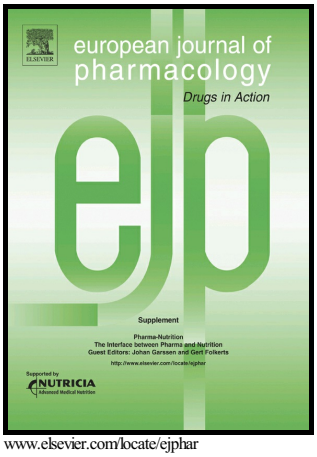

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SYP-5, a novel HIF-1 inhibitor, suppresses tumor cells invasion and angiogenesis

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Abstract

Hypoxia-inducible factor-1 (HIF-1) plays an essential role in carcinogenesis. The overexpression of HIF-1 induced by hypoxia is closely associated with metastasis, poor prognosis and high mortality. In this study, a novel HIF-1 inhibitor SYP-5 was first observed by the luciferase reporter assay. Western blots results showed SYP-5 inhibited hypoxia-induced upregulation of HIF-1. Moreover, the proteins of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP)-2 that are targets of HIF-1, were down-regulated by SYP-5. Furthermore, in the tube formation assay, SYP-5 suppressed angiogenesis induced by hypoxia and VEGF *in vitro*. Additionally, using Transwell and RTCA assays, we found that SYP-5 also retarded the Hep3B and Bcap37 cells migration and invasion induced by hypoxia and FBS. Last, we also detected the upstream pathways related to HIF-1 and found both PI3K/AKT and MAPK/ERK were involved in the SYP-5 mediated invasive inhibition of Bcap37 cells. These results indicates that SYP-5 inhibits tumor cell migration and invasion, as well as tumor angiogenesis, which are mediated by suppressing PI3K/AKT- and MAPK/ERK-dependent HIF-1 pathway. It suggests that SYP-5 might be a potential HIF-1 inhibitor as an anticancer agent.

Keywords: HIF-1, SYP-5, angiogenesis, invasion, PI3K/AKT pathway, MAPK pathway.

1. Introduction

Oxygen is essential for survival, while lack of oxygen will result in a strong stress. In order to adapt to hypoxia, cells regulate the expression of certain proteins and genes via oxygen sensor and signal transduction pathway (Hochachka et al., 2001). The existence of micro-environmental hypoxia is an important and continuous pathological feature of solid tumors (Gillies et al., 2007). Hypoxia in solid tumors is associated with resistance to radiation therapy and chemotherapy, selection of more invasive and metastatic clones and poor survival (Karakashev et al., 2015; Semenza et al., 2012; Masoud et al., 2015).

Semenza et al (Semenza et al., 1991) discovered for the first time that hypoxia-inducible factor 1 (HIF-1) could be induced by hypoxia and possess DNA binding activation. This transcription factor, which consists of HIF-1α and HIF-1β, is a master regulator for hypoxic adaptation (Semenza et al., 2003). As a result of insufficient blood supply caused by the rapid proliferation of tumor cells in hypoxia environment, HIF-1 is usually high expressed in tumor tissues. The overexpression of HIF-1α has been reported in numerous tumor types, including breast, liver, gastric, lung, prostate and colon (Kaya et al., 2012; Gaballah et al., 2014; Bhattacharyya et al., 2010; Wang et al., 2014; Ranasinghe et al., 2015; Nagaraju et al., 2015). HIF-1 plays a pivotal role in the regulation of key survival pathways in cancer cells. It activates the transcription of genes that are involved in angiogenesis, cell survival, glucose

metabolism, migration, and invasion (Nagaraju et al., 2015; Yang et al., 2015). HIF-1, therefore, has been identified as a potential drug target for cancer treatment.

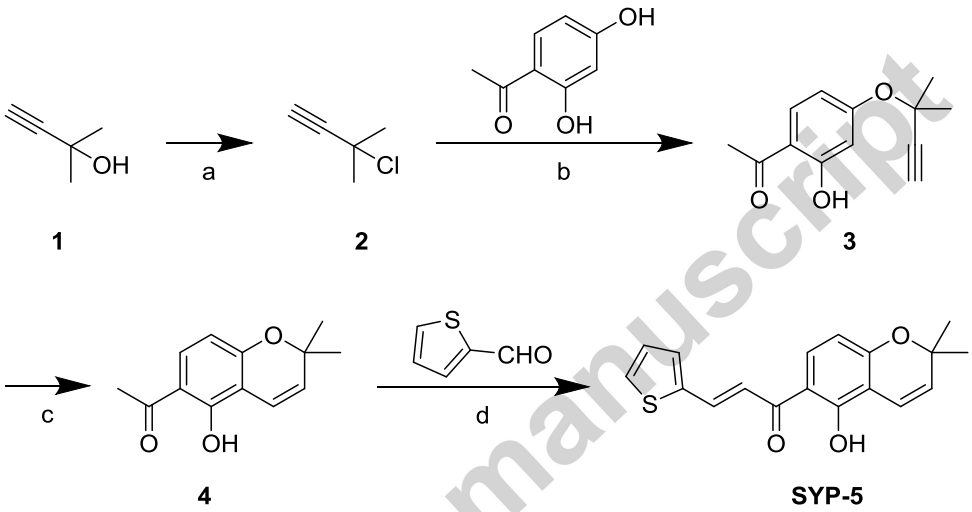
Currently, there are a fraction of small molecules originally designed or discovered as HIF-1 inhibitors (Hu et al., 2013), including a few chalcone-based compounds. Recently, the results of ours and others showed that chalcone is a privileged template to develop HIF-1 inhibitor (Srinivasan et al., 2011; Wang et al., 2015a). In the present study, we showed that the novel chalcone-based compound SYP-5 is a potential HIF-1 inhibitor. We selected the HUVEC, Hep3B and Bcap37 cell lines to investigate the antitumor activity of SYP-5 targeting HIF-1. It obviously suppressed angiogenic and invasive potential. The above results indicate that SYP-5, a novel chalcone-based compound, might be a potential antitumor drug targeting HIF-1.

2. Materials and methods

2.1 Preparation of SYP-5

(*E*)-1-(5-hydroxy-2,2-dimethyl-2*H*-chromen-6-yl)-3-(thiophen-2-yl)prop-2-en-1-one(SYP-5) was synthesized as shown in **Scheme 1**. A key intermediate (**4**) was prepared according to our previous work (Wang et al., 2015a). Chlorination of the commercially available 2-methyl-3-butyn-2-ol(**1**) with HCl in presence of copper powder afforded to 3-chloro-3-methylbut-1-yne(**2**), which was then reacted with 1-(2,4-dihydroxyphenyl)ethanone in the presence of potassium carbonate and potassium iodide to yield the 4-position alkylation product(**3**). The cyclisation of intermediate(3) refluxed in *N,N*-dimethylaniline produced only 3-position product

rather than 5-position one which can be confirmed by ^{1}H-nuclear magnetic resonance (NMR). The target compound(SYP-5) was obtained by reacting intermediate(**4**) with thiophene-2-formaldehyde catalyzed by piperidine in toluene with moderate yield(43%, m.p. 105-107 ^{o}C). The structure of SYP-5 was confirmed by the nuclear resonance magnetic (^{1}H-NMR) and shown in Fig. 1A.



Reagents and conditions; (a) HCl/$CaCl_2$, Cu/CuCl -15^{o}C 0.5h, then 0 ^{o}C 2 h; (b) K_2CO_3/KI PEG-600 reflux 48h; (c) *N,N*-dimethylaniline reflux 2 h; (d) toluene reflux 4 h

Scheme 1 The synthetic route of target compound.

2.2 Biology Regents

The primary antibodies against HIF-1β, MMP-2, p-EGFR, EGFR, p-AKT, AKT, p-ERK, ERK, p-4E-BP1, 4E-BP1 and β-actin were got from Cell Signaling Technology (Danvers, MA). The primary antibodies against HIF-1α and VEGF were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The PD98059, MEK inhibitor, and LY294002, AKT inhibitor, were purchased from Beyotime Biotechnology (CHN).

2.3 Cell Culture and Exposure to Hypoxia

Hep3B (human hepatocellular carcinoma cell line, American Type Culture Collection, ATCC) and Bcap37 (human breast cancer cell line, Institute of Biochemistry and Cell Biology, Shanghai, CHN) were routinely cultured in DMEM (high glucose) with 10 % heat-inactivated FBS (Gibco BRL, Grand Island, NY) and maintained at 37°C in a humidified incubator with 5% CO_2. Primary human umbilical vascular endothelial cells (HUVECs) were purchased from ATCC and were cultured in Roswell Park Memorial Institute 1640 supplemented with 10% fetal bovine serum (FBS). For cells culture under hypoxia, cells were grown in a microprocessor-based oxygen controller (Coy Laboratory Products) containing 1% O_2, 5% CO_2 and 94% N_2 at 37 ℃.

2.4 Luciferase reporter assay

U251-HRE cells (human glioma cell line U251 stably expressing a hypoxia regulatory element (HRE)-luciferase pGL2 construct) and U251-pGL3 cells (U251 cells stably expressing a pGL3 construct) were kindly provided by Dr. Giovanni Melillo (NCI, USA). The cells were grown in RPMI 1640 with 5% heat-inactivated FBS, 200 μg/ml G418. The experiments were performed according to previous published paper (Rapisarda et al., 2002). Briefly, the cells (1×10^5 cells/ml) were seeded into 96-well Opti-plates (Packard Instrument, Inc., Meriden, CT). After overnight incubation, the cells were pretreated with various concentrations of agents for 1 h. Then cells were grown in the microprocessor-based oxygen controller for 16 h. The Luciferase reporter assays were tested by Bright Glo luciferase assay reagents (Promega Inc, Madison, WI).

2.5 Cell viability assay

The cells (1×10^5 cells/ml) were seeded into 96-well culture plates. After overnight incubation, the cells were treated with various concentrations of SYP-5 for 24 h. Then 10 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (2.5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 h at 37 ^{o}C. After centrifugation (2500 rpm, 10 min), the medium containing MTT was aspirated, and 100 μl DMSO was added. The optical density of each well was measured at 570 nm with a SpectraMax Paradigm Reader(Molecular Devices).

2.6 Western blot assay

About 1×10^7 Hep3B and Bcap37 cells were gathered after treatment by SYP-5 concurrently under hypoxia. In order to avoid interaction with SYP-5, the inhibitors were pretreated for 1 h. Western blot was performed as previously described (Wang et al., 2014). In brief, equal amount from cultured cells were fractionated by 8-15% SDS-PAGE and electrically transferred onto polyvinylidene difluoride (PVDF) membranes. Mouse or rabbit primary antibodies and horseradish peroxidase (HRP)-conjugated appropriate secondary antibodies were used to detect the designated proteins. Membrane-bound secondary antibodies were reacted with ECL detection reagents (Thermo Scientific, MA, USA) and exposed in dark room. Results were normalized to the internal control β-actin.

2.7 Tube formation assay

Tube formation assay was performed as previously described (Wang et al., 2015). In

brief, 50 μl matrigel was placed into each well of the 96 well-plate and allowed to polymerize by incubation at 37 ℃ for 30 min. The HUVECs were seeded onto the matrigel layer at a density of 5×10^4 cells with or without SYP-5, followed by hypoxia treatment or addition 20 ng/ml VEGF. After 12 h, tubular structure of endothelial cells was stained by Calcein-AM (Molecular Probes, Eugene, OR) and photographed using high content screening system ImageXpress Micro (Molecular Devices, CA, USA), and tube formation (branches/field) was examined and quantified. Three independent experiments were performed.

2.8 RTCA (Real-time cell analysis) migration assay

RTCA was performed as previously described (Wang et al., 2015b). In brief, The CIM-plate contains 16 modified Boyden chambers, which can be used independently to measure cell migration in real time through 8 μm pores of a polyethylene terephthalate membrane onto gold electrodes on the underside of the membrane using the xCELLigence analyser system (ACEA Biosciences, CA, USA). Experiments were set up according to the manufacturer's instructions. A chemotactic signal for movement was provided by inoculating various concentrations of SYP-5 treated 50,000 cells in serum-free medium in the upper chamber and supplying 15% FBS or 20 ng/ml VEGF in the lower chamber. Cell index (electrical impedance) was monitored every 5 min for the duration of the experiment. The cell index reflects the tumor cell's migratory capacity. And the slope of the curve can be related to the migration velocity of tumor cells.

2.9 Transwell Assay

Transwell Assay was performed as previously described (Wang et al., 2015b). Cells ($1\text{-}2\times10^5$) were plated in RPMI 1640 medium in the upper chamber of the Trans-well (8 μm pore, Corning Costar, Corning, NY), which were pre-coated with or without matrigel (Becton-Dickinson, Bedford, MA). Different concentrations of SYP-5 were added to the lower chamber followed by incubating under normoxia or hypoxia for 16 hours at 37 ℃. The non-migrating cells and the collagen gel from the interior side of the inserts were gently removed using a cotton-tipped swab. The cells that migrated through the gel insert to the lower surface of the membrane were stained by Calcein-AM and photo- graphed using a high-content screening system (Molecular Devices). The number of migrated cells was quantified by counting cells number at X 4 objectives.

2.10 Statistical analysis

Statistical analysis was performed using SPSS11.5 software package for Windows (SPSS, Chicago, IL). Data were presented as the mean±s.e.m. Statistical significance was calculated using a student's t-test, with a probability level of $P<0.05$ considered to be statistically significant.

3. Results

3.1 SYP-5 inhibits HIF-1 and downstream gene expression in Hep3B and Bcap37 cells

U251-HRE and U251-pGL3 cells (a HIF-1α inhibitor screening model) were used to detect the ability of SYP-5 to inhibit HIF-1 activity. In this model, U251-HRE cell

line, which stably transfected with pGL2 plasmid with 3 copies of the hypoxia-responsive element (HRE), was used to measure the HIF-1 activity within the cells under various conditions. Parallel U251-pGL3 cell line, which stably transfected with pGL3 plasmid, was used to exclude compounds that inhibited luciferase expression in a nonspecific fashion. As shown in Fig. 1B, SYP-5 specifically inhibited hypoxic induction of luciferase expression in U251-HRE but not in U251-pGL3. To evaluate how SYP-5 inhibits HIF-1α activity, we investigated the expressions of HIF-1α and HIF-1β in protein levels using western blot analysis. SYP-5 was found to inhibit HIF-1α and HIF-1β protein levels under hypoxic condition in a concentration-dependent manner in both Hep3B and Bcap37 cells (Fig. 1C).

As a transcription factor, the activation of HIF-1 would lead to upregulation of target genes that are involved in many aspects of cancer progression, such as vascular endothelial growth factor (VEGF) which is the key factor in angiogenesis (Ahluwalia et al., 2012). HIF-1 also regulates the expression of genes encoding matrix metalloproteinase 2 (MMP2), which plays an established role in the pathophysiology of invasion (Zhao et al., 2014).To investigate whether the inhibitory effect of SYP-5 on HIF-1α affects VEGF and MMP2 expression, we assessed the level of VEGF and MMP2 in both cells. We found that treatment with SYP-5 displayed significant inhibition on hypoxia-induced overexpression of VEGF and MMP2 in both cell lines (Fig. 1C). Taken together, our results showed that SYP-5 could inhibit the activation of HIF-1 through downregulating HIF-1α and HIF-1β protein levels, and suppress its target genes.

3.2 SYP-5 inhibits microvessel formation induced by hypoxia (95%N2+5%CO2) and VEGF in vitro

Angiogenesis, the formation of new blood vessel, is essential for cancer progression. Under hypoxia, HIF-1 has been proved to be a direct transcriptional activator of VEGF (Ahluwalia et al., 2012). HUVECs can spontaneously form three dimensional capillary-like tubular structures when cultured on matrigel. Tube formation assay represents a classical model for studying inhibitors of angiogenesis. Next we studied the effects of SYP-5 on angiogenesis in HUVECs. Firstly, we have detected the cell viability using MTT assay before conducting tube formation assay. The data was showed in the Supplementary material Fig. 1. After treated with HUVEC cells with various concentrations of SYP-5 for 24 h, the concentrations lower than 50 μM of SYP-5 have no significant effect on cell viability (Cell viability more than 80%). Thus, to exclude the effect of cell cytotoxicity of SYP-5 on tube formation assays, we choose 50 μM as the maximum concentration of SYP-5. As shown in Fig. 2, SYP-5 was confirmed to significantly inhibit HUVECs tube formation induced by hypoxia. To further address whether SYP-5 inhibits angiogenesis by targeting VEGF, tube formation induced by VEGF was performed. Treatment with SYP-5 resulted in significant reduction in the number and the continuity of microvessel formation of HUVECs induced by VEGF (10 ng/ml) in a concentration-dependent manner. The above data suggesting that SYP-5 could retard angiogenesis induced by either hypoxia or VEGF.

3.3 SYP-5 suppresses migratory and invasive potential of HUVEC induced by VEGF

in vitro

Endothelial cell migration is one of the most important and early event during the process of angiogenesis (Zecchin et al., 2015). To explore the effect of SYP-5 on HUVECs in migration and invasion, RTCA and transwell assay were performed. As shown in Fig. 3, SYP-5 was confirmed to significantly inhibit HUVECs migration and invasion induced by VEGF (10 ng/ml) at a concentration dependent manner, suggesting SYP-5 displayed a potential to inhibit endothelial cell migration.

3.4 SYP-5 inhibits Hep3B and Bcap37 cells migration and invasion induced by hypoxia and FBS

The occurrence of tumor cell metastasis is the primary factor causing poor prognosis of patients with cancer. When tumors encounter low oxygen tension, overexpression of HIF-1α enhances tumor invasion and metastasis through up-regulating MMPs (Ding et al., 2009). In the present study, we found that SYP-5 at un-cytotoxic concentrations (Supplementary materials Fig. 1) not only suppressed the expression of MMP-2 under hypoxic conditions (Fig. 1B), but also significantly inhibited hypoxia-induced migration and invasion of Hep3B and Bcap37 cells (Fig. 4A). Furthermore, our data showed that FBS-induced cell migration and invasion of both cell lines could be also dramatically suppressed by SYP-5(Fig. 4B and C). Taken together, our data demonstrates that SYP-5 owns the potential against multiple factors-induced migration and invasion of cancer cells.

3.5 SYP-5 inactivates hypoxia-induced PI3K-AKT and MAPK-ERK1/2 pathways

Previous studies have shown that growth factors related pathways activation, mainly

including PI3K-AKT and MAPK-ERK1/2, is necessary to regulate VEGF and HIF-1 expression in human cancer cells(Masoud et al., 2015; Jiang et al., 2008), so we next detect the effect of SYP-5 on both pathways. Our data indicated that hypoxia could induced the activation of EGFR, which is consistent with previous report (Swinson et al., 2008), and its downstream protein phosphorylation, including AKT, 4E-BP1, and ERK, whereas, administration of SYP-5 inhibited the activation of EGFR and its downstream pathways in Bcap37 cells (Fig. 5). These results demonstrated that hypoxia related PI3K-AKT and MAPK-ERK1/2 pathways might also be regulated by SYP-5.

3.6 PI3K-AKT and MAPK-ERK pathways involve into the inhibitory effect of SYP-5 on cell invasion

In order to explore the role of PI3K-AKT and MAPK-ERK1/2 pathways in SYP-5 caused inhibitory effect on cell invasion, we applied the specific inhibitors for MAPK/ERK and PI3K/AKT pathways and analyzed cell invasion. As shown in Fig. 6 A and B, single treatment with PD98059 (a specific inhibitor of MAPK/ERK) and LY294002 (a specific inhibitor of PI3K/AKT) induced a slight inhibition of hypoxia-induced invasion in Bcap37 cells. Interestingly, in contrast to PD98059, pretreatment with LY294002 combined with SYP-5 resulted in a dramatically suppression of invasive cells induced by hypoxia, indicating that the PI3K/AKT pathway play a synergistic role in regulating SYP-5-mediated invasive inhibition of Bcap37 cells.

Furthermore, the effects of SYP-5, LY294002, and PD98059 alone or combination on

the signaling pathways and invasion related protein MMP-2 were also assessed in Bcap37 cells. As expected, single treatment with LY294002 or PD98059 could result in the inhibition of AKT pathway and ERK pathway, respectively. In consistent with the cell invasion data, LY294002 and PD98059 treatment also led to the downregulation of MMP2. Interestingly, LY294002 treatment slightly inhibited ERK phosphorylation, meanwhile the addition of PD98059 also resulted in the inhibition of AKT pathway, suggesting the crosstalk between ERK and AKT pathways. Importantly, we found the combination of SYP5 and LY294002 or PD98059 displayed an enhanced inhibition of MMP2 expression, suggesting both pathways are involved in the inhibitory effect of SYP-5 on cell invasion.

4. Discussion

Hepatocellular carcinoma (HCC) and breast cancer share the character of tissue hypoxia, especially when the tumor grows quickly but angiogenesis fails to catch up with the speed of tumor growth. The key hypoxic regulator HIF-1α, has been shown to be highly expressed in HCC and breast cancer. Meanwhile, evidence suggests that HIF-1 is involved with invasion, metastasis and angiogenesis (Tu et al., 2014; Bos et al., 2005).

In recent decades, the discovery and development of novel small molecules targeting HIF-1 has been an exciting area of developmental therapeutics (Xia et al., 2012). The role of HIF-1 inhibitors in cancer therapy remains speculative. It has been reported that quite a number of chalcone-based compounds reveal moderate HIF-1 inhibitory

activity and many chalcone-based compounds prove promising anticancer activities in various animal models (Srinivasan et al., 2011; Wang et al., 2015). It indicates that chalcone might be a template to develop HIF-1 inhibitor. Here, we identified a novel chalcone-based compound, SYP-5, which displayed specifically inhibit HIF-1 activity.

In this study, our results show that SYP-5 decreased the HIF-1α and HIF-1β protein levels under hypoxic condition. As HIF-1 regulates multiple genes expression involved in tumorigenesis, we further investigated its downstream gene expression. The complex process of angiogenesis begins when cells respond to hypoxia by increasing generation of VEGF, which has been widely known as one of the most important factors stimulating angiogenesis in solid tumors. In our study, protein of VEGF was up-regulated in both Hep3B and Bcap37 cells under hypoxic condition. We speculated that SYP-5 suppressed the hypoxia-mediated VEGF expression by down-regulating HIF-1 expression. Furthermore, we found that SYP-5 could reduce the HUVECs tube formation induced by hypoxia and VEGF *in vitro*, which further confirmed the inhibitory action of SYP-5 on HIF/VEGF signaling axle.

MMP-2, a critical protease, degrades many kinds of extracellular matrix and plays a critical role in invasion. HIF-1α enhances tumor invasion and metastasis through up-regulating MMP-2(Jing et al., 2012). Presently, we observed a significant reduction of MMP-2 after treatment with SYP-5. Moreover, we discovered that SYP-5 inhibited hypoxia-induced migration and invasion in Hep3B and Bcap37 cells, indicating that SYP-5 could influence the both cell lines migration and invasion

through HIF-1 pathway.

It has been reported that various signaling pathways are involved in the expression of HIF-1α. Among them, EGFR related PI3K/AKT and MAPK/ERK pathways appears to play a major role in HIF-1α expression (Masoud et al., 2015; Bos et al., 2005). Here we found that administration of SYP-5 could reverse hypoxia induced activation of EGFR and its downstream pathways, PI3K/AKT and MAPK/ERK, in Bcap37 cells. Further confirming results showed that treatment with PI3K/AKT inhibitor combined with SYP-5 resulted in an enhanced suppression of migratory cells induced by hypoxia. Additionally, western blot data showed that the combination of SYP5 and LY294002 or PD98059 displayed an enhanced inhibition of migration related protein MMP2. These results demonstrate that both PI3K/AKT pathway and MAPK/ERK pathway play a role in regulating SYP-5-induced migratory inhibition.

In summary, this study shows for the first time that a novel chalcone-based compound SYP-5 inhibited the tumor angiogenesis and cell invasion *in vitro*. Mechanistically, SYP-5 decreased HIF-1α and HIF-1β expression in Hep3B and Bcap37 cells through inhibiting EGFR/PI3K/AKT and MAPK/ERK pathways.

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the U251-HRE and U251-PGL3 cells.

Conflict of interest

The authors have declared no conflict of interest.

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Fig. 1 Effects of SYP-5 on HIF-1 activity and downstream gene expression. (A) The structure of SYP-5. (B) Hypoxic induced luciferase expression in U251-HRE cells was detected by luciferase assay. U251-pGL3 cells was considered as control, and the luciferase unit in normoxia U251-pGL3 cells was considered as 100%. (C) The HIF-1 and VEGF, MMP2 proteins were assessed by western blot analyses in Hep3B and Bcap37 cell line after SYP-5 (2, 10, 50 μM) treated for 48 h under hypoxic condition. β-actin expression was used as a loading control. All error bars are S.E.M. $^{###}$Denotes a significant difference compared to normoxia treated U251-HRE cells, $P<0.001$; ***Denotes a significant difference compared to hypoxia treated U251-HRE cells, $P<0.001$.

Fig. 2 The effects of SYP-5 on hypoxia or VEGF-induced tube formation of HUVEC cells. (A) HUVECs tube formation induced by hypoxia or VEGF (20 ng/ml) was measured after SYP-5 (2, 10, 50 μM) treated for 12 h. The experiments were performed as described in Materials and Methods. **(B)** Quantification of the effect of SYP-5 on tube formation is shown in column diagram. Con represents the group without treatment hypoxia or VEGF. All error bars are S.E.M. *Denotes a significant difference compared to hypoxia or VEGF treated control, $P<0.05$; ***Denotes $P<0.001$.

Fig. 3 The effects of SYP-5 on VEGF-induced migration and invasion of HUVEC cell. (A) HUVEC cells migration was measured by RTCA. The cells were treated SYP-5 at concentrations of 2, 10, 50 μM, as well as VEGF(20 ng/ml). (B) HUVEC

cells invasion was measured by transwell assay after SYP-5 (2, 10, 50 μM) treated for 16 h. VEGF(20 ng/ml) was added in the lower chamber. (C) Quantification of the effect of SYP-5 on cell invasion is shown in column diagram. All error bars are S.E.M. **Denotes a significant difference compared to VEGF single treated control, P<0.01; ***Denotes P<0.001.

Fig. 4 The effects of SYP-5 on hypoxia or FBS-induced migration and invasion of Hep3B and Bcap-37 cells. (A) Hep3B cells and Bcap37 cells migration and invasion induced by hypoxia were measured by transwell assay after SYP-5 (2, 10, 50 μM) treated for 16 h. (B) Hep3B cells and Bcap37 cells migration induced by FBS were measured by RTCA after SYP-5 (2, 10, 50 μM) treated for 24 h. (C) Hep3B cells and Bcap37 cells invasion induced by FBS were measured by transwell assay after SYP-5 (2, 10, 50 μM) treated for 16 h.

Fig. 5 The effects of SYP-5 on EGFR, PI3K/AKT and MAPK/ERK pathways. The p-EGFR, EGFR, p-AKT, AKT, p-ERK, ERK, p-4E-BP1, and 4E-BP1 proteins were assessed by western blot analyses in Bcap37 cell line after SYP-5 (2, 10, 50 μM) treated for 48 h under hypoxic condition. β-actin expression was used as a loading control.

Fig. 6. The effects of specific inhibitor of PI3K/AKT or MAPK/ERK on SYP-5 mediated invasive inhibition in Bcap37 cells. (A) Bcap37 cells invasion was measured by transwell assay after treatment with SYP-5(10 μM) and/or specific inhibitors (PD98059 10 μM; LY294002 10 μM). (B) Quantification of cell invasion is shown in column diagram. (C) The p-ERK, ERK, p-AKT, AKT, and MMP-2 proteins

were assessed by western blot analyses in Bcap37 cell line as the above described drug treatment condition. β-actin expression was used as a loading control. Con represents the group without hypoxia treatment. All error bars are S.E.M. *Denotes a significant difference compared to hypoxia control, $P<0.05$.

Supplementary materials Fig. 1. The effects of SYP-5 on cell viability in HUVEC, Hep-3B and Bcap37 cell lines. The cells were treated with SYP-5 for 24 h, and detected by MTT assay.

Figure 1

Fig.1.

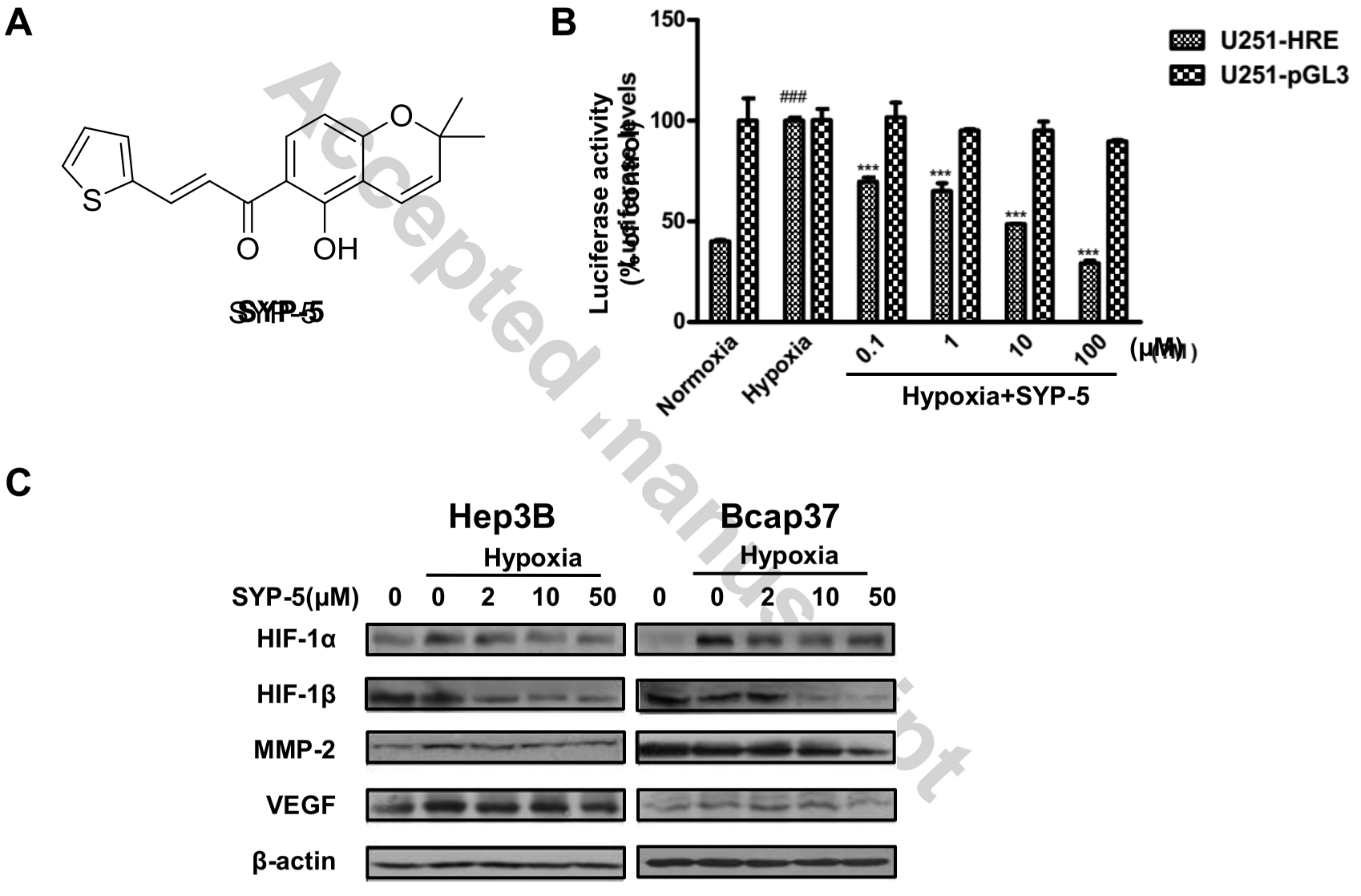

Figure 2

Fig.2.

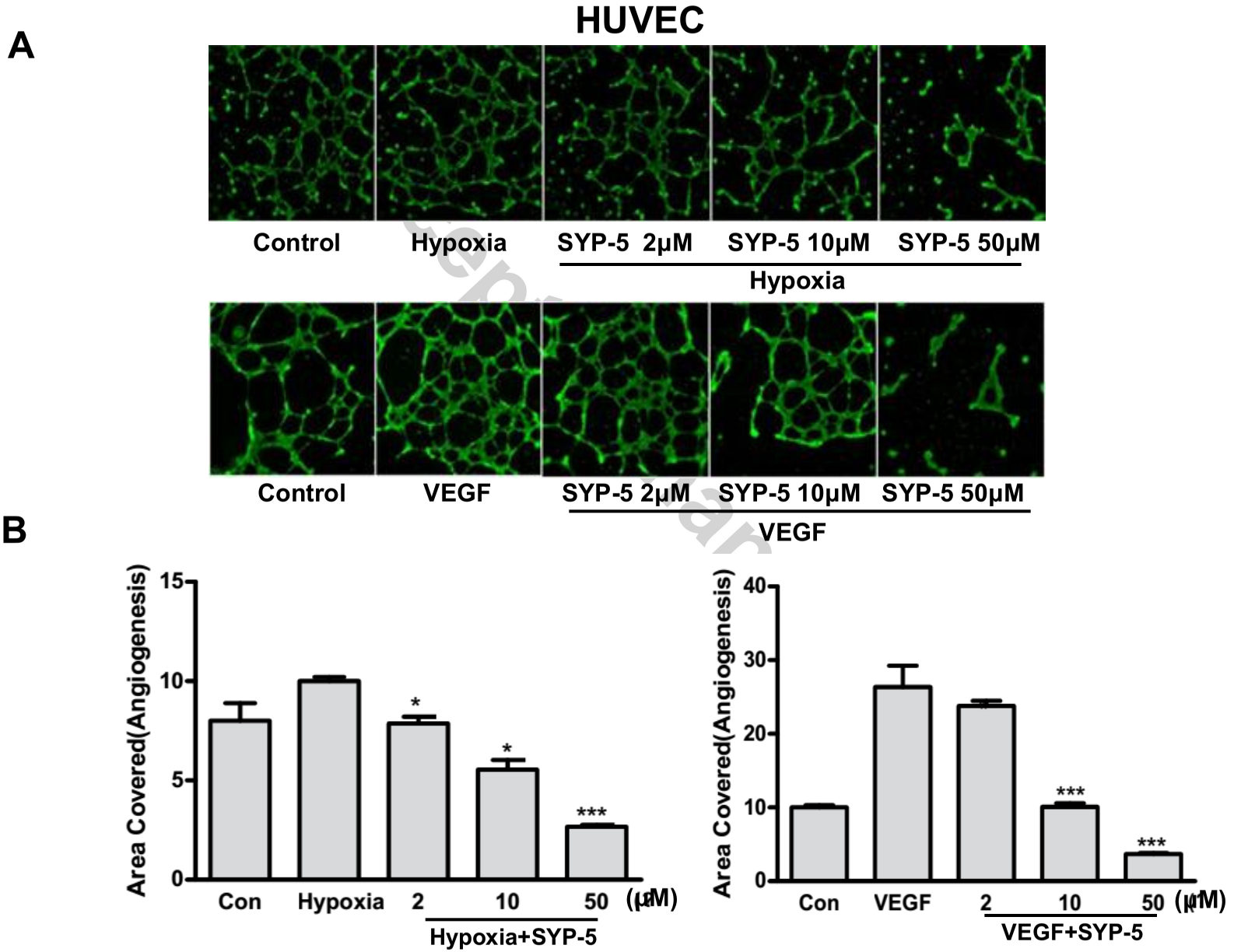

Figure 3

Fig.3.

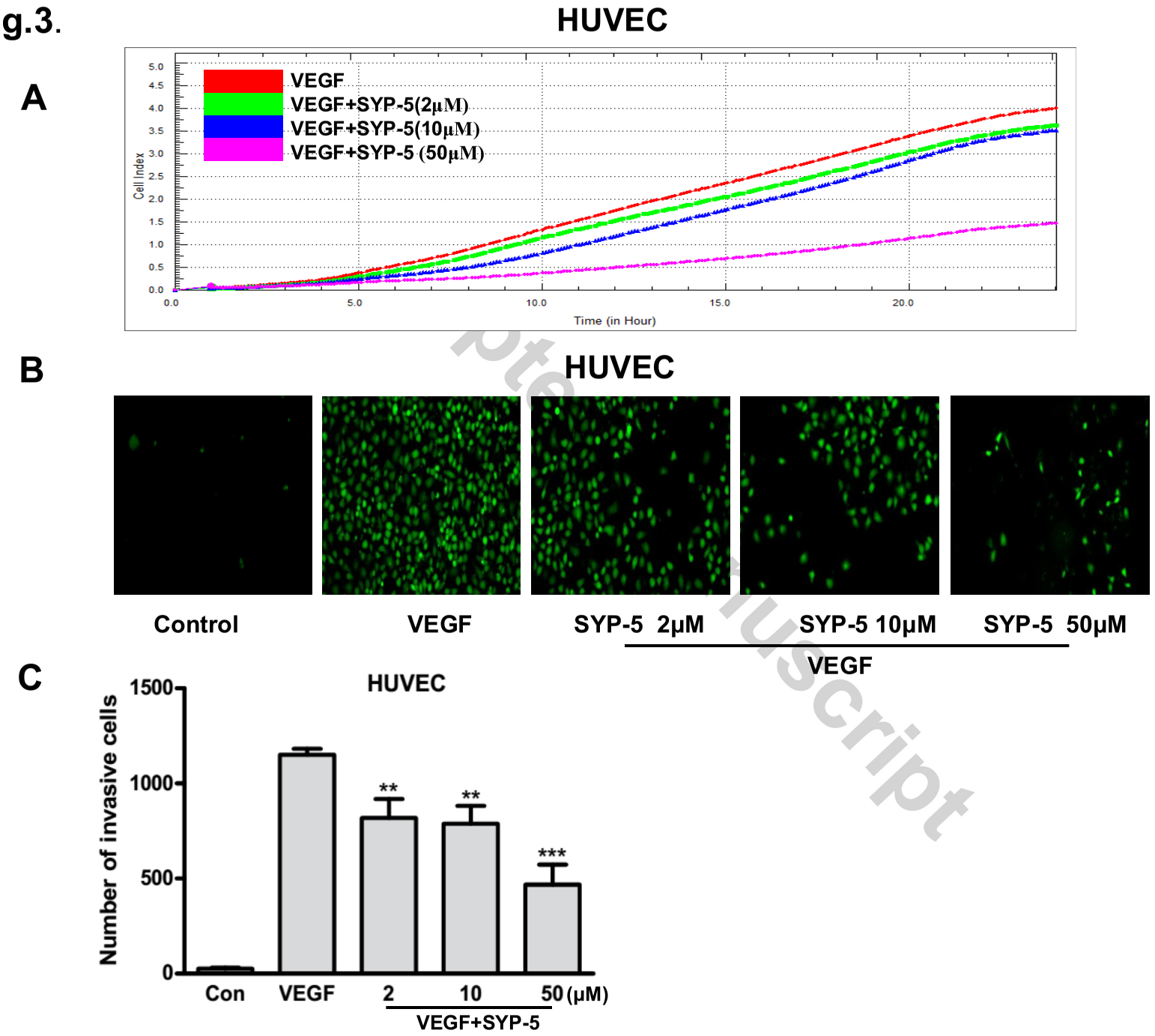

Figure 4

Fig.4.

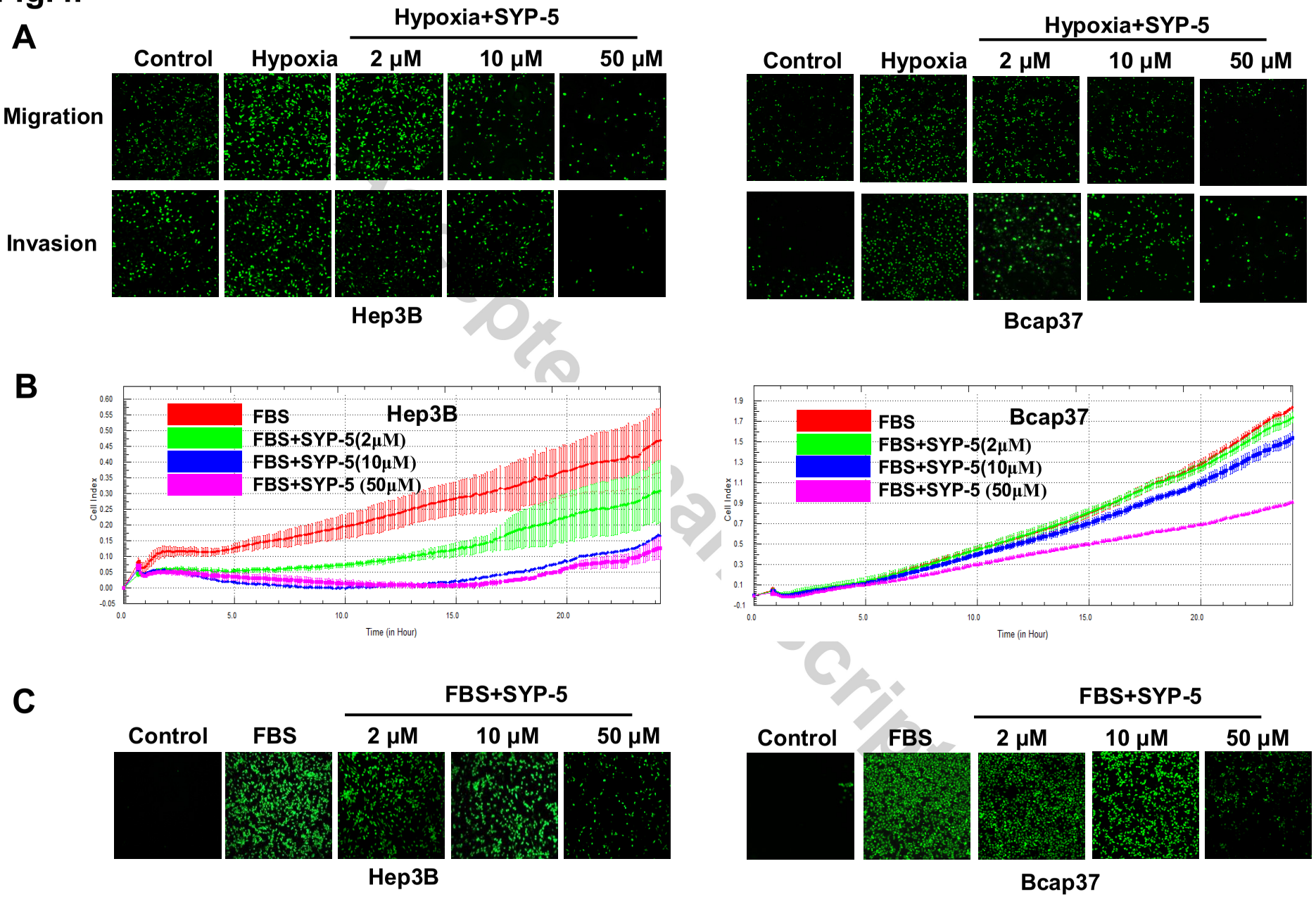

Figure 5

Fig.5

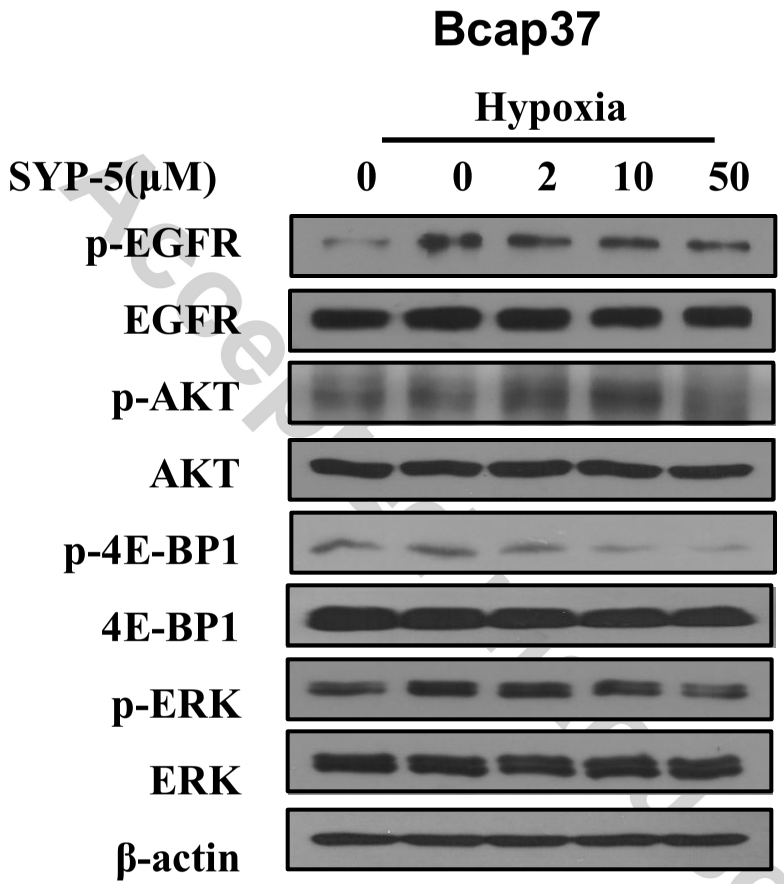

Fig.6