Epoxyquinol B shows antiangiogenic and antitumor effects by inhibiting VEGFR2, EGFR, FGFR, and PDGFR.

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Short title: Antiangiogenic and antitumor effects of epoxyquinol B

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Abstract

Angiogenesis is the development of new blood vessels to provide oxygen and nutrients and is indispensable for solid tumor growth. Therefore, the inhibition of angiogenesis is an important modality for cancer chemotherapy. Here we report the antiangiogenic mechanism and antitumor effects of epoxyquinol B (EPQB), which was isolated from fungal metabolites. Short term treatment of EPQB resulted in the reduction of tumor growth and the number of blood vessels directed to the tumor in a murine xenografts model. Furthermore, EPQB inhibited vascular endothelial growth factor (VEGF) -induced migration and tube formation in human umbilical vein endothelial cells (HUVECs) without cytotoxicity in vitro. VEGF-stimulated phosphorylation of VEGF receptor 2 (VEGFR2), phospholipase C γ-1 (PLCγ1), and p44/42 MAP kinases (ERK) was inhibited by EPQB in a dose-dependent manner, and *in vitro* assay using kinase domain of VEGFR2 showed that EPQB covalently bound and inhibited the VEGFR2 kinase. Its binding site on VEGFR2 was different from SU5614, a well-known VEGFR2 kinase inhibitor. Interestingly, EPQB inhibited growth factor-induced activation of not only VEGFR2 but also epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor receptor (PDGFR), suggesting that EPQB is a novel multiple kinase inhibitor. These findings suggest that EPQB would be

a good lead compound for the development of potent antiangiogenic and anti-tumor drugs.

Key words: antiangiogenesis, antitumor, epoxyquinoid, VEGF

Introduction

Angiogenesis, the formation and growth of new blood capillaries from pre-existing vessels, is a vital function for the growth of normal tissues during embryogenesis, as well as for the malignant growth of solid tumors. The process of angiogenesis in endothelial cells consists of several distinct and sequential steps such as degradation of the basement membrane by proteolytic enzymes, migration (chemotaxis) toward the stimulus, proliferation, formation of vascular loops, maturation of neovessels, and neosynthesis of basement membrane constitute. However, abnormal angiogenesis often occurs in pathological conditions such as a malignant tumor, rheumatoid arthritis, diabetic retinopathy, and other chronic inflammatory diseases [(1)].

An important step in the development of pathological angiogenesis is thought to involve the signaling pathway of angiogenesis growth factors, among which VEGF is a key regulator of malignant tumor growth [(2)]. VEGF binds and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1). VEGFR2 has especially strong tyrosine kinase activity, and it transduces the major signals for angiogenesis in endothelial cells [(3-5)]. VEGFR2 has two major VEGF-dependent autophosphorylation sites, Y1175 and Y1214, and Y1175 is an important target for the VEGF-VEGFR2 signaling pathway [(6)]. However, several reports suggest that antiangiogenic therapy

with inhibition of VEGF signaling alone has a limited effect against tumor growth [7]. With continued anti-VEGF signaling therapy, tumors show a drug resistance. In addition, tumors induce different angiogenesis growth factors, such as EGF, FGF, and PDGF [8, 9]. Therefore, it is more important for the inhibition of tumor growth to block the signaling of several angiogenesis growth factors.

Recently, various angiogenesis inhibitors have been developed to target vascular endothelial cells and block the signaling of angiogenesis factors as a cancer therapy. For example, bevacizumab (Avastin; Genentech and Roche) is a humanized monoclonal antibody that binds VEGF and prevents receptor binding and signal transduction [10]. In addition, several angiogenesis inhibitors, ZD6474, BAY 43-9006, SU5416, and AG-013736, have been developed for clinical trial [7]. Therefore, new angiogenesis inhibitors, which have unique chemical structures or biological activities, could become lead compounds in significant antiangiogenesis drugs and could be used to dissect the multiple stages leading to angiogenesis. In this regard, we have discovered several novel anti-tumor and antiangiogenic compounds using cell-based screening systems from microbial metabolites; i.e., epoxyquinol A and B [11, 12], epoxytwinol A [13], azaspirene [14], and RK-805 [15]. All these compounds inhibit the VEGF-induced migration of HUVECs, but the inhibition mechanisms have not yet been clarified.

Here, we focus the antiangiogenic effect and the target molecule(s) of epoxyquinol B (EPQB). EPQB is the first compound we have found that covalently binds receptor tyrosine kinases involved in angiogenesis.

Materials and Methods

Cells and reagents

HUVECs were cultured in HuMedia-EG2 (KURABO, Osaka, Japan) containing 2% fetal calf serum (FCS) in 5% CO_2 at 37°C. HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (FCS) (JRH Bioscience, Lenexa, KS), 50 units/ml penicillin, and 50 μg/ml streptomycin (Sigma). Recombinant human VEGF, EGF, and bFGF were obtained from R&D. Systems (Minneapolis, MN). Recombinant human PDGF BB was obtained from Peprotech Inc. (Rocky Hill, NJ). SU5614 and U73122 were obtained from Merck Biosciences. EPQB was isolated from the culture broth of a producing fungal strain using bioassay-guided purification procedures (11, 12). Recombinant human His-tagged VEGFR2 kinase, GST-tagged EGFR kinase, GST-tagged FGFR1 kinase, and His-tagged PDGFRβ kinase were obtained from Upstate Biotechnology (Charlottesville, VA).

Antibodies

Anti-human VEGFR2 antibody (C-1158) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-VEGFR2 (Y1175) antibody (#2478), anti-phospho-PLCγ1 (Y783) antibody (#2821), anti-p44/42 MAP kinase antibody (#9102),

anti-phospho-p44/42 MAPK (T202/Y204) antibody, and anti-PDGFRβ (#3175) were obtained from Cell Signaling Technology (Beverly, MA). Anti-PLCγ1 antibody was obtained from Upstate Biotechnology. Anti-GST antibody was obtained from Amersham Pharmacia Biotech (Amersham, UK).

In vivo assay for anti-tumor and antiangiogenesis effects

Balb/c mice were used as a subcutaneous model. These mice were maintained under a constant temperature, humidity, and light-controlled environment with free access to food and water. After the implantation of Renca cells (1×10^6 cells/site), mice (n=5) were separated into a control group, an EPQB group, and a paclitaxel group as a positive control. These mice received either EPQB (3.16 or 10 mg/kg, intraperitoneal (i.p.) injection, every other day) or paclitaxel (6 or 20 mg/kg, i.p., daily). One week after the chemical administration, tumor volume, number of blood vessels supplying the tumor, and changes of body weight were measured. Tumor volume (TV) was calculated using the formula $TV = W^2 \times L/2$ (with W being the shortest diameter and L being the longest diameter). The statistical analysis was performed using Student's t-test; p-values of < 0.05 were interpreted as statistically significant.

Migration assay

The migration assays were performed in a 24-well modified Chemotaxicell chamber (KURABO, Osaka, Japan). The chambers were placed in a 24-well plate containing 500 μl HuMedia-EG2 with 0.1% FCS. Next, HUVECs (1×10^5 cell/well) were incubated on the upper chambers for 30 min and treated with or without EPQB for 1 h. After VEGF (3.1 ng/ml) stimulation, the cells were allowed to migrate from the upper to the lower chamber for 9 h in 5% CO_2 at 37°C. Non-migratory cells were removed from the upper chamber by wiping the upper surface with a swab. Migrated cells were fixed for 30 min with methanol and stained with hematoxin. After H_2O washing, the number of migrated cells was counted in four different fields under a microscope.

Tube formation assay

To determine the effect of EPQB on the formation of tube-like structures, we incubated HUVECs on growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) for 1 h with or without EPQB. After pre-treatment, cells were incubated for 16 h and capillary-like tubes were photographed under a microscope. Capillary-like tubes and all side branches were counted.

Western blot analysis

The cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 5% non-fat dry milk or 5% albumin from bovine serum (BSA) (Sigma) buffer and probed with specific antibodies. The proteins were visualized using enhanced chemiluminescent substrate detection reagents (Pierce, Rockford, IL). The quantitative evaluation was measured using Scion image (http://www.scioncorp.com/pages/scion_image_windows.htm).

In vitro kinase assay

We assessed the results of an *in vitro* VEGFR2 kinase assay using europium-labeled tyrosine phosphorylation antibody and performed measurements using a time-resolved-fluorescence method. Each antibody, enzyme, and substrate was obtained from Cell Signaling (Beverly, MA). Human recombinant VEGFR2 kinase was diluted in kinase buffer (60 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 mM Na_3VO_4, 50 μM ATP, and with or without dithiothreitol (DTT)) and treated with or without EPQB. After the addition of substrate buffer containing 1.5 μM peptide substrate biotin-gastrin precursor (Cell Signaling), the reaction solutions were incubated on ice for 30 min. Next,

after adding 50 mM EDTA (pH 8.0) to stop the kinase reaction, we placed the reaction solutions in a 96-well streptavidin-coated plate (Perkin Elmer) and incubated the plate at room temperature for 1 h. The wells were washed three times with PBS and probed with phospho-tyrosine monoclonal antibody with PBS diluted with 1% BSA. After being incubated at room temperature for 30 min and washed three times with PBS diluted with 1% BSA, europium-labeled anti-mouse IgG, was added to each well. The wells were incubated at room temperature for 30 min and washed five times with PBS. Finally, after adding DELFIA enhancement solution, we measured kinase activities by 615 nm fluorescence emission with an appropriately time-resolved plate reader.

In vitro binding assay

For the *in vitro* binding assay, recombinant His-VEGFR2, GST-EGFR, GST-FGFR1, and His-PDGFRβ kinase protein (100 ng/sample) were pretreated with or without EPQB at 0.1 and 1 mM for 1 h at 37°C as the competitor in the reaction buffer. In addition, recombinant His-VEGFR2 was pretreated with or without cysteine, glutathione, and serine at various concentrations under the same conditions. Next, each sample was treated with biotinylated-EPQB (Bio-EPQB) at 0.5 mM for 1 h at 37°C. These samples were separated by SDS-PAGE and detected by Western blotting.

Results

EPQB inhibits tumor growth and tumor-induced angiogenesis *in vitro* and *in vivo*

To investigate that EPQB inhibits tumor growth or tumor-induced angiogenesis *in vivo,* we tested *in vivo* assay using a Balb/c mouse subcutaneous model. Mice (n=5) received either EPQB (3.16 or 10 mg/kg, i.p. every other day) or paclitaxel (6 or 20 mg/kg, i.p. daily) after inoculation of Renca cells (1×10^6 cells/site). The number of blood vessels supplying tumors, tumor volume, and change of body weight were assessed at day 7. EPQB reduced the number of blood vessels supplying tumors and tumor volume in the 3 and 10 mg/kg groups without significant toxicity (Fig. 1b, c). These results suggest that EPQB contributes to the reduction of tumor growth and tumor-induced blood vessels.

EPQB inhibits migration and tube formation of HUVECs

As EPQB reduced tumor-induced blood vessels, we examined its inhibitory effect on migration and tube formation of HUVECs for antiangiogenic effect. In a migration assay, VEGF treatment significantly induced the migration of HUVECs for 9 h. The induction of migration by VEGF was inhibited by EPQB in a dose-dependent manner without significant cell toxicity as well as by SU5614, a potent VEGFR2/PDGFR kinase inhibitor (Fig. 2a). We next performed a capillary-like tube formation assay on matrigel

matrix. After the 18 h incubation on the matrigel matrix, HUVECs significantly formed a capillary-like tube. EPQB inhibited the tube formation of HUVECs. Vessel branch-points, which were an indication of the vessel network, were inhibited with EPQB treatment in a dose-dependent manner (Fig. 2b). These results suggest that EPQB has an antiangiogenic effect in HUVECs.

EPQB inhibits VEGF-induced VEGFR2 activation and signaling

To characterize the effect of EPQB on the angiogenesis signaling pathway, we investigated the VEGF-induced phosphorylation of VEGFR2, PLCγ1, and ERK. HUVECs (2×10^5 cell/well) were starved with M199 medium for 18 h and treated with various concentrations of EPQB for 2 h. After VEGF (3.1 ng/ml) stimulation for 5 min, the cells were lysed, and analyzed by Western blotting. VEGFR2, PLCγ1, and ERK were not phosphorylated after 24 h serum starvation, but they were significantly phosphorylated after VEGF addition (Fig. 3a). In contrast, EPQB inhibited VEGF-induced their phosphorylations in a dose-dependent manner.

Next, we examined the activation of VEGFR2 kinase *in vitro*. The *in vitro* VEGFR2 kinase assay used human recombinant VEGFR2 kinase as described in Materials and Methods. The tyrosine kinase activity of VEGFR2 was dose-dependently inhibited

without DTT (IC_{50} = 6.85 μM) but not with DTT (Fig. 3b). These results suggest that EPQB inhibits VEGFR2 kinase directly and blocks the VEGF signaling pathway.

EPQB covalently binds VEGFR2 kinase domain

It is well known that several compounds covalently bind to and inhibit their target molecules [16-24]. Since EPQB also has the reactive functional groups epoxide and α, β-unsaturated ketone (Fig. 1a), we anticipated that EPQB covalently bound to VEGFR2. To test this possibility, we synthesized Bio-EPQB and investigated EPQB binding on VEGFR2 (Fig. 4a). Synthesized Bio-EPQB also inhibited the tube formation of HUVECs, however its activity was weaker than that of EPQB. In a binding assay, recombinant VEGFR2 kinase domain was pre-treated with or without EPQB for 1 h as the competitor, and then incubated with Bio-EPQB for 1 h at 37°C. Protein samples were separated by SDS-PAGE and analyzed by Western blotting with anti-VEGFR2 antibody and HRP-avidin. As shown in Fig. 4b, Bio-EPQB covalently bound to VEGFR2 kinase domain, and its binding was countered by EPQB in a dose-dependent manner, but not by SU5614, a potent inhibitor for VEGFR2 and PDGFR activation.

To obtain information about the possible amino acid residues of EPQB binding, we next conducted a competition assay. EPQB didn't inhibit the kinase activity of VEGFR2 in

the presence of DTT, a potent reducing agent (Fig. 3b). Therefore, we investigated whether thiol groups such as a cysteine residue in VEGFR2 were targets of EPQB. Recombinant VEGFR2 kinase domain was pre-treated with or without cysteine, glutathione, and serine for 1 h as the competitor and then incubated with Bio-EPQB for 1 h at 37°C. Protein samples were analyzed under the conditions shown in Fig. 4b. As shown in Fig. 4c, the binding between VEGFR2 and Bio-EPQB was inhibited by cysteine and glutathione, but not serine. These results suggest that EPQB covalently binds VEGFR2 through the cysteine residue and that its binding mechanism is different from that of SU5614.

EPQB binds receptors of other growth factors and inhibits their signaling

We next investigated whether EPQB inhibited the signal transduction of other growth factors, i.e., EGF, FGF, and PDGF. HUVECs (2×10^5 cell/well) were starved with M199 medium for 18 h and treated with various concentrations of EPQB for 2 h. After the stimulation by EGF (10 ng/ml) for 5 min, FGF (25 ng/ml) for 10 min, and PDGF (10 ng/ml) for 10 min, the cells were lysed and analyzed by Western blotting for ERK phosphorylation. Without pretreatment with EPQB, each growth factor induced ERK phosphorylation. EPQB inhibited the growth factor-induced phosphorylation of ERK in

a dose-dependent manner (Fig. 5a). This result suggests that EPQB also inhibits the EGF, FGF, and PDGF signaling pathways.

Next, we investigated the physiological binding between EPQB and EGFR, FGFR, and PDGFRβ. Recombinant EGFR, FGFR, and PDGFRβ kinase domain were pre-treated with or without EPQB for 1 h as the competitor and then incubated with Bio-EPQB for 1 h at 37℃. Protein samples were separated by SDS-PAGE and analyzed by Western blotting. As shown in Fig. 5b, Bio-EPQB covalently bound all the receptors we tested, and the binding between those receptors and Bio-EPQB was countered by EPQB, respectively, in a dose-dependent manner. These results suggest that EPQB is a multiple receptor kinase inhibitor of the angiogenic growth factors.

Discussion

The angiogenic signaling in mammalian endothelial cells is carried out mainly by growth factors that interact with receptors containing a tyrosine kinase activity domain. The interaction of the growth factors with the receptors activates signaling pathways and induces tumor angiogenesis. Therefore, the inhibition of tyrosine kinase by small molecule inhibitors is a critical strategy for the treatment of tumor angiogenesis, and several tyrosine kinase inhibitors have been tested in clinical trials [6, 25]. In this study,

we showed that EPQB inhibits growth factor-induced angiogenesis in endothelial cells by binding tyrosine kinase receptors and blocking their signaling.

We revealed that EPQB showed a decreasing of tumor growth and tumor-induced blood vessels (Fig.1 b,c). Moreover, EPQB dose-dependently inhibited the migration and tube formation in HUVECs without apparent cytotoxicity (Fig. 2). To identify the molecular target of EPQB on the antiangiogenic effect, we first focused on the VEGF signal transduction cascade. Pretreatment with EPQB resulted in the inhibition of VEGF-stimulated phosphorylation of VEGFR2, PLCγ, and ERK (Fig. 3a), and of interaction between VEGFR2 and PLCγ1 (supplemental data Figs. S.2a, b) in HUVECs. These results strongly suggested that EPQB inhibits VEGFR2 activation. To confirm this possibility, we next tested the inhibitory activity of EPQB on the VEGFR2 kinase using recombinant VEGFR2 kinase domain. EPQB inhibited the kinase activity of VEGFR2 in a dose-dependent manner (IC_{50} = 6.85 μM), suggesting that EPQB inhibits angiogenesis *in situ* via VEGFR2 inhibition. However, though the significant inhibition on kinase phosphorylation was observed mainly 10-30 μM (Fig. 5a), 3 μM EPQB inhibited migration and tube formation of HUVECs (Fig. 2a and 2b). Because migration and tube formation assays were examined at 9 and 18 h after VEGF stimulation, there is a possibility that, even if the inhibition is weak to kinases, continuous inhibition of

receptor kinase is sufficient to inhibit the migration and tube formation of HUVECs.

Interestingly, the inhibitory effects of EPQB on VEGFR2 kinase activity were completely cancelled by DTT addition. There are several reports that compounds containing epoxides or α, β-unsaturated ketones with concomitant opening of the epoxide are highly reactive to the addition of nucleophiles such as thiols [26-28]. Since EPQB has α, β-unsaturated ketone and epoxide, it is possible that EPQB covalently binds to VEGFR2 kinase domain directly. Therefore, we synthesized biotinylated EPQB (Bio-EPQB) and examined whether Bio-EPQB covalently binds VEGFR2. *In vitro* binding assay clearly showed that Bio-EPQB bound VEGFR2 kinase domain, and its binding was countered not only by EPQB but by the thiol-containing compounds cysteine and glutathione (Fig. 4). On the other hand, the binding between Bio-EPQB and VEGFR2 was not countered by SU5614 (Fig. 4b). These results strongly suggest that EPQB covalently binds to VEGFR2 through the cysteine residues and that its binding mechanism is different from that of SU5614.

Recently, resistance to anti-VEGF therapy has been noted in human and animal models [6]. The mechanism of tumor resistance is not yet clear, but several hypotheses have been reported. Drevs et al reported that increases of treatment response in VEGF and other angiogenic factors could contribute to tumor escape from antiangiogenic

treatment [29]. Huang et al reported that maturation of the tumor vasculature might render vessels relatively resistant to anti-VEGF signaling therapy [30]. Furthermore, when tumors were treated with potent antiangiogenic drugs aimed at a single target, tumors produced new proangiogenic growth factors to induce the development of robust blood vessels [7, 8]. Therefore, the multiple target inhibitors that act on several angiogenic factors might be a more effective remedy against solid tumor. Given that, we next investigated whether EPQB inhibited the signaling pathway not of only VEGF but also other growth factors such as EGF, FGF, and PDGF. As shown in Fig. 5, EPQB inhibited the EGF, FGF, and PDGF induced phosphorylation of ERK by covalent binding. In addition, the binding between these receptors and Bio-EPQB was countered by EPQB in a dose-dependent manner. These results showed that EPQB is a multiple kinase inhibitor. Several reports recently suggested that compounds with broad selectivity would be more effective than compounds with a narrow selectivity *in vivo* [31-33]. Different from these multiple kinase inhibitors, EPQB covalently binds and inhibits receptor kinases. Therefore, it is expected to develop more potent kinase inhibitors and effective antiangiogenic compounds from EPQB. Our study revealed that EPQB would be a good lead compound for the development of potent antiangiogenic and anti-tumor drugs.

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Figure legends

Fig. 1　EPQB inhibits tumor-induced angiogenesis *in vivo*.

(a) Structure of epoxyquinol B (EPQB). (b) Balb/c mice (n=5) received either EPQB (3.16 or 10 mg/kg, i.p. every other day) or paclitaxel (6 or 20 mg/kg, i.p. daily) after inoculation of Renca cells (1×10^6 cells/site). Arrows indicates blood vessels supplying tumors. (c) The number of blood vessels supplying tumors, and tumor volume (mm^3) in Fig. 1b, and change of body weight (g) at day 7 were measured. None means control group. P values were calculated with respect to the control using Student's *t*-test. *, more significant than controls ($p < 0.05$).

Fig. 2　EPQB inhibits migration and tube formation of HUVECs.

(a) EPQB inhibits VEGF-induced migration of HUVECs. HUVECs (1×10^5 cell/well) were treated with various concentrations of EPQB for 1 h. After VEGF (3.1 ng/ml) stimulation, the cells were incubated for 9 h. After incubation, the migrated cells were counted under a microscope. Cell viability was measured by trypan blue assay. Bar indicates 250 μm. (b) HUVECs (1×10^5 cell/well) were incubated with various concentrations of EPQB for 18 h on matrigel matrix. After incubation, the vessel branch-points of tube formation were counted under a microscope. Bar indicates 1 mm.

SU5614 was an inhibitor of VEGFR and PDGFR activation. *: more significant than None EPQB controls (p<0.05).

Fig. 3　EPQB inhibits VEGF-induced VEGFR2 activation and signaling.

(a) HUVECs (2×10^5 cell/well) were starved with M199 medium for 18 h and treated with various concentrations of EPQB for 2 h. After VEGF (3.1 ng/ml) stimulation for 5 min, the cells were lysed and analyzed by Western blotting for VEGFR2, PLCγ1, and ERK phosphorylation. The quantitative evaluation about phosphorylation of VEGFR2 was measured using Scion image. The phosphorylation rate (p-VEGFR2 intensity/VEGF intensity) of each concentration of EPQB was represented as the percentage of that of control (VEGF +). (b) In an *in vitro* VEGFR2 kinase assay, the samples were treated with various concentrations of EPQB for 30 min with or without DTT. The assay was performed using europium-labeled tyrosine phosphorylation antibody and measured using the time-resolved-fluorescence method.

Fig. 4　EPQB binds to VEGFR2 and attacks cysteine residues.

(a) A structure of Bio-EPQB. (b) Recombinant His-VEGFR2 kinase domain was pre-treated with or without EPQB at 0.5 mM for 1 h as the competitor and then

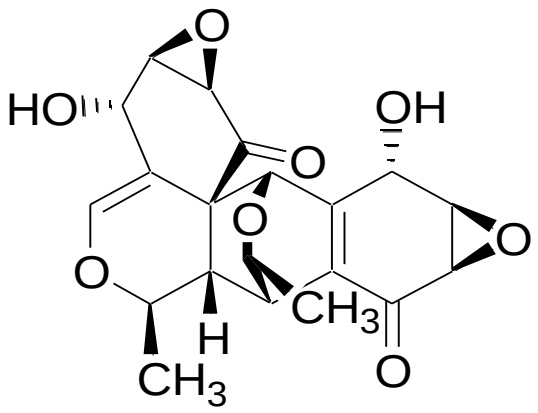
incubated with Bio-EPQB at 0.1 and 1 mM for 1 h at 37°C. Protein samples were separated by SDS-PAGE and analyzed by Western blotting for VEGFR2 and HRP-avidin. (c) Recombinant His-VEGFR2 kinase domain was pre-treated with or without cysteine, glutathione, and serine at various concentrations for 1 h as the competitor and then incubated with Bio-EPQB at 0.5 mM for 1 h at 37°C. Protein samples were separated by SDS-PAGE and analyzed by Western blotting.

Fig. 5　EPQB binds EGFR, FGFR, and PDGFR and inhibits their signaling.

(a) HUVECs (2×10^5 cell/well) were starved with M199 medium for 18 h and treated with various concentrations of EPQB for 2 h. After EGF (10 ng/ml), FGF (25 ng/ml), and PDGFβ (50 ng/m) stimulation, the cells were lysed and analyzed by Western blotting for ERK phosphorylation. (b) Recombinant GST-EGFR, GST-FGFR1, and His-PDGFRβ kinase proteins were pre-treated with or without EPQB at 0.1 and 1 mM for 1 h as the competitor and then incubated with Bio-EPQB at 0.5 mM for 1 h at 37°C. Protein samples were separated by SDS-PAGE and analyzed by Western blotting.

Fig.1a

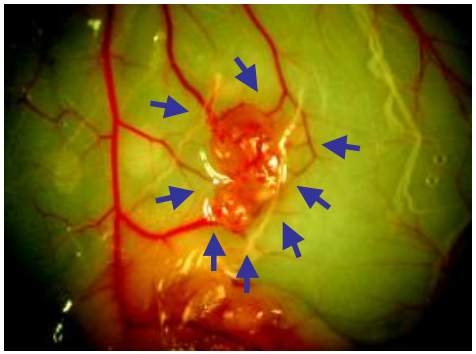
(a)



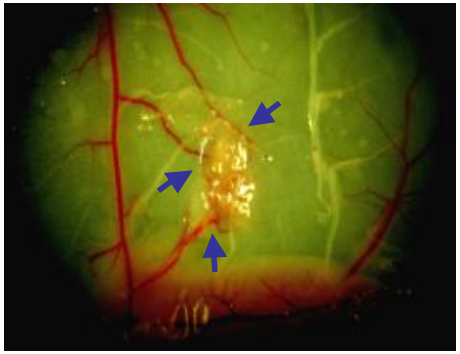
Epoxyquinol B

Fig. 1b

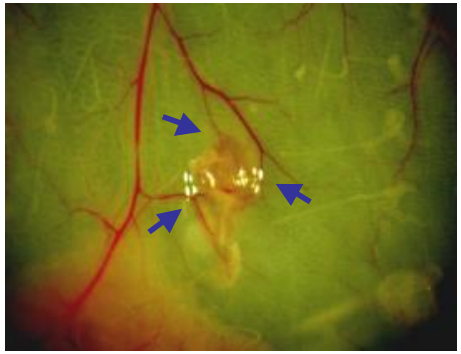
(b)



vehicle



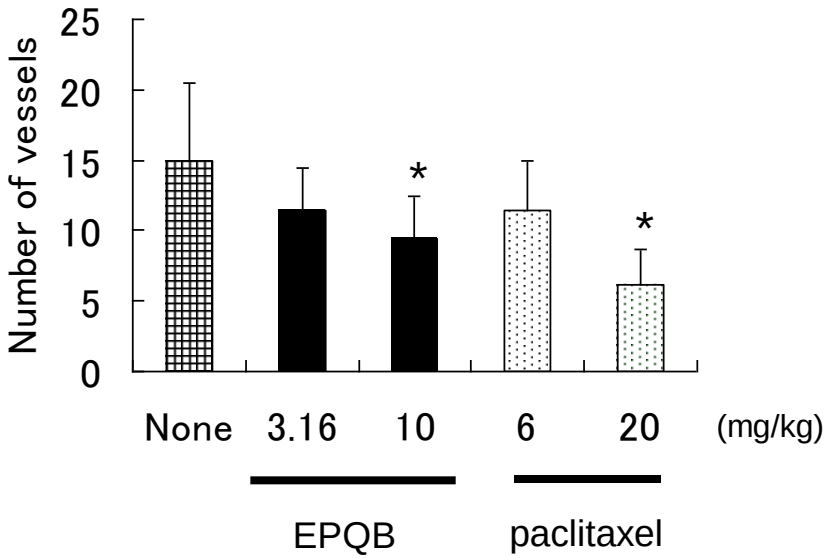
EPQB (10 mg/kg)

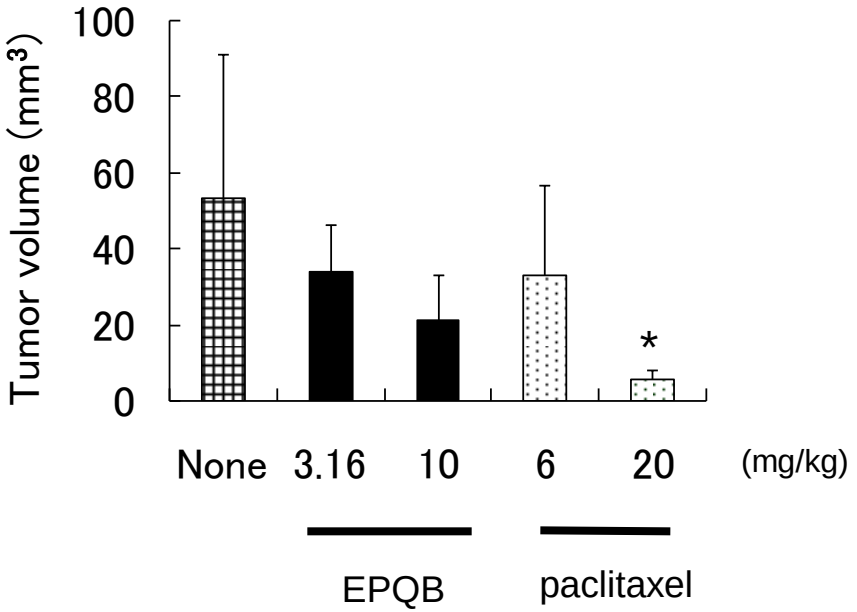


paclitaxel (6 mg/kg)

Fig. 1c

(c)


Number of vessels
25
20
15
10
5
0
*
*
None
3.16
10
6
20
(mg/kg)
EPQB
paclitaxel


Tumor volume (mm³)
100
80
60
40
20
0
*
None
3.16
10
6
20
(mg/kg)
EPQB
paclitaxel

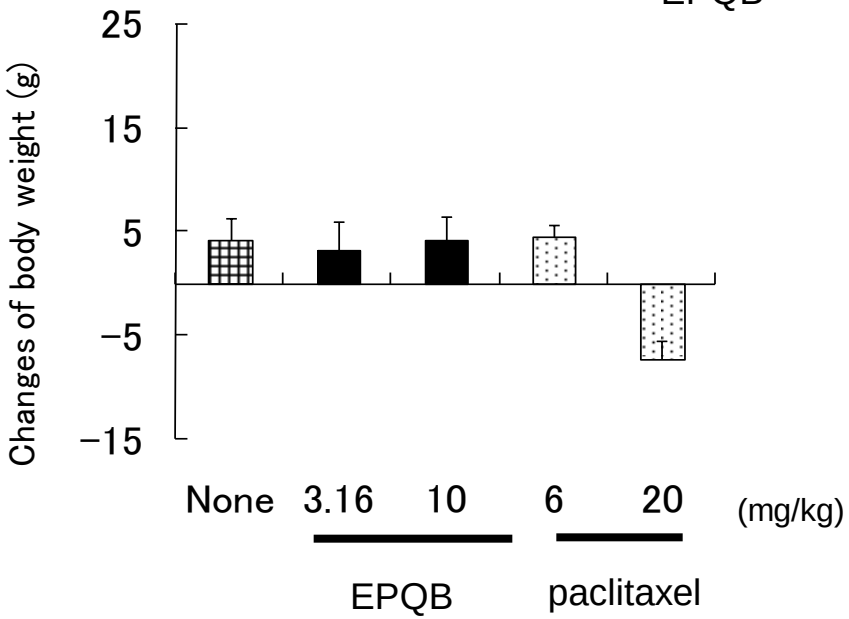

Changes of body weight (g)
25
15
5
−5
−15
None
3.16
10
6
20
(mg/kg)
EPQB
paclitaxel

Fig.2a

(a)

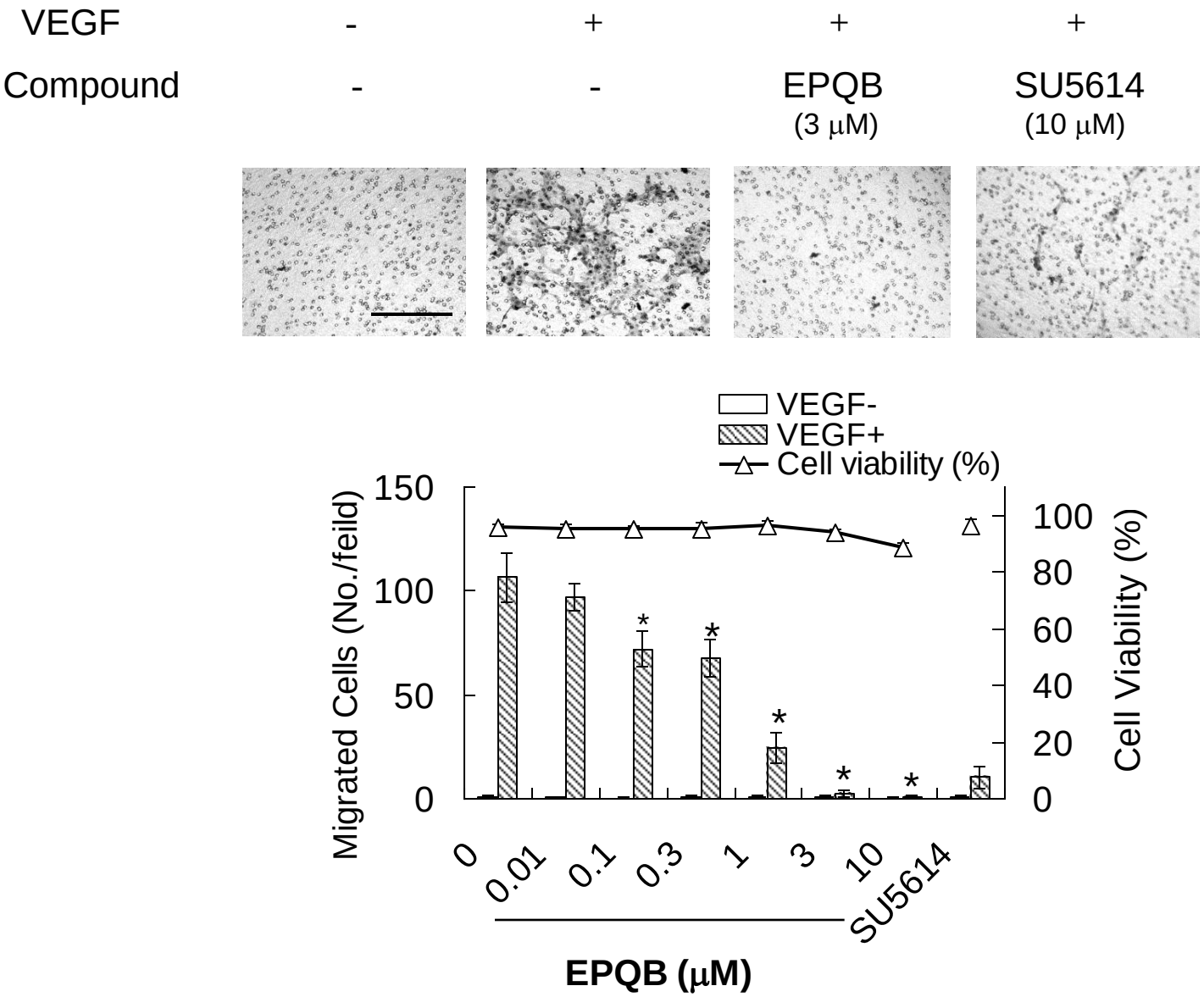

VEGF - + + +
Compound - - EPQB (3 μM) SU5614 (10 μM)
VEGF-
VEGF+
Cell viability (%)
Migrated Cells (No./feild)
Cell Viability (%)
150
100
50
0
100
80
60
40
20
0
*
0 0.01 0.1 0.3 1 3 10 SU5614
EPQB (μM)

Fig.2b

(b)

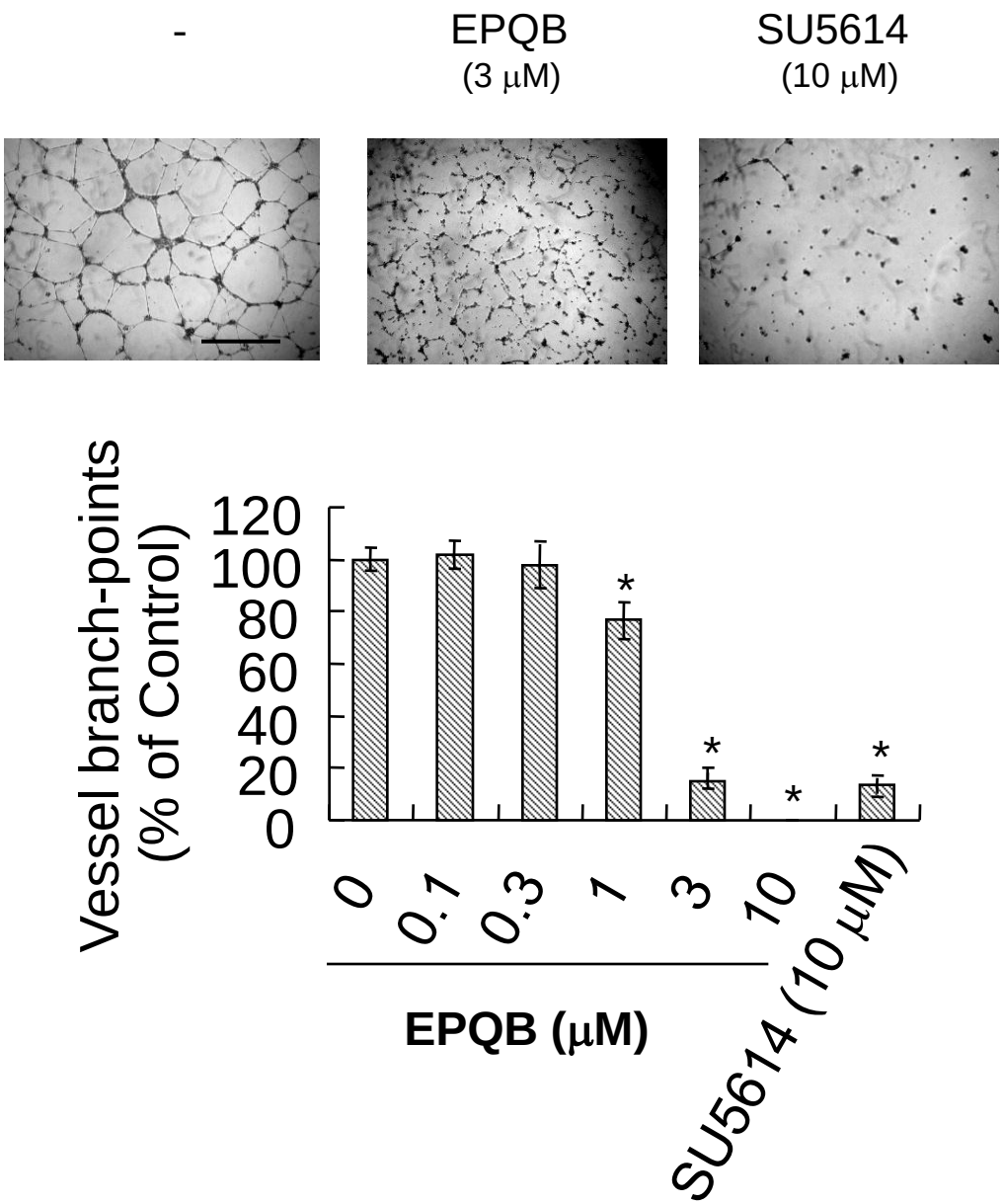
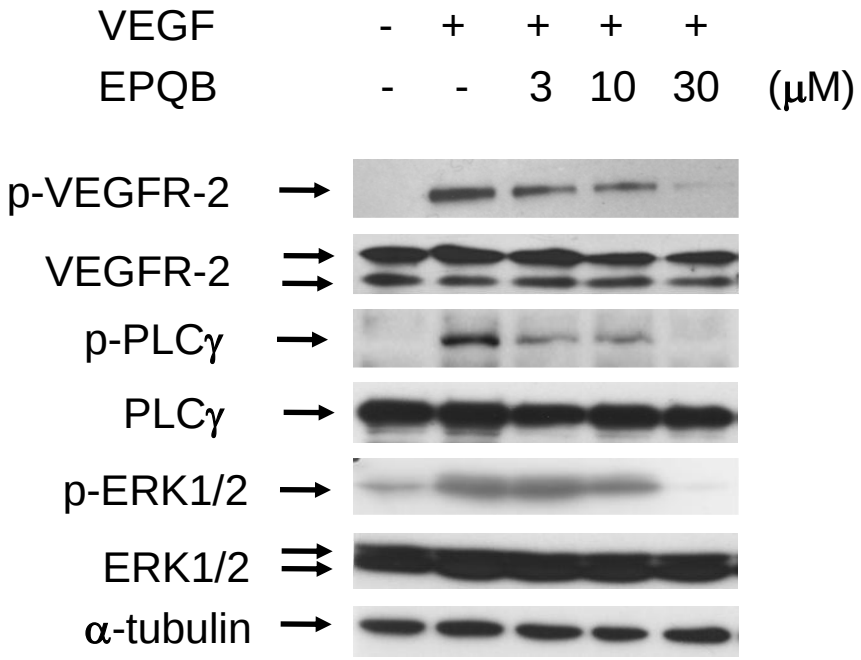

Compound
-
EPQB
(3 μM)
SU5614
(10 μM)
Vessel branch-points
(% of Control)
120
100
80
60
40
20
0
*
*
*
*
0
0.1
0.3
1
3
10
EPQB (μM)
SU5614 (10 μM)

Fig.3a

(a)


VEGF - + + + +
EPQB - - 3 10 30 (μM)
p-VEGFR-2
VEGFR-2
p-PLCγ
PLCγ
p-ERK1/2
ERK1/2
α-tubulin

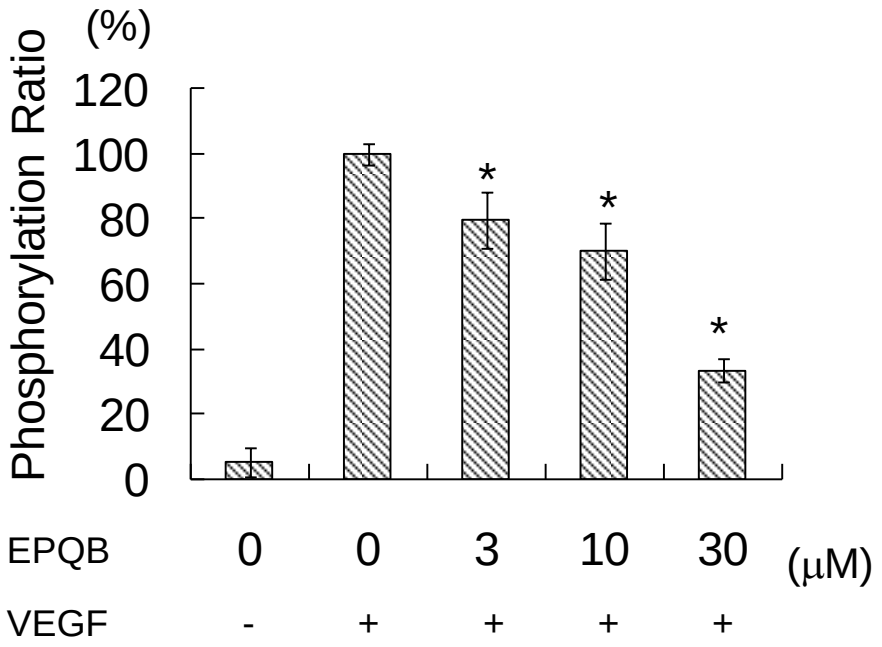

Phosphorylation Ratio
(%)
120
100
80
60
40
20
0
*
*
*
EPQB 0 0 3 10 30 (μM)
VEGF - + + + +

Fig.3b

(b)

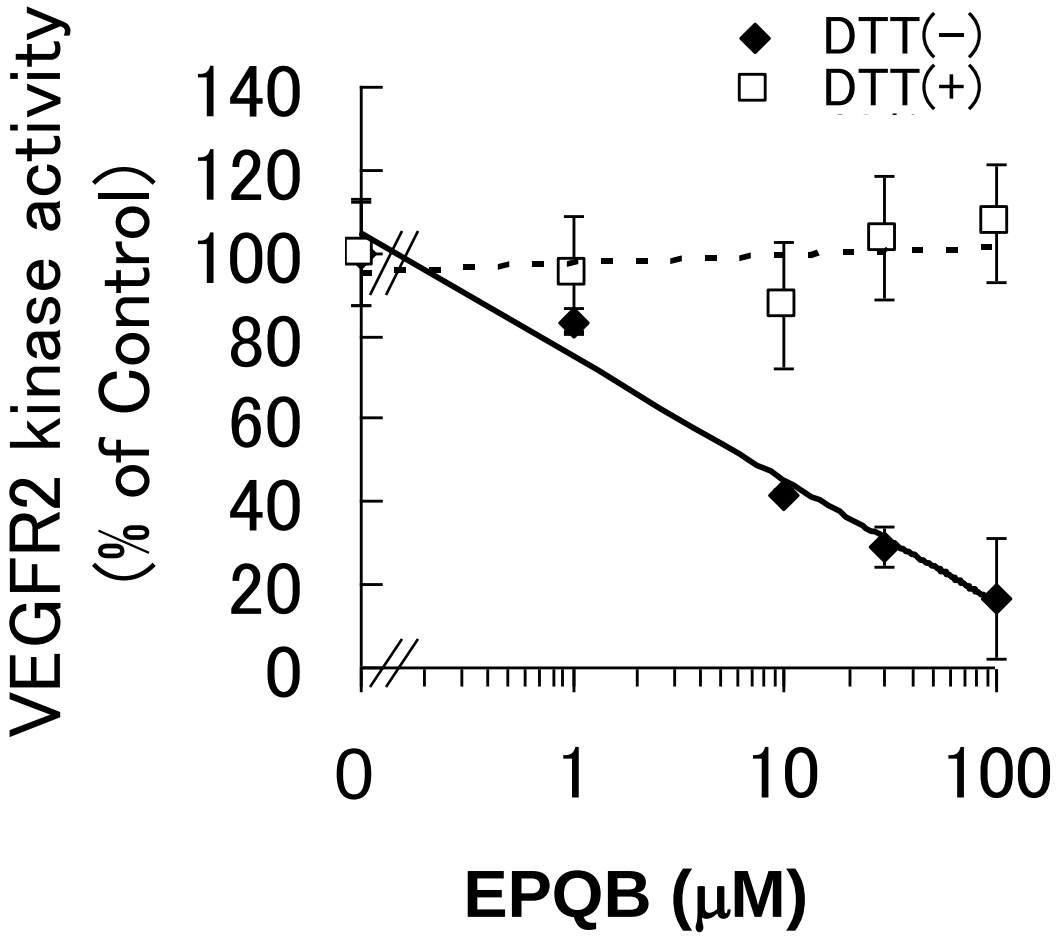
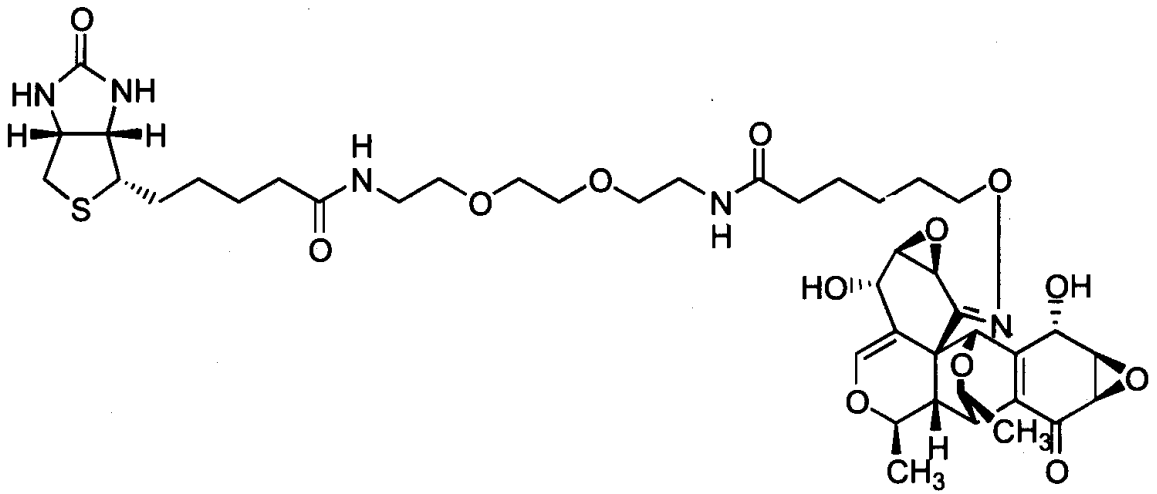

DTT(−)
DTT(+)
VEGFR2 kinase activity
(% of Control)
140
120
100
80
60
40
20
0
0
1
10
100
EPQB (μM)

Fig. 4a

(a)



Biotinylated-Epoxyquinol B (Bio-EPQB)

Fig. 4b

(b)

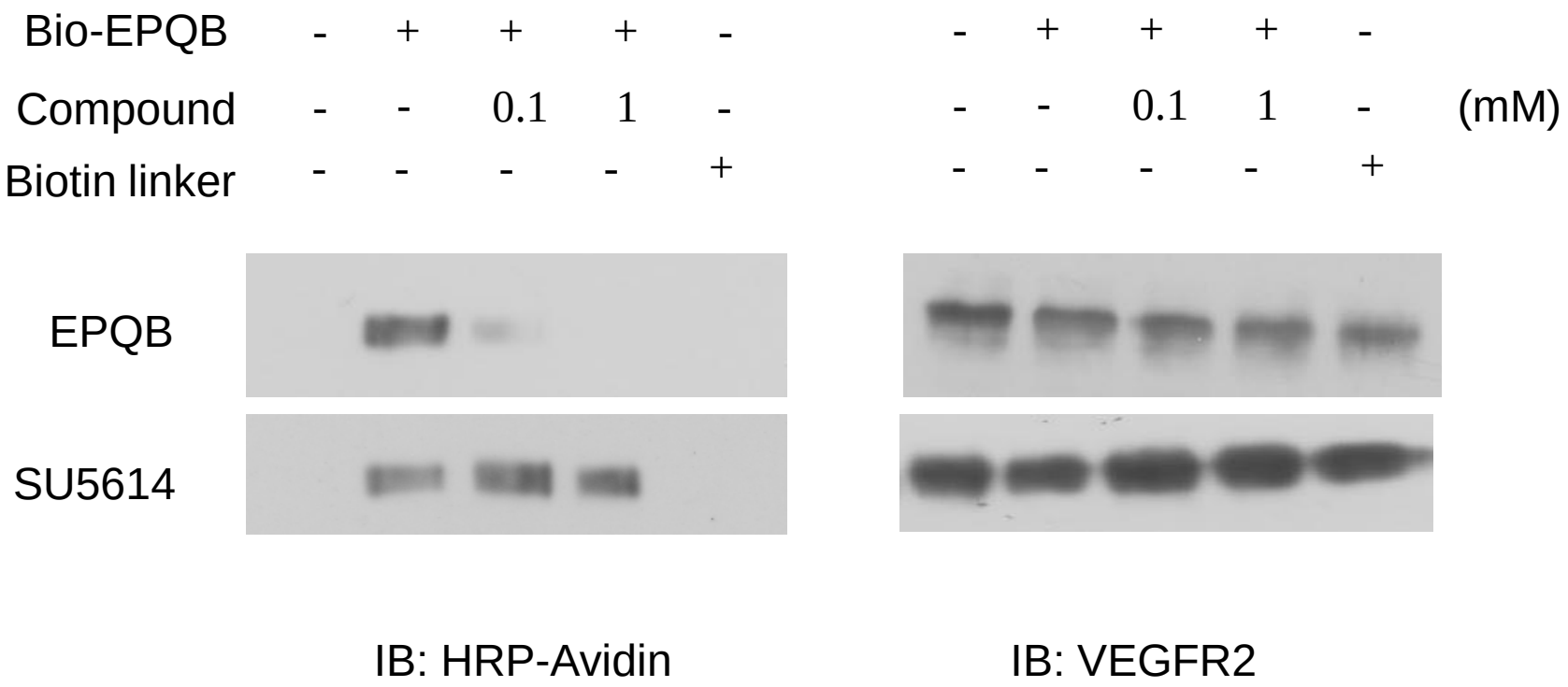

Bio-EPQB - + + + - - + + + -
Compound - - 0.1 1 - - - 0.1 1 - (mM)
Biotin linker - - - - + - - - - +
EPQB
SU5614
IB: HRP-Avidin
IB: VEGFR2

Fig. 4c

(c)

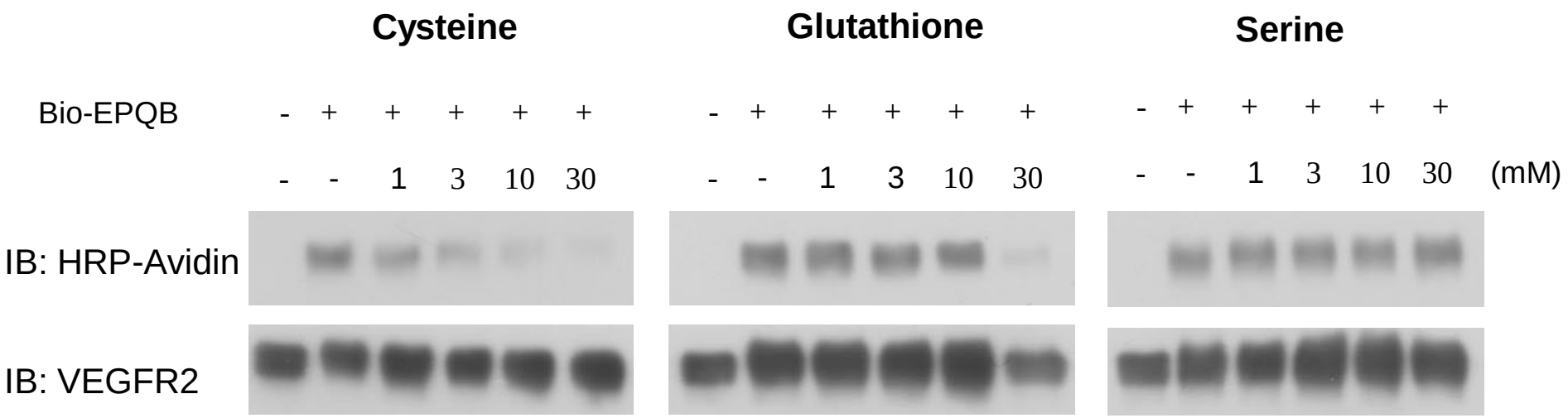

Cysteine
Glutathione
Serine
Bio-EPQB
- + + + + +
- - 1 3 10 30
- + + + + +
- - 1 3 10 30
- + + + + +
- - 1 3 10 30
(mM)
IB: HRP-Avidin
IB: VEGFR2

Fig. 5a

(a)

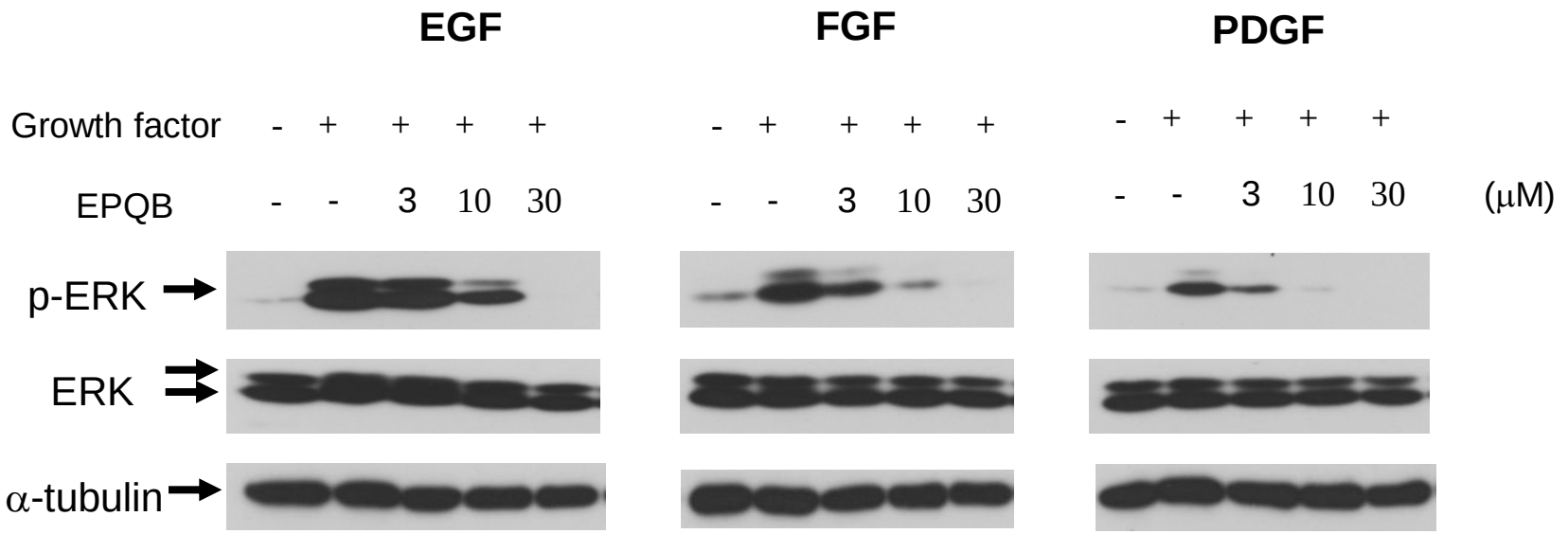
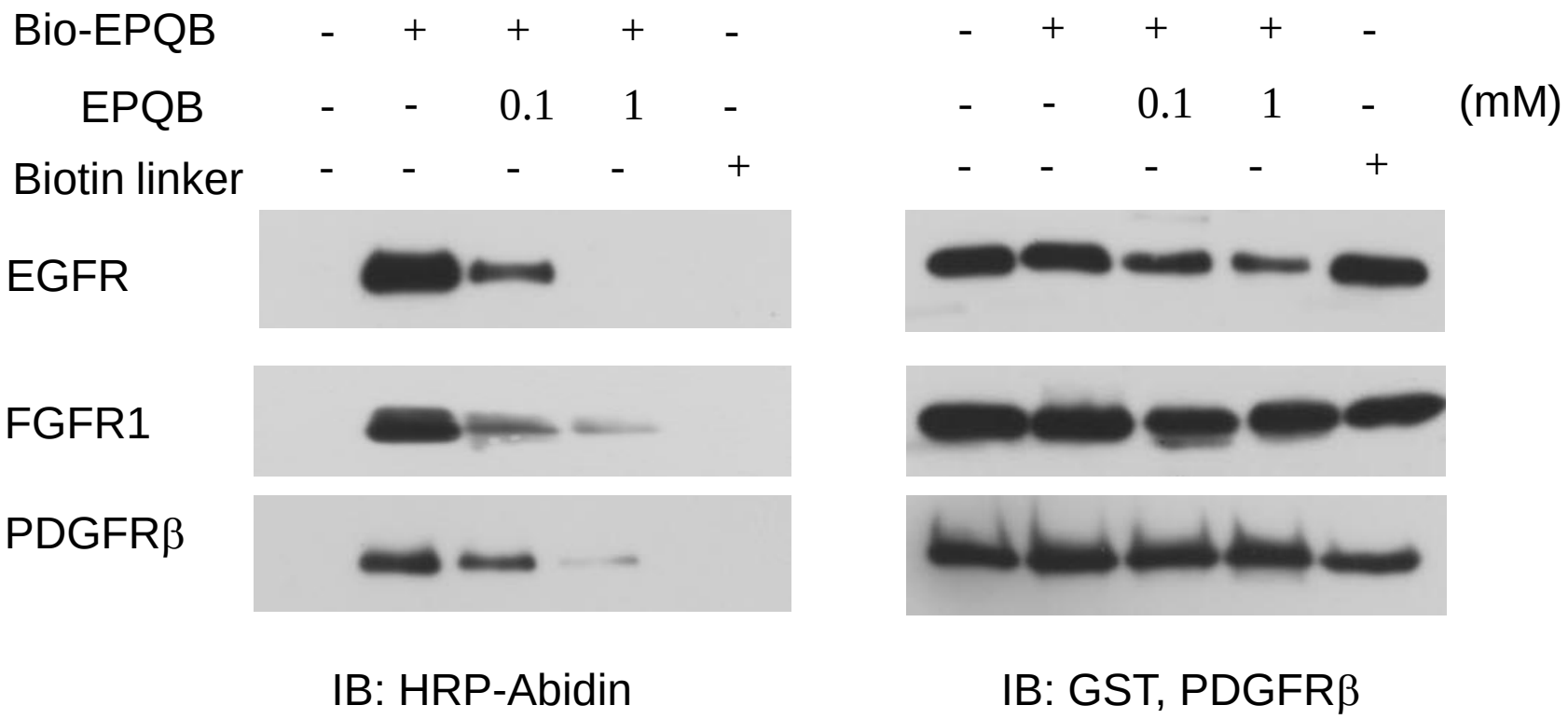

EGF
FGF
PDGF
Growth factor - + + + + - + + + + - + + + +
EPQB - - 3 10 30 - - 3 10 30 - - 3 10 30 (μM)
p-ERK
ERK
α-tubulin

Fig. 5b

(b)


Bio-EPQB - + + + - - + + + -
EPQB - - 0.1 1 - - - 0.1 1 - (mM)
Biotin linker - - - - + - - - - +
EGFR
FGFR1
PDGFRβ
IB: HRP-Abidin
IB: GST, PDGFRβ

Supplemental data

Materials and Methods

Western blot analysis

Western blot analysis and quantitative evaluation were described in Materials and Methods of manuscript. The statistical analysis was performed using Student's *t*-test; *p*-values of < 0.05 were interpreted as statistically significant.

Immunoprecipitation

HUVECs (2×10^5 cell/well) were starved in Medium 199 (Invitrogen) for 18 h and treated with or without EPQB for 2 h. After VEGF (3.1 ng/ml) stimulation for 5 min, cells were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer (20 mM HEPES, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM $Na_4P_2O_4$, 50 mM NaF, 10% glycerol, 0.2% Triton X-100, 2 mM Na_3VO_4, 1 mM PMSF, the protease inhibitor mixture (Complete; Roche Diagnostics)). The cell lysate was immunoprecipitated with anti-VEGFR2 antibody together with protein A-agarose (Amersham Biosciences) overnight. The agarose beads were then washed four times with the lysis buffer. Proteins were separated by SDS-PAGE and detected by Western blotting.

Results

EPQB inhibits the VEGF signaling and binding between VEGFR2 and PLCγ1.

To investigate the VEGF signaling to PLCγ1 from VEGFR2, we examined the phosphorylation of PLCγ1 and the binding between VEGFR2 and PLCγ1. As shown in Figs. S1a and S1b, the phosphorylation of PLCγ1 and binding between VEGFR2 and PLCγ1 were activated by VEGF stimulation, and its phosphorylation and binding were inhibited by EPQB (S.2a, 2b). These results suggest that EPQB inhibits VEGF signaling between VEGFR2 and PLCγ1.

U73122 is an activation inhibitor of PLCγ1, and SU5614 is an activation inhibitor of VEGFR2 and PDGFR.

Figure legends

Fig. S.1　EPQB inhibits the VEGF signaling and binding between VEGFR2 and PLCγ1. (a) HUVECs were treated with inhibitors of PLCγ1 or VEGFR2 activation for 2 h. After

VEGF (3.1 ng/ml) stimulation, the cells were lysed and analyzed by Western blotting for VEGFR2 and PLCγ1 phosphorylation. (b) The association between VEGFR2 and PLCγ1 was analyzed by immunoprecipitation (IP). The lysate was immunoprecipitated by anti-VEGFR2, and we detected an association with immunoblotting (IB) using anti-PLCγ1 antibody and anti-VEGFR2 antibody. EPQB inhibited the association and phosphorylation between VEGFR2 and PLCγ1. U73122 was an inhibitor of PLCγ1 activation.

Fig. S1a

(a)

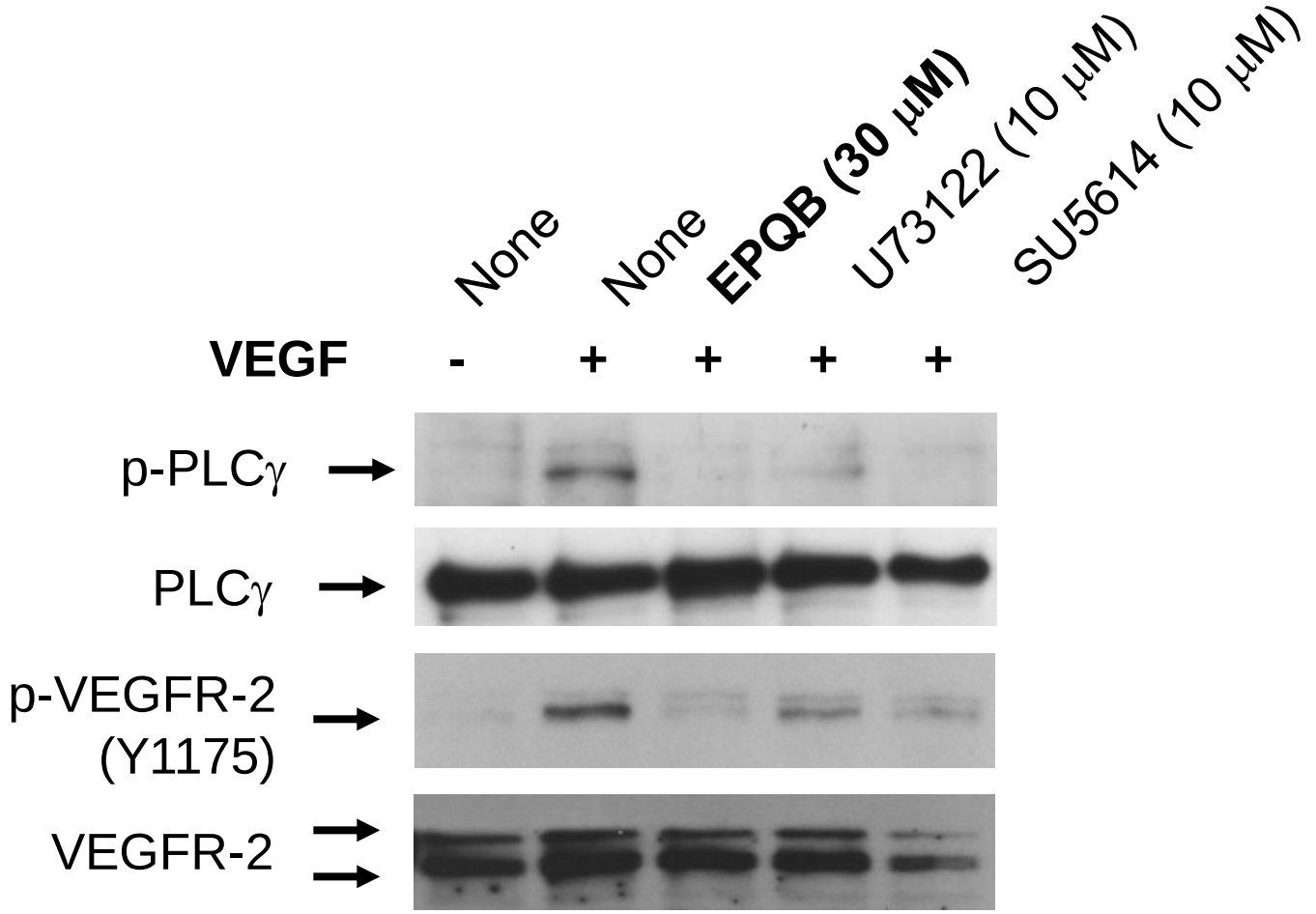
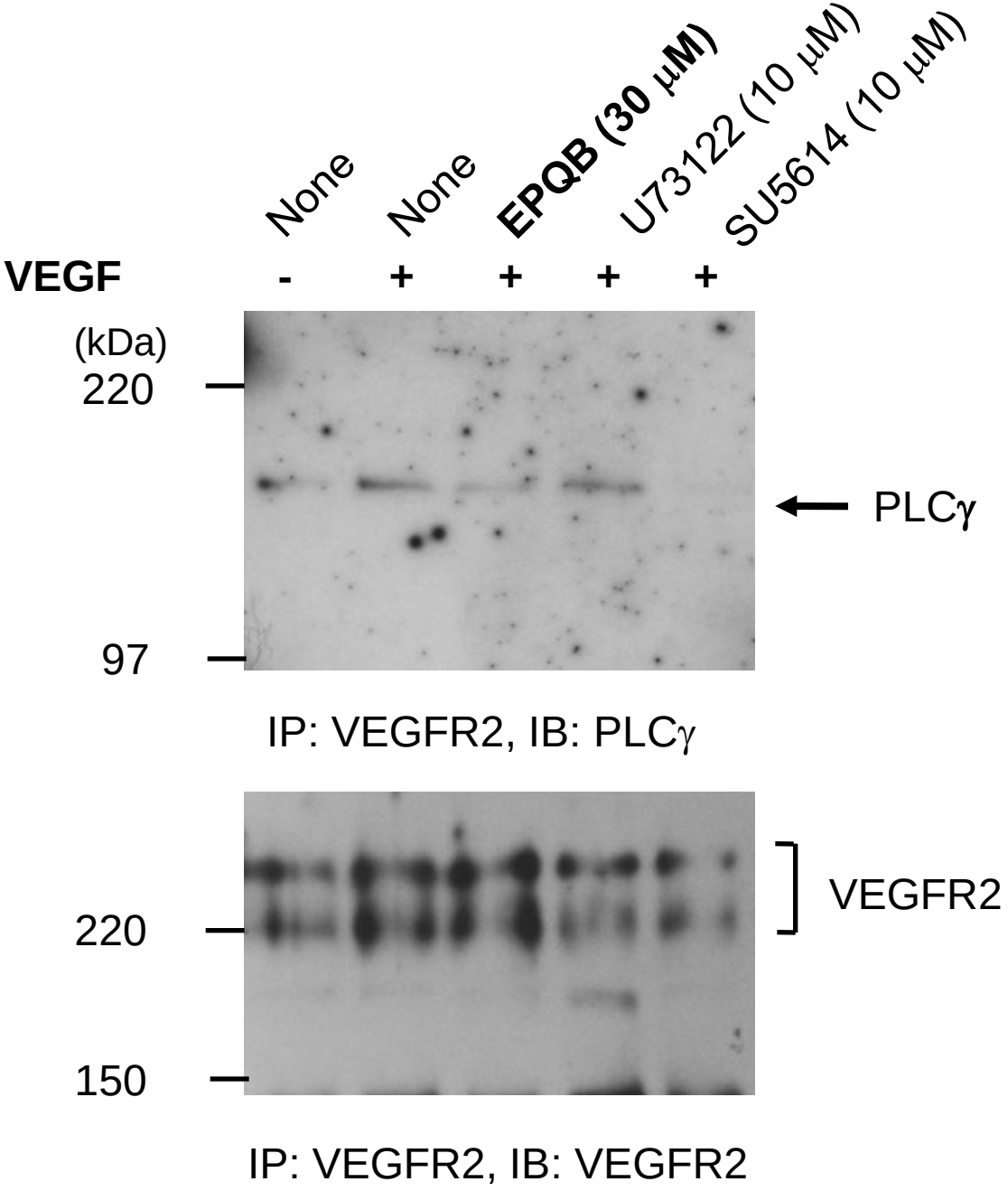

None
None
EPQB (30 μM)
U73122 (10 μM)
SU5614 (10 μM)
VEGF - + + + +
p-PLCγ
PLCγ
p-VEGFR-2 (Y1175)
VEGFR-2

Fig. S1b

(b)


None
None
EPQB (30 μM)
U73122 (10 μM)
SU5614 (10 μM)
VEGF
-
+
+
+
+
(kDa)
220
97
PLCγ
IP: VEGFR2, IB: PLCγ
220
150
VEGFR2
IP: VEGFR2, IB: VEGFR2