IC-4, a new irreversible EGFR inhibitor, exhibits prominent anti-tumor and anti-angiogenesis activities

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Abstract
Accumulating evidence suggested that the irreversible tyrosine kinase inhibitors (TKIs) have potential to override the acquired resistance to target-based therapies. Herein, we reported IC-4 as a novel irreversible TKI for epidermal growth factor receptor (EGFR). IC-4 potentially suppressed proliferation, induced apoptosis and a G2/M cell cycle arrest in breast cancer cells, correlating with inhibition of EGF-induced EGFR activation, but independent of DNA damage. In addition, IC-4 exhibited anti-angiogenic activities both in vitro and in vivo. It suppressed cell viability and proliferation induced by various growth factors in human umbilical vein endothelial cells (HUVECs). IC-4 also inhibited HUVECs migration and tube formation. In transgenic zebrafish embryo model, IC-4 was shown to suppress formation of intersegmental vessel and development of subintestinal vessels. Taken together, these results demonstrated that IC-4 is a new irreversible EGFR-TKI, exhibiting potent anti-breast cancer and anti-angiogenic effects.

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1. Introduction
Breast cancer is one of the most common malignancies in women and the second most common cause of female cancer-related deaths in the world [1]. Despite significant progress has been achieved in developing early diagnosis and treatment, acquired resistance to current chemotherapies and failure of endocrine-targeted therapy in some patients make exploring new therapeutic agents for breast cancer a great clinical need. The important role of epidermal growth factor receptor (EGFR) in breast cancer formation and progression gained considerable attention [1–3]. By binding with ligands, EGFR family, including EGFR, HER-2, HER-3 and HER-4, forms activated homodimers or heterodimers and participates in tumorigenesis and progression, including cell proliferation, survival, migration and angiogenesis. EGFR family is observed to be dysregulated in various human malignancies including breast cancer. EGFR overexpression was observed in 16–48% of the human breast tumors. Furthermore, a negative relationship was indicated between EGFR and steroid receptor (estrogen receptor (ER) and progesterone receptor (PR)) status [4,5]. Therefore, it is likely that EGFR-based targeting treatment is a strategy to overcome resistance to current chemotherapy and endocrine-targeted therapy.

A series of small molecule tyrosine kinases inhibitors (TKIs) targeting the intracellular kinase domain have been approved for clinical use, such as lapatinib, erlotinib and gefitinib. These reversible TKIs have shown promising therapeutic potential in treatment of breast cancer. However, problems like the intrinsic and acquired resistance have been emerging [2]. Treatment with reversible TKIs provided benefits to a rather small subset of patients with certain clinical characteristics such as EGFR-activating mutations [6]. Genetic mutations like T790M mutation and activation of oncogenes like VEGFR also lead to chemoresistance to reversible TKIs [7,8]. In order to delay or prevent the resistance to these first-generation EGFR TKIs, a number of irreversible EGFR TKIs and agents with activity against multiple targets have been developed. It was reported that irreversible TKIs such as Neratinib (HKI-272) [9,10] and PF00299804 [11,12] demonstrated beneficial effects in EGFR T790M mutated cancer models which developed resistance to first-line reversible TKIs, providing rationale to explore new irreversible TKIs.

In our previous study [13], novel dithiocarbamates IC-4 and IC-5 (Fig. 1A) were identified to exhibit potent antitumor activities. Herein, we demonstrated that IC-4 is a novel irreversible EGFR TKI. IC-4 could potentially inhibit growth of breast cancer cells, induce a G2/M arrest of the cell cycle, and inhibit EGFR activation. Moreover, IC-4 showed anti-angiogenesis effects on HUVECs and zebrafish. Our data suggested that IC-4 represented a novel compound exhibiting prominent anti-tumor and anti-angiogenesis activities in breast cancer.

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Fig. 1. Effects of IC-4 and IC-5 on cancer cells viability. (A) Chemical structures of IC-4 and IC-5. (B) MDA-MB-468, MDA-MB-231 and MCF-7 were treated with IC-4 or IC-5 (0.25–12 μM) for 72 h, then MTT assay was used to assess the effect of agents on cell viability. Results were presented as mean ± SD of three independent experiments. (C) EGFR expression levels in (1) MCF-7, (2) MDA-MB-468 and (3) MDA-MB-231 cells.

2. Materials and methods

2.1. Chemical

IC-4 (2-(benzylsulfinyl)ethyl pyridin-3-ylmethylcarbamodithioate) and IC-5 (2-cyanoethyl pyridin-3-ylmethylcarbamodithioate) were prepared by our group (Fig. 1A) were confirmed by 1H NMR, 13C NMR, elemental analysis. The purity of them as measured by high performance liquid chromatography (HPLC) was >99%.

2.2. Reagents

Kaihns's modification of Ham's F12 medium (F-12K), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin, streptomycin, and 0.25% (w/v) trypsin/1 mM EDTA were purchased from Invitrogen. Endothelial cell growth supplement (ECGS), epidermal growth factor (EGF), heparin and gelatin were purchased from Sigma. Recombinant human vascular endothelial growth factor (VEGF) was purchased from R&D Systems. MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide] was purchased from Sigma-aldrich. Antibodies were purchased from Cell Signaling Technology. RIPA lysis buffer, phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail and BCA protein assay kit were purchased from Pierce Biotechnology. ECL advanced Western blotting detection kit was purchase from Amersham.

2.3. Cell culture

Human breast carcinoma cell lines MDA-MB-468, MDA-MB-231 and MCF-7 were purchased from American Type Culture Collection, and maintained at 37 °C and 5% CO2 in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection, and maintained at 37 °C and 5% CO2 in F-12K medium supplemented with 2 mM l-glutamine, 1.5 g/L sodium bicarbonate, 30 mg/L ECGS, 10% (v/v) FBS, 100 mg/L heparin, 100 U/ml penicillin and 100 μg/ml streptomycin.

All experiments were performed with cells between passages 2–5.

2.4. Cell proliferation assessment

Effects of agents on cancer cell viability and proliferation were assessed by MTT assay. Cells were seeded in 96-well plates and incubated overnight. After corresponding treatment, cells were incubated with 100 μM DMEM medium containing 0.5 mg/ml MTT. After 4 h incubation at 37 °C, cell supernatants were discarded, MTT crystals were dissolved in 100 μl DMSO and absorbance was measured at 570 nm using a Multilabel counter (Perkin Elmer, 1420 Multilabel Counter Victor3, Wellesley, USA).

Effects of agents on HUVECs viability and proliferation were examined using the Cell Proliferation Kit II (XTT, Roche Diagnostics GmbH) in according to manufacturer’s instruction. Briefly, after incubation with the XTT reagent for 4 h, the absorbance was measured at 490 nm with the reference wavelength at 690 nm using a Multilabel counter (Perkin Elmer, 1420 Multilabel Counter Victor3, Wellesley, USA).

2.5. Cell cycle analysis

Cells were incubated in DMEM containing 0.5% FBS overnight for synchronization, and then treated with indicated concentration of IC-4 or IC-5. Cells were collected and fixed in 1 ml of cold 70% ethanol at −20 °C overnight. After that, cell pellet was collected by centrifugation, followed by PBS washes. Finally, 0.5 ml of propidium iodide (PI) staining solution [20 μg/ml of PI and 8 μg/ml of DNase-free RNase] was added to the samples, and incubated for 30 min at room temperature. After staining, the samples were immediately analyzed using flow cytometry. The results were analyzed with Mod Fit LT 3.0 software (Verity Software House, Topsham, USA).

2.6. Wound healing assay

Wound healing assay was performed to assess the migration of HUVECs as described previously [14,15]. Cells were seeded in 24-well plates and allowed to form confluent monolayers. Then cells were cultivated in medium containing 0.5% FBS overnight and exposed to different concentration of agents. The monolayers were then scratched horizontally using a sterile 100 μl pipette tip. Cells were washed with PBS and cultivated in DMEM medium. Bright field images of the randomly selected views along the scraped line were taken at 0, 6 and 10 h post-scratch. Cell migration rate was quantified by measuring the ratio of migration distance to total distance of the wound gap.

2.7. Transwell migration assay

Cell migration was assessed using 24-well polycarbonate membrane inserts system (8 μM pores, BD Falcon) as described previously [16]. The chambers were filled with 600 μl medium containing 1% FBS and 20 ng/ml VEGF. HUVECs (5 × 10^3) were resuspended in 100 μl medium containing 0.5% FBS and different concentration of agents, and seeded onto the upper side of the insert. Cells were
then incubated for 6 h to allow cell migration through the membrane to the lower side of the insert. After washed with PBS, cells were fixed in 1% paraformaldehyde. The non-migrated cells on the upper surface of the membrane were removed using cotton swabs. The migrated cells were stained with Hoechst 33342 and observed by fluorescent microscope.

2.8. Tube formation assay

Matrigel Matrix (BD, Biosciences) were thawed at 4 °C overnight. 24-well plate was coated with 250 μl Matrigel per well and incubated at 37 °C for 20 min for polymerization. HUVECs (1 × 10^6) were resuspended in 500 μl medium containing 10% FBS and different concentration of agents, and seeded onto the Matrigel-coated plate. After 6–8 h incubation, HUVECs in control group formed tube-like structure, which were defined as endothelial cord formations that connected at both ends. Tube formation was quantified by counting the number of branching points in three randomly selected fields.

2.9. Western blot analysis

Protein was extracted using RIPA lysis buffer containing 1% PMSF and 1% protease inhibitor. Lysates were centrifuged at 12,000 × g for 20 min at 4 °C and the supernatant was collected. Equal amounts of protein were subjected to SDS–PAGE. After repeated washes with PBS, proteins were visualized using an ECL advanced Western blot detection kit. Photos of protein bands were taken by a Molecular Imager ChemiDoc XRS (Biorad). Densitometric measurements of band intensity in the Western blots were performed using Quantity One Software.

2.10. Molecular modeling

The crystal structure of EGFR kinase domain in complex with lapatinib (PDB entry 3F7A; Fig. 3B) was used as EGFR target for molecular docking simulation [17]. Docking studies using the Glide program were performed to predict the binding of IC-4 to EGFR. The evaluation with the lowest binding energy was used to analyze ligand pose and the interaction model of IC-4/EGFR.

2.11. Morphological observation of zebrafish

Tg(fli1-EGFP) transgenic zebrafish were maintained as described before [18] and all animal experiments were conducted in accordance with the ethical guidelines of the Institute of Chinese Medical Sciences, Macau SAR. Zebrafish embryos were generated by natural pair-wise mating (3–12 months old). Healthy, transparent and regular embryos were selected and placed into a 24-well microplate with 8–10 embryos in each well. Embryos were exposed to IC-4 followed by stimulation with its ligand EGF. The immunoblot results in Fig. 3A demonstrated that 50 ng/ml EGF stimulated EGFR phosphorylation, whereas pretreatment of IC-4 for 60 min suppressed EGF-induced EGFR phosphorylation in a dose-dependent manner. In order to explore whether this inhibition is reversible, cells were washed with 200 μl Matrigel/per well and incubated at 37 °C for 20 min for polymerization. HUVECs (1 × 10^5) were resuspended in 500 μl medium containing 10% FBS and different concentration of agents, and seeded onto the Matrigel-coated plate. After 6–8 h incubation, HUVECs in control group formed tube-like structure, which were defined as endothelial cord formations that connected at both ends. Tube formation was quantified by counting the number of branching points in three randomly selected fields.

3. Results

3.1. Inhibition of IC-4 and IC-5 on cell viability of breast cancer cells

Cell lines expressing high level of EGFR including MDA-MB-468 and MDA-MB-231, and MCF-7 cells with a low level of EGFR expression were used in the study (Fig. 1C). The effects of IC-4 and IC-5 on these human breast carcinoma cell viabilities were assessed by MTT assay. IC-4 and IC-5 significantly reduced cell viability in a dose-dependent manner in all three cell lines (Fig. 1B). The IC_{50} for IC-4 in MDA-MB-468, MDA-MB-231 and MCF-7 were 2.3, 3.5 and 6.0 μM, respectively, whereas the IC_{50} for IC-5 in these cells were 7.6, 9.7 and 5.2 μM, respectively (Fig. 1B).

3.2. Effects of IC-4 and IC-5 on cell cycle distribution

Since MDA-MB-468 cells were more sensitive to IC-4- and IC-5-induced cell death, the effects of IC-4 and IC-5 on cell cycle distribution in MDA-MB-468 cells were determined by flow cytometry. As shown in Fig. 2, treatment with IC-4 and IC-5 for 24 h led to obvious dose-dependent changes in the cell cycle profile. A significant accumulation of cells in G2/M phase with concomitant loss in S and G0/G1 phase were observed with treatment of IC-4 in MDA-MB-468 cells.

3.3. Inhibition of IC-4 on EGF-induced EGFR phosphorylation

To assess the effect of IC-4 on EGFR tyrosine kinase activation, MDA-MB-468 cells were treated by IC-4 followed by stimulation with its ligand EGF. The immunoblot results in Fig. 3A demonstrated that 50 ng/ml EGF stimulated EGFR phosphorylation, whereas pretreatment of IC-4 for 60 min suppressed EGF-induced EGFR phosphorylation in a dose-dependent manner. In order to explore whether this inhibition is reversible, cells were washed with 200 μl Matrigel/per well and incubated at 37 °C for 20 min for polymerization. HUVECs (1 × 10^5) were resuspended in 500 μl medium containing 10% FBS and different concentration of agents, and seeded onto the Matrigel-coated plate. After 6–8 h incubation, HUVECs in control group formed tube-like structure, which were defined as endothelial cord formations that connected at both ends. Tube formation was quantified by counting the number of branching points in three randomly selected fields.

2.12. Statistics

IC_{50} was calculated by Nonline Fit of EC_{50} shift by GraphPad Prism software. Data were analyzed by Student’s t-test. P < 0.05 was considered to be statistically significant.
to remove the unbounded compounds after treatment with IC-4, and incubated in fresh medium for another 6 h prior to stimulation with EGF. It is interesting to find that the inhibition of IC-4 on EGF-induced EGFR activation was still remained (Fig. 3B). These observations suggested that IC-4 could irreversibly inhibit EGF-induced EGFR activation. Furthermore, pretreatment of IC-4 for 3 h also dose-dependently inhibited EGF-increased phosphorylation of ERK1/2 and Akt, which have been reported to be the major downstream signaling routes of EGFR [19,20](Fig. 3C). Using a molecular docking method and molecular mode of EGFR and lapatinib, we docked IC-4 into the ATP-binding pocket of EGFR kinase domain. As shown in Fig. 4A and B, IC-4 probed deeply into the ATP-binding pocket of EGFR, which is very similar to the binding mode of lapatinib (Fig. 4C and D). In a zoomed-in view (Fig. 4B), IC-4 was hydrogen-bonded to Thr854. Therefore, it is predicted that IC-4 blocked the engagement of ATP with EGFR, thereby act as ATP competitive inhibitory agent to EGFR. Taken together, these observations suggested that IC-4 could irreversibly suppress EGF-induced activation of EGFR.

3.4. Effects of IC-4 on cell viability and proliferation of HUVECs

Apart from the closely correlation with malignant progression and prognosis, aberrant EGFR activation has been also found to contribute to angiogenesis, which is the essential process in tumor growth and metastasis [21,22]. Thus, we examined the effects of IC-4 on HUVECs proliferation upon stimulation of different growth factors including the ligand of EGFR. As seen in Fig. 5A, HUVECs were exposed to F-12K medium containing 0.5% FBS and different concentration of IC-4, and a decrease in cell viability was observed dose-dependently by treatment of IC-4. Moreover, IC-4 could inhibit the proliferation of HUVECs driven by different growth factors including 10% fetal bovine serum, 20 ng/ml VEGF and 20 ng/ml EGF (Fig. 5B–D).

3.5. Effects of IC-4 on cell migration and tube formation of HUVECs

Effects of IC-4 on cell migration and tube formation were further examined at the dosages which showed no significant inhibition on cell viability of HUVECs. The results of wound healing assay demonstrated that, at 10 h after wounding, the migration rate in control group were 76.5 ± 3.8% of wound gap, whereas migration rates were significantly reduced to 38.7 ± 3.8% and 29.6 ± 6.8% by treatment with IC-4 for 0.25 and 0.5 µM, respectively, the concentrations which were not cytotoxic for HUVECs (Fig. 6A and B). Consistently, transwell migration results also demonstrated that HUVECs migrated to the lower side of the insert were reduced by treatment with IC-4 (Fig. 6C). Furthermore, tube formation assay was used to assess the effect of IC-4 on HUVECs differentiation on Matrigel. As seen in Fig. 7, cells in control group formed a network of tube-like structures in Matrigel. However, inhibitions on tube formation were observed upon treatment with IC-4. Overall, these results suggested that IC-4 exhibited anti-angiogenic activities in vitro.

3.6. Effects of IC-4 on angiogenesis in zebrafish

The anti-angiogenic effects of IC-4 were further evaluated using transgenic zebrafish Tg(jhi1-EGFP), which express enhanced green fluorescent protein (EGFP) in their endothelial cells [23]. After 30 h treatment of IC-4, the viability and morphological changes of embryos were observed. IC-4 did not show noticeable toxicity. In addition, as shown in Fig. 8, zebrafish in control group formed intersegmental vessel (ISV) (Fig. 8A) and developed a smooth basket-like structure subintestinal vessels (SIV) (Fig. 8C). IC-4 showed inhibitory effects on ISV formation and SIV basket growth (Fig. 8B and D), compared to the vehicle-control treated with 0.1% DMSO.

4. Discussion

Small molecular EGFR-TKIs demonstrated great potential in cancer treatment. They are generally orally bioavailable and well tolerated. Their small sizes allowed them to easily penetrate blood–brain barrier. Most importantly, these TKIs exhibited efficiency to overcome resistance to current chemotherapy and endocrine-targeted therapy [1,24–26]. However, intrinsic and acquired resistance to target inhibition seems inevitable as experienced with target EGFR and HER-2 inhibitors. Thus, there is a great clinical need to explore new agents. In the present study, we obtained several lines of evidence that IC-4 is a novel irreversible EGFR inhibitor, exhibiting prominent anti-tumor and anti-angiogenesis activities.

Fig. 3. IC-4 irreversibly inhibited the phosphorylation of EGFR and downstream molecules in MDA-MB-468 cells. Duplicate sets of cells were treated with the IC-4 for 60 min with the indicated concentrations. One set of cells was then stimulated with EGF (50 ng/ml) for 15 min (A). The other set of cells was stimulated with EGF (50 ng/ml) after PBS washouts and an additional 6 h recovery (B). Then cell lysates were collected and analyzed by Western blot using anti-phospho-EGFR or anti-EGFR antibodies. (C) Cells were preincubated with IC-4 for 3 h with the indicated concentrations prior to stimulation with EGF, then cell lysates were collected and analyzed by Western blot for phosphorylation and total level of ERK and Akt.
Our data demonstrated that IC-4 exhibited anti-proliferation effects in a panel of breast cancer cells including MDA-MB-468, MDA-MB-231 and MCF-7 cells. In addition, IC-4 was shown to induce apoptosis (Fig. S2) and a G2/M growth arrest in MDA-MB-468 and MDA-MB-231. Previous evidence suggested that genomic stability is an important factor leading to G2/M arrest in cell cycle and/or apoptosis [27]. In order to examine whether IC-4-induced G2/M arrest is attributed to DNA damage, DNA double-strand breaks and the level of phosphorylated histone γ-H2AX was examined. However, it was no significant DNA damage was observed (Fig. S3). Treatment of IC-4 failed to increase the phosphorylation level of γ-H2AX comparing with DMSO control group. Meanwhile, exposure to IC-4 could not produce typical comet tail, a widely used marker of DNA double-strand damage, until up to the concentration ten times higher than its IC50 (Fig. S4). These results confirmed that IC-4-induced G2/M cell cycle arrest and apoptosis were not due to DNA damage.

In the three cell lines which were used to assess the anti-proliferation ability of IC-4, MDA-MB-468 and MDA-MB-231 cells express high level of EGFR, while the EGFR level is relatively lower in MCF-7 cells (Fig. 1C). Interestingly, MDA-MB-468 and MDA-MB-231 were more sensitive to IC-4 than MCF-7 in cell proliferation. As EGFR is strongly associated with breast cancer cell growth, it suggested that inhibitory effects of IC-4 may be mediated through modulating EGFR activity. In order to confirm this speculation, EGF-induced EGFR activation was examined in EGFR high expressed MDA-MB-468 cells. As shown in Fig. 3, EGFR phosphorylation was increased by presence of its ligand EGF for 15 min. However, IC-4 decreased the EGF-induced EGFR activation in a concentration-dependent manner and almost completely block the EGFR phosphorylation at 4 μM without affecting EGFR levels. RAS/RAF/MEK/ERK and PI3 K/Akt are major downstream effectors following EGFR activation [20]. IC-4 treatment also decreased the phosphorylation of ERK and Akt, providing further evidence of the inhibitory effect of IC-4 on EGFR activity. Furthermore, it was noteworthy that a prolonged inhibitory effect of IC-4 on EGFR activation was observed. Cells were treated with IC-4 for 60 min and were washed to remove the unbounded compounds. After that, cells were incubated in fresh medium for an additional 6 h prior to EGF stimulation. It is interesting to find that the inhibition of IC-4 on EGF-induced EGFR activation was still exhibited (Fig. 3B). Docking studies were performed to predict the binding of IC-4 to EGFR. The evaluation with the lowest binding energy was used to analyze ligand pose and the interaction model of IC-4/EGFR. It was found that IC-4 probed deeply into the ATP-binding pocket of EGFR, which is very similar to the binding mode of lapatinib. In a zoomed-in view (Fig. 4B), IC-4 was hydrogen-bonded to Thr854. Therefore, it is predicted that IC-4 blocked the engagement of ATP with EGFR, thereby act as ATP competitive inhibitory agent to EGFR. These observations suggested that IC-4 could irreversibly inhibit EGF-induced EGFR activation.

Investigating agents that targeting multiple molecules also provided a new therapeutic intervention to enhance anti-cancer capability and prevent TKIs resistance by suppress unsuspected feed-backloops or cross-talk between different cell signaling pathways [28,29]. It was well accepted that targeting on tumor angiogenesis is a useful strategy to suppress tumor growth and metastasis [30,31]. In addition, accumulating evidence suggested...
Fig. 5. IC-4 inhibited viability and proliferation of HUVECs induced by different growth factors. (A) HUVECs were treated with IC-4 (0.25–10 μM) for 24 or 48 h, then XTT assay was used to assess the effect of IC-4 on cell viability. (B–D) HUVECs were treated with IC-4 (0.5–10 μM) in the presence or absence of 10% FBS (B), 20 ng/ml VEGF (C) or 20 ng/ml EGF (D) respectively for 24 or 48 h, followed by XTT examination. Results were presented as mean ± of three independent experiments.

Fig. 6. IC-4 suppressed the migration of HUVECs. (A and B) Wound healing assay was performed to assess the migration of HUVECs. Cells were cultivated to form confluent monolayers. Then cells were starved and exposed to different concentration of IC-4. Representative photographs were shown (A) and the cell migration rate was quantified by measuring the ratio of migration distance to total distance of the wound gap. Results were presented as mean ± SD of three independent experiments (B). *P < 0.01, vs. control cells 6 h post-scratch, #P < 0.01 vs. control cells 10 h post-scratch. (C) Effect of IC-4 on 3D migration of HUVECs was assessed by transwell assay. Cells were seeded onto the upper side of the insert and incubated for 6 h to allow cell migration through the membrane to the lower side of the insert. After remove of non-migrated cells, the migrated cells were stained and observed by fluorescent microscope.
that there are complicated interactions between factors that involved in angiogenesis such as VEGFR and EGFR pathways [29]. Therefore, development of agents that targeting EGFR and angiogenesis might represent a promising strategy in cancer treatment. In our study, in addition to the inhibitory effect on EGFR activation, IC-4 also demonstrated potent anti-angiogenic effect in zebrafish.
HUVECs and zebrafish model. IC-4 could dose-dependently inhibit endothelial cell viability. Besides, cell proliferation induced by different growth factors including FBS, EGF and VEGF was also suppressed by treatment of IC-4. Next, concentrations with no significant cytotoxic effects on HUVECs were used in further study. Results using various assays demonstrated that IC-4 could suppress cell growth, migration and tube formation at the concentration of 0.5 and 1 μM. Furthermore, transgenic zebrafish embryo expressing EGF in vasculature was used as an in vivo model to assess the effect of IC-4, which represents a powerful model for the study of angiogenesis [32,33]. The results also indicated that IC-4 inhibited ISV formation and SIV basket growth. These results suggested that IC-4 could combat angiogenesis both in vitro and in vivo. However, it was reported that the ligand of EGRF could also stimulate VEGF production [34]. Additionally, a decrease in VEGF expression was found following exposure of anti-EGFR monoclonal antibodies like cetuximab [35]. In this case, further study is needed to confirm whether the anti-angiogenic effects of IC-4 was attribute to its inhibitory on EGRF activity or other mechanisms.

Taken together, herein we reported IC-4 as a novel irreversible EGF TKI with promising anti-cancer and anti-angiogenic activities. IC-4 processes novel chemical structure and therefore could be a potent candidate for development of a series of agents to prevent resistance of current first-line EGRF-TKIs in treatment of breast cancer.

Conflict of Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary material

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References