB-231 cells treated with ZD6474 along with control. (D) Western blotting of breast cancer cells treated with ZD6474 for the indicated time in hours. Representative figures of three independent experiments.

It is speculated that ZD6474 could inhibit angiogenesis more efficiently than treatment with a selective anti-VEGFR agent, because in addition to a direct inhibitory effect on VEGFR-2 signaling, it would also have an indirect effect on angiogenesis via blockade of EGFR-induced paracrine production of angiogenic growth factors such as VEGF, bFGF and TGFα by cancer cells. Moreover, breast tumor formation and progression depends not only on tumor cells, but also on microenvironment consisting of additional infiltrating cells mainly macrophages, endothelial cells, and hematopoetic cells, at the site of neovascularization. It is also speculated that ZD6474 could block tumor proliferation more efficiently than selective anti-EGFR agents, because in addition to inhibiting EGFR, it will also have an indirect effect on cell proliferation by depriving tumors of growth factors and nutrients normally supplied by the endothelial cells. The antitumor and anti-angiogenesis role of ZD6474 is mainly responsible for enhanced anticancer activity of ZD6474 as evident from both in vivo as well as in vitro experiments.

The phosphorylation status of two major downstream targets of EGFR and VEGFR pathways, Akt and MAPK, is downregulated in vitro as well as in vivo after ZD6474 treatment. This finding is in agreement with previous studies.[31] Expression of phosphorylated Akt at Ser-473 in breast tumors is significantly associated with HER-2, ER and proliferating index Ki-67,[32] and in patients prone to relapse with distant metastases.[33] Inhibition of Akt phosphorylation by a novel Akt inhibitor KP-372-1 successfully suppressed cellular growth and induced apoptosis in thyroid cancer cells.[34] The expression level of MAPK1/2 increases markedly in breast cancer tissues in comparison to normal tissues and is strongly related with axillary lymph node metastasis.[35] MAPK is a prognostic and predictive factor even in cases of triple negative breast cancers and the elevated level of activated MAPK is associated with shorter overall survival (OS).[36] Moreover, in vitro activated pERK/MAPK is expressed in highly metastatic potential cells in comparison to non-metastatic MCF-7 cells.[37] MAPK inhibitors like U0126 and CI-1041 decreased phosphorylated

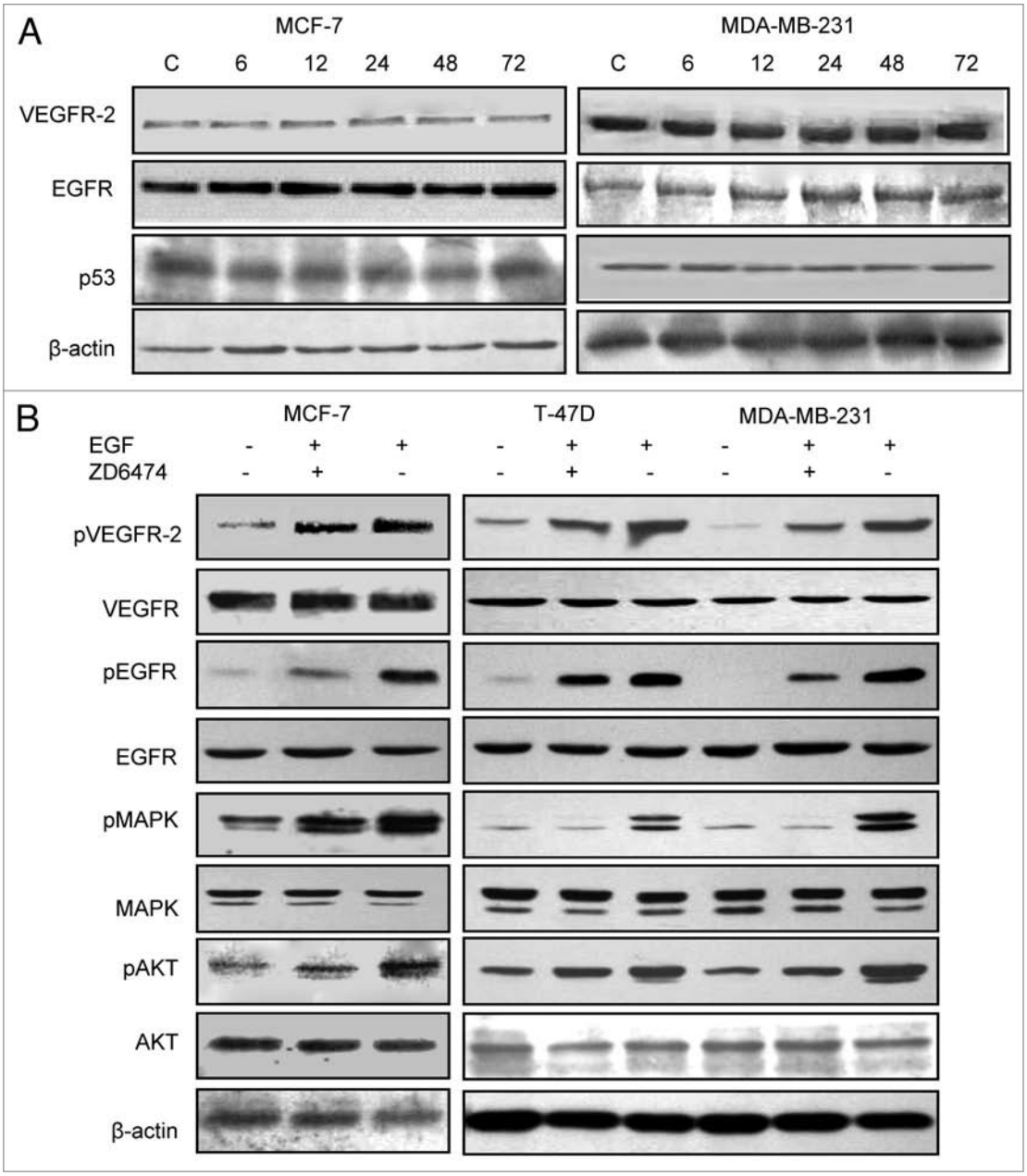

Figure 3. ZD6474 inhibits EGFR and VEGFR signaling pathways. (A) Western blotting of MCF-7 and MDA-MB-231 cells treated with ZD6474 for the indicated time and probed with anti-EGFR, VEGFR-2 and p53. (B) Autophosphorylation of EGFR, VEGFR-2, MAPK and Akt was evaluated in MCF-7, MDA-MB-231 and T-47D breast cancer cells growing in vitro in serum-free medium and stimulated with recombinant human EGF (25 ng/mL) for 30 min in the presence or absence of ZD6474. β-actin protein expression was used as an internal probe for equal loading. Representative of three independent experiments.

MAPK and reduced cell proliferation of follicular thyroid cancer and breast cancer cells in vitro and in vivo.[38,39] Our results confirm that ZD6474 can be used to inhibit the MAPK/ERK and Akt pathways responsible for human breast cancer tumorigenesis and progression.

The downstream targets of ERK/MAPK and Akt/PI3K pathways are cyclin and cyclin-dependent kinases (Cdk), and apoptosis regulators. The balance between cell division and apoptosis controls the fate of a tumor when exposed to an anticancer agent. Our results suggest that ZD6474 is both a cytostatic and cytocidal agent. It inhibits transition from G_1 to S phase as confirmed by flow cytometry and western blotting. There is a decrease in both cyclin D1 and cyclin E. Cyclin D1 and cyclin E are associated with breast cancer progression, early relapse and poor prognosis. Cyclin D1 null mice are resistant to mammary carcinomas induced by the *neu* and *ras* oncogenes, indicating an essential role for cyclin D1 in the development of some mammary cancers.[40] Therefore, blocking cyclin D1 expression by ZD6474 may represent a more specific and less toxic means of treating breast cancer.

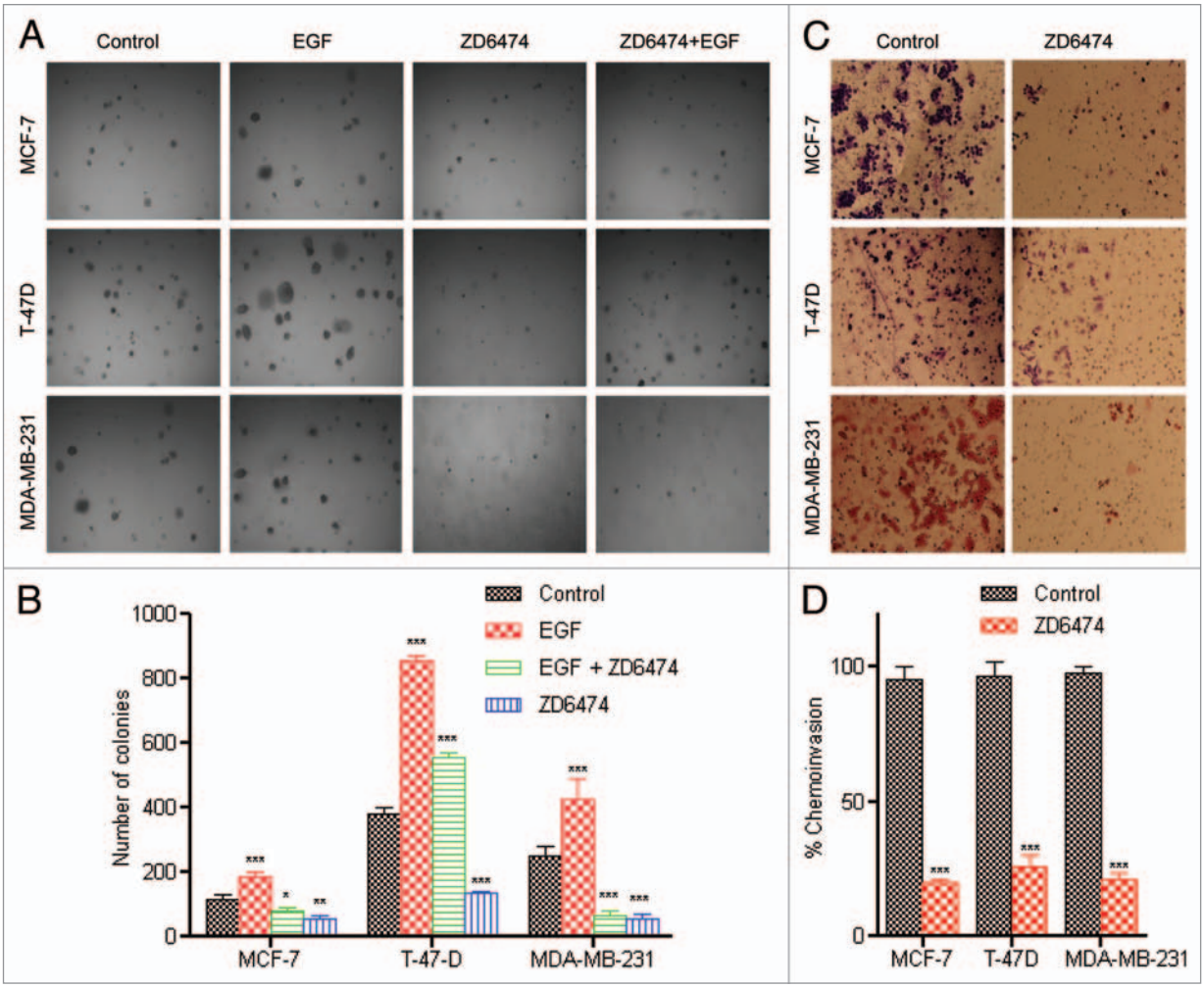

Figure 4. ZD6474 inhibits anchorage-independent cell proliferation, chemoinvasion and migration. MCF-7, T-47D and MDA-MB-231 cells were treated with ZD6474. (A) Representative figure of soft agar growth of breast cancer cells treated with ZD6474 and/or EGF along with control (without EGF and ZD6474). (B) Data represent the average (±SD) number of colonies of three different experiments, each performed in triplicate. (C) Representative figure of Boyden chamber assay for breast cancer cells treated with ZD6474 along with control. (D) Data represent the average percentage of cells (±SD) invading the Boyden chamber inserts coated with Matrigel of three different experiments, each performed in triplicate. Bars, SD. *, ** and *** represent level of significance with p (<0.05) with respect to control.

Apart from its inhibitory effect on cell cycle regulatory proteins, ZD6474 also induces apoptosis in breast cancer cells. Apoptosis is regulated by Bcl-2 gene family members. We observed an increase in the expression of Bax and a downregulation in the expression of Bcl-2. ZD6474 also decreased the expression of Bcl-x_L (data not shown) in breast cancer cells. An increase in Bcl-2 shifts the balance in favor of cell survival and can mediate resistance to chemotherapy. The tumorigenic potential of Bcl-2 has been shown in animal models[41,42] and is supported by the finding of overexpression of Bcl-2 in a variety of solid organ tumors and in lymphomas.[43,44] Moreover overexpression of Bcl-2 is associated with metastatic potential of breast cancer cells, since Bcl-2 transfectants or Bcl-2 overexpressing clones secrete MMP-9 and MMP-2, leading to an increase in invasion and metastasis.[45] There are also reports suggesting that ZD6474 enhances the antitumor effects of taxanes and ionizing radiation in glioblastoma, breast, colon and ovarian cancers.[18]

In summary, we show that simultaneously blocking EGFR and VEGFR signaling by ZD6474 can significantly inhibit cell proliferation and induce apoptosis in breast cancer cells. ZD6474 also inhibits angiogenesis and invasion in vitro. Moreover, ZD6474 significantly reduced tumor size and mass in nude mice bearing human breast cancer xenografts. These data support further development of ZD6474 for clinical use for the treatment of breast cancer patients expressing higher level of EGFR and VEGFR. By inhibiting both signaling pathways simultaneously, a greater than additive growth inhibitory and antitumor effect will be generated in breast cancers, thereby resulting in improved clinical efficacy.

Materials and Methods

Cell lines. Human breast cancer cell lines MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's Modified

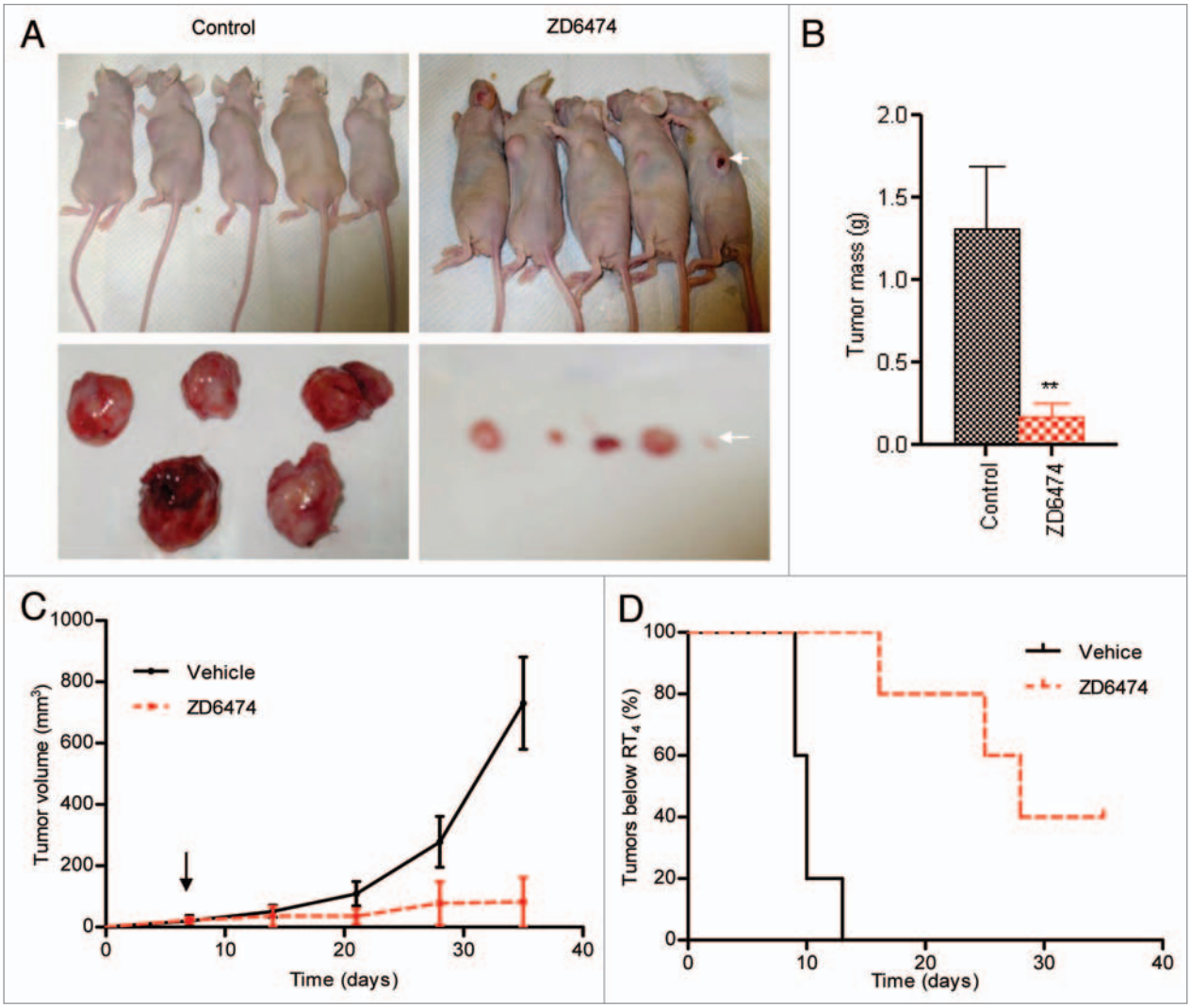

Figure 5. Antitumor activity of ZD6474 in MDA-MB-231 human breast carcinoma xenografts. Mice bearing MDA-MB-231 xenografts were treated with ZD6474 (50 mg/kg body weight) once orally in alternative days starting 1 week after tumor cell implantation and continued for 5 weeks. (A) Tumor volume was prominent and large in case of vehicle vs. the treatment groups. Mice were sacrificed and tumor masses of vehicles and in treatment groups were weighed. (B) Bar graph with SE represents tumor mass in grams after 5 w of tumor cells implantation. There was significant difference ($p = 0.0002$, $n = 5$; unpaired t-test) in tumor mass between control and treatment groups. Antitumor effect of ZD6474 was compared by tumor response curves. (C) Data points represent mean (±SE) tumor volume for vehicle and treatment groups ($n = 5$). (D) Diagrammatic representation of ZD6474 treatment on the time taken for each individual tumor within the treatment groups to achieve RT_4, along with control groups.

Eagle's Medium: nutrient mixture F-12 (Ham) (D-MEM/F-12) with 15 mM HEPES buffer, L-glutamine, pyridoxine hydrochloride, supplemented with 1.2 g sodium bicarbonate, antibiotics (10,000 U/L penicillin and 10 mg/L streptomycin) and 10% FBS. ZR-75-1 and T-47D cells were grown in RPMI-1640, supplemented with 10% FBS. Cells were incubated at 37°C in a 5% CO_2 and 95% humidified incubator.

Reagents. A stock solution of 20 mM of ZD6474 was dissolved in DMSO, stored at -20°C, and diluted in fresh medium just before use. For western blot analysis, the following antibodies were used: polyclonal rabbit anti-EGFR, anti-VEGFR-2, anti-pEGFR, anti-pVEGFR-2, monoclonal rabbit anti-pAkt, anti-pMAPK, anti-Akt and anti-MAPK (Cell Signaling Technology, Beverly, MA), monoclonal rabbit anti-PARP, anti-cytochrome-*c*, anti-Bax, anti-caspase-3, mouse anti-p53, anti-Bcl2, anti-caspase-7, horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG, alkaline phosphatase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Chemiluminescent peroxidase substrate, BCIP/NBT Propidium iodide (PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO). Stock solutions of PI and MTT were prepared by dissolving 1 mg of each compound in 1 mL PBS in incomplete medium, respectively. The solution was protected from light, stored at 4°C, and used within 1 mo. Stock concentrations of 10 mg/ml RNase A (Sigma) were prepared and kept at -20°C.

Cell viability. Cell viability was determined by MTT-dye reduction assay as described by Mosmann.[19] The cell suspensions were

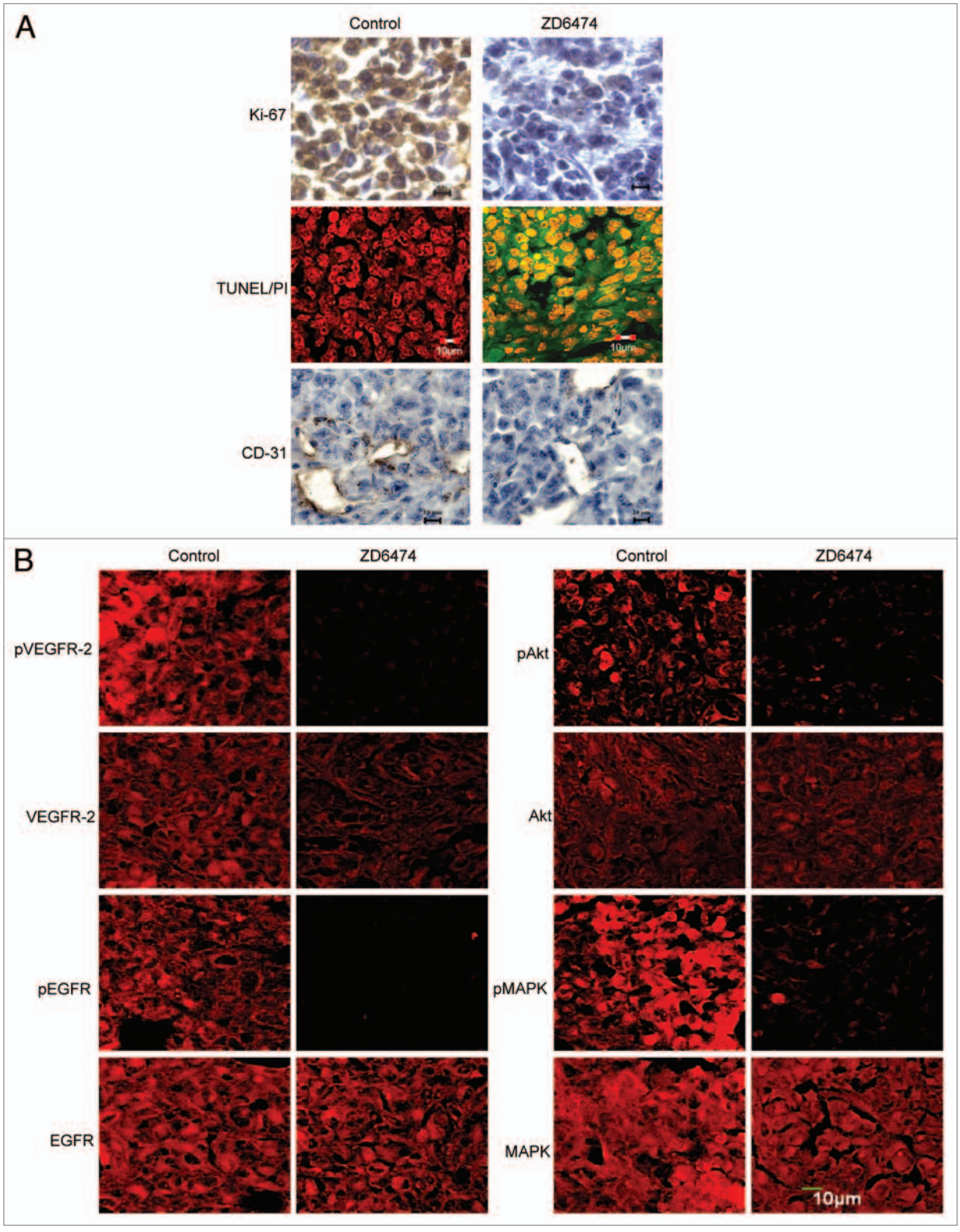

Figure 6. Immunohistochemistry of ZD6474-treated MDA-MB-231 human breast carcinoma xenografts. Paraffin-embedded sections of MDA-MB-231 bearing tumors in nude mice were processed and IHC was done. (A) IHC of Ki-67, CD-31 and TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assays were performed. (B) IHC of pEGFR (Tyr1068), pVEGFR-2 (Tyr1175), pMAPK (Thr42/22), pAkt (Ser473) along with total EGFR, VEGFR-2, MAPK and Akt. Representative of three independent experiments. Bar represents 10 μm.

Table 2. Influence of ZD6474 on MDA-MB-231 xenograft tumor growth

Treatment	RT_2 (days ± SE)*	RT_4 (days ± SE)	Growth delay (days ± SE)
Vehicle	5.06 ± 0.30	10.11 ± 0.61	NA
ZD6474 (50 mg/kg)	14.71 ± 2.71	ζ 29.41 ± 5.43	19.89 ± 5.56

*Doubling times were calculated by using the exponential growth equation of each individual tumor with in the treatment group. ζ Based upon n = 5; one tumor didn't achieve RT_4 (four times that of initial tumor volume at the start of the treatment) with in 28 d after treatment with ZD6474, and with almost complete loss of tumor mass.

dispensed (200 μl) in quadruplicate into 96-well tissue culture plates at an optimized concentration of 1 x 10^4 cells/well in complete medium. Twenty-four hours after seeding, cells were treated with 10 nM to 50 μM of ZD6474 or 0.1% DMSO (control), and incubated for 72 h. Cell viability was measured at 540 nm.

Cell cycle and apoptosis. Cells were treated at their respective IC_{50} values 24 h after seeding in 60-mm tissue culture plates. Temporal response curves were determined from 6–24 h for cell cycle study and 6–72 h for apoptosis study, along with the control (0.1% DMSO). After treatment, cells were collected and washed in phosphate buffer saline (PBS) and incubated in 70% ethanol for 45 min at 4°C or kept at -20°C overnight for fixation. Cells were centrifuged, washed and then incubated with PI solution (40 μg/ml PI, 100 μg/ml RNase A in PBS) at 37°C for 1 h. Apoptotic cells were determined by their hypochromic sub-diploid staining profiles. The distribution of cells in the different cell cycle phases was analyzed from the DNA histogram using Becton-Dickinson FACSCaliber flow cytometer and Cell Quest software.

DNA laddering. For the DNA fragmentation assays, 2 x 10^6 cells were seeded in 100-mm plates and incubated for 24 h in 10% FBS medium. Cells were treated with ZD6474 at their respective IC_{50} concentration for 6–72 h, along with control, in incomplete medium. Both floating and attached cells were collected, washed with phosphate-buffered saline, and resuspended in 0.5 mL of lysis buffer (20 mM Tris HCl [pH 8], 10 mM EDTA [pH 8] and 0.5% Triton X-100) and low molecular weight DNA was isolated as described previously.[20] DNA was resuspended in Tris-EDTA buffer (TE), treated with RNase A (1 mg/ml) at 37°C for 1 h, and total DNA was analyzed using 1.5% agarose gel and visualized by ethidium bromide staining of the gel.

Western blot analysis of growth regulatory proteins and apoptosis proteins. Cells were treated with ZD6474 at their IC_{50} dose for the indicated time periods in hours. The cells were then scraped and lysed in Nonidet P-40 lysis buffer [(50 mM Tris HCl (pH 8.0)], 137 mM sodium chloride, 10% glycerol, 1% Nonidet P-40, 50 mM sodium fluoride, 10 mM EDTA] containing 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and protease cocktail inhibitor. Cell extracts were separated on sodium dodecyl sulfate-polyacrylamide electrophoretic gel (SDS-PAGE) and transferred to nitrocellulose membranes, which were blocked with 2% BSA and probed with the appropriate antibodies and secondary antibodies. Membranes were then developed using enhanced chemiluminescence or alkaline phosphatase-based colorimetric methods.

Western blot analyses of pEGFR/EGFR, pVEGFR-2/VEGFR-2, pMAPK/MAPK, pAkt/Akt. Cells were plated in a six-well plate at a concentration of 4 x 10^5 cells per well and incubated in 10% FBS medium overnight. The next day, the cells were washed and incubated in serum-free medium for 24 h. The experimental wells were treated with ZD6474 at a concentration of 10 μM, whereas the control wells were treated with 0.1% DMSO for 1 h. Then, cells were activated with recombinant human EGF (25 ng/mL) for 30 min, washed with PBS, and scraped with lysis buffer as described previously. The proteins (50 μg) were resolved on SDS-PAGE and western blotting was performed.

Colony formation assays. Cells (2 x 10^4 cells/well) were suspended in 1 ml of 0.36% bactoagar, supplemented with complete culture medium. This suspension was layered over 1 ml of 0.6% agar-medium base layer in 6-well plates, and treated with ZD6474 at respective IC_{50} dose alone and/or in combination with 25 ng/μl EGF, along with the untreated control (0.1% DMSO) for 3 d. After 14 to 21 d, cells were stained with crystal violet (0.005%) and colonies >0.05 mm were counted as described previously.[21]

Boyden chamber assay. To test the invasive behavior of treated cells, 8-μm filters were coated with Matrigel (20 μg/filter) and placed in Boyden chambers. Cells (1 x 10^5), suspended in DMEM/F-12 containing 0.1% BSA and treated with ZD6474, were added to the top chamber. Conditioned medium from mouse fibroblast NIH3T3 cells was used as a source of chemoattractant and placed in the bottom compartment of the Boyden chambers. After 24 h incubation at 37°C, non-invaded cells were scraped off, and the cells that had migrated to the lower surface of the filter inserts were fixed with 100% methanol for 10 min and stained with H&E.[22] The results are expressed as the percent of migrated cells as compared to the control (untreated cells). Each experiment was performed three times with triplicate samples.

In vivo xenografts. Tumor response to ZD6474 was studied using a nude mouse model of breast cancer. The animal research protocol was approved, and mice were maintained in accordance with the institutional (IACUC) guidelines of Virginia Commonwealth University, School of Medicine, and mice were acclimatized at the Virginia Commonwealth University Animal Facility for 1 w prior to injection with MDA-MB-231 cells. Exponentially growing MDA-MB-231 cells were harvested and a tumorogenic dose of 2.5 x 10^6 cells in Matrigel (0.5 mg/ml) were injected s.c. in 6–7 w-old female athymic BALB/c (nu^+/nu^+) mice. Tumors were allowed to grow for 7 d; all of the mice then were weighed, and all of the tumors were measured using microcalipers.

Tumor volume was calculated using the formula (A) (B^2) π/6, where A was the length of the longest aspect of the tumor, and B was the length of the tumor perpendicular to A. All mice were randomized into two groups, containing five mice per group. Group 1, the control group received 1% poysorbate resuspended in deionized water, and the second group was treated with ZD6474 (50 mg/kg body weight) in alternative days by mouth at 0.1 ml/10 g body weight for 4 w. The dose was selected based on previous experiments.[17,18] Tumors were measured at the end of

every week. After 4 w of treatment, mice were euthanized, and the tumors were measured again. During the experiment, mice were examined twice weekly for weight loss.

Immunohistochemistry (IHC). Immunohistochemistry was performed with the following antibodies: rabbit anti-pEGFR (Tyr1068), anti-EGFR, anti-pVEGFR-2 (Tyr1175), anti-VEGFR-2, anti-pMAPK (Thr202/Tyr204), anti-MAPK, anti-pAKT (Ser473) and anti-AKT and monoclonal mouse anti-CD31 (Cell Signaling Technology), mouse anti-Ki-67 (Santa Cruz). For antibody staining, paraffin-embedded sections were first deparafinized in xylene. Excess xylene was removed by washing the slides in a series of ethanol solutions. Antigen unmasking was done by boiling the tissue with 10 mM Sodium citrate (pH 6.0), followed by maintaining the temperature at sub-boiling 90°C for 30 min. The slides were cooled at RT for 30 min, and washed thrice with dH_2O. Endogenous peroxidase blocking was done with 3% H_2O_2 followed by protein blocking using 2% BSA in TBST (protein-blocking solution). After blocking, the primary antibody (1:200 dilutions) was added and incubated for 1 h at RT. The slides were then washed with TBST, and incubated with rhodamine-conjugated anti-rabbit IgG (1:400 dilutions, Santa Cruz) for 1 h at RT in dark or biotinylated secondary antibody (Vectastain Elite ABC kit, dilutions were made according to the manufacturer's instructions), followed by addition of ABC reagent and developed using DAB as a substrate. The slides were washed again in TBST twice for 10 min, dehydrated by using ethanol, followed by washing in xylene, and mounted with D.P.X mountant (MERCK).

Immunofluorescence studies were done using a confocal microscope (Olympus FluoView FV1000, Version 1.7.1.0). Excitation wavelength at 543 nm, emission wavelength at 578 nm, 30% transmissivity, 596 (V) PMT voltages were set for image acquisition in LSM mode. Images were captured digitized using FLUOVIEW 1000 (Version 1.2.4.0) imaging software.

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