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ORIGINAL ARTICLE

RY10-4, a novel anti-tumor compound, exhibited its anti-angiogenesis activity by down-regulation of the HIF-1α and inhibition phosphorylation of AKT and mTOR

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Abstract
Purpose To assess the anti-angiogenesis potential and mechanism of RY10-4, a derivative of protoapigenone, which was verified the broad-spectrum anti-tumor activities by previous study.
Methods RY10-4 and RY10-3 were synthesized according to the procedure described. Breast cancer cells MCF-7 and MDA-MB-231 that got the best performance in the previous anti-tumor activity screening were selected for further anti-cancer mechanism research. Firstly, cells proliferation assay of RY10-4 and RY10-3 was used to demonstrate the fact that the 4-hydroxy-2,5-cyclohexadien-1-one system would be the efficient pharmacophore of RY10-4. Then, a series of assays such as human umbilical vein endothelial cells (HUVECs) proliferation assay, HUVECs migration, tube network formation and morphological observations of zebrafish were applied to confirm its anti-angiogenesis activity. Upon RY10-4 treatment, the HIF-1α and VEGF were analyzed by western blot in normoxic and hypoxic conditions, meanwhile the PI3K-AKT-mTOR pathway-related protein such as AKT, p-AKT, mTOR and p-mTOR was also analyzed.
Results In the MCF-7, MDA-MB-231 and HUVECs proliferation assay, RY10-4 that has 4-hydroxy-2,5-cyclohexadien-1-one system showed distinct advantage compared with RY10-3. Tests had verified the anti-angiogenesis capability of RY10-4. Down-regulation of the HIF-1α and inhibition phosphorylation levels of AKT and mTOR were found to be the pathway that RY10-4 exerts its functions on anti-angiogenesis.
Conclusions The structure of 4-hydroxy-2,5-cyclohexadien-1-one should be the effective pharmacophore of RY10-4. RY10-4 got fine performance in anti-tumor and anti-angiogenesis assay, and thus, the quinol compound will be the new hot-spot for further anti-tumor agency development.

Keywords RY10-4 · Anti-angiogenesis · VEGF · HIF-1α · AKT · mTOR

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Introduction

Breast cancer is the most common primary cancer with poor prognosis among the gynecology cancers. Although localized breast cancer can be cured by surgery, breast cancer often has a high mortality rate primarily due to the frequent metastasis [1, 2]. Metastatic breast cancer is considered incurable, and surgery has only limited superiority in the treatment of this disease [3]. The metastasis of tumor means the chemotaxis to the nutrition and survival space. Vessels around the tumor tissue play a key role in the transportation of nutrients and oxygen. Thus, the anti-tumor compound that has both low IC_{50} to tumor cell lines and anti-angiogenesis function is regarded as a promising compound for further development.

Protoapigenone, a natural derivative of apigenin, has been proven to have potent anti-tumor activity in vivo and in vitro [4]. Its unusual non-aromatic B-ring, which is different from apigenone, is regarded as an efficient pharmacophore. The synthetic work of the probably minimal pharmacophore named RY10-4 that contains the 1-hydroxycyclohexa-2,5-dien-4-one group had been done

by our laboratory. The novel compound exhibited more powerful capability than protoapigenone to suppress multiple human cancer cell lines such as HepG2 and HepG3 (liver), MDA-MB-231 and MCF-7 (breast), MDAH-2774 and SKOV3(ovary), LNCap (prostate) and A549 (lung) with IC_{50} values of 0.94–5.59 μM [5]. A surprising advantage of RY10-4 contrasted with protoapigenone was that no distinct impairment to hematopoiesis or any immune functions were observed. Furthermore, regarding that the breast tumor cells MCF-7 and MDA-231 got the best performance among the eight kinds of human tumor cell lines mentioned above [5], so MCF-7 and MDA-231 were adopted to evaluate the possible mechanism how RY10-4 exerts its effects on the human tumor cell lines. The compound 2-(4-Hydroxyphenyl)-4H-pyran-4-one named RY10-3, which is similar to RY10-4 but do not have non-aromatic B-ring, was used as a reference compound. The structure of RY10-4 and RY10-3 was displayed in Fig. 1.

Angiogenesis refers to the formation procedure of new blood vessels from pre-existing ones in sites or from distant sites. The whole procedure includes degradation of the extra-cellular matrix, migration and proliferation of endothelial cells, and the formation and sprouting of new vessels [6]. Under normal conditions such as wound recovery, embryonic development and organ or tissue regeneration, the angiogenesis process switches between on and off at the proper time as a result of precise regulation caused by positive or negative factors [7]. While in the malignant tumor tissues, not only is the proliferation rate of vessels faster than normal tissues, but also the size and shape are leaky and abnormal. A tumor tissue is impossible to grow to 1 mm^3 unless there are new vessels to supply blood and nutrition [8]. Therefore, anti-angiogenesis therapy had become a hot-spot in cancer chemotherapy. Animal experiments proved that a combination of anti-angiogenesis drugs is more powerful than single agent, similar to experience with the clinical chemotherapy [9, 10]. The VEGF family that is most known as a series of potent angiogenesis factors is thought to act as an angiogenesis switch for expansion of quiescent tumor tissues [11, 12]. The VEGF-related pathway has become an attractive target because some VEGF inhibitors had already shown potent anti-tumor effects in vivo and in vitro models. These include anti-sense VEGF RNA, antibodies to VEGF or its receptors and small molecule compounds such as SU5416 [13].

In our study, the RY10-4 was found to have fine anti-angiogenesis capabilities, which was verified by the zebrafish model in vivo and HUVECs model in vitro. The expression of HIF-1α and VEGF in both MFC-7 and MDA-231 also was obviously suppressed by the RY10-4 in a dose-dependent manner. The PI3K/AKT/mTOR pathway that can either activate or suppress the function of several downstream pathways [14] was found to be the one of pathways which the RY10-4 affects the VEGF expression. So the RY10-4 that has the low IC_{50} to several tumor cells and anti-angiogenesis capability got a promising future as a new anti-cancer drug.

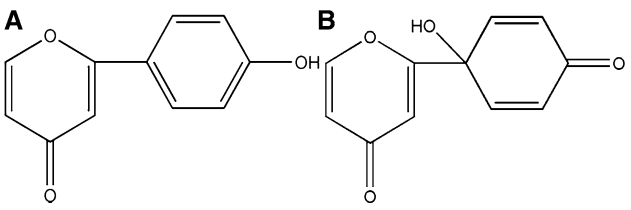

Fig. 1 The structures of **a** RY10-3 and **b** RY10-4

Materials and methods

Chemicals and materials

Compound 2-(1-Hydroxy-4-oxo-cyclohexa-2,5-dienyl)-pyran-4-one (RY10-4) and 2-(4-Hydroxyphenyl)-4H-pyran-4-one (RY10-3) were synthesized according to the procedure described by Yuan et al. [5]. The structure was identified by H-NMR, C-NMR and MS, and purity (>98 %) was tested by HPLC–UV. RY10-4 and RY10-3 were dissolved in dimethyl-sulfoxide (DMSO) to produce a 100-mM stock solution. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), SU5416, DMSO were purchased from Sigma Chemical (St. Louis, MO, USA). Culture medium 1640, DMED-HG, M199 and tissue culture reagents were obtained from Hyclone. Fetal bovine serum (FBS) was provided by Sijiqing Biologic (Hangzhou, China). The other reagents unless specified were all analytical reagents.

Cell lines and establishment of hypoxic culture condition

Human umbilical vein endothelial cells (HUVECs), MCF-7 and MDA-MB-231 were obtained from Tongji hospital, Wuhan, China. HUVECS, MCF-7 and MDA-MB-231 were separately grown in M199, DMEM, RPMI 1640. All of which were supplemented with 10 % FBS and 100 U/mL penicillin–streptomycin in an atmosphere of 5 % CO_2, 37° in a humidified incubator. Experiments under hypoxic conditions (1 % O_2, 5 % CO_2) were achieved using the hypoxia incubator.

Viability assay

The survival rates of HUVECs, MCF-7 and MDA-MB-231 were determined by MTT assay [15]. Cells were seeded in a 96-well plate of 10^4 cells per well. RY10-4 and SU5416 were dissolved in DMSO and diluted with appropriate culture medium at the concentrations from 0.01 to 10 μM.

RY10-3 was prepared for the final concentrations among 0.1–100 μM. The cells were incubated with RY10-4, RY10-3 and SU5416 for 24 h. The viability was represented by the absorbance at 540 nm, which was read by microplate reader (Power-Wave XS, Bio-Tek). The inhibition rate was the percentage rate to the vehicle control (0.1 % DMSO). Each treatment was performed in triplicate.

Chemotaxis assay

HUVECs migration assay was achieved by using a transwell system (Costar Corporation) equipped with 8-μm pore size polyvinylpyrrolidone-free polycarbonate filters (diameter, 13 mm). When the cells grew to 80 % confluence, HUVECs were trypsinized and cultured with a 1 % BSA-free culture medium. 200 uL of cell suspension that contains RY10-4 (0.1 μM, 0.5 μM, 2.5 μM) and 1 μM SU5416 were added into upper layer of transwells thrice. Culture mediums containing 10 % BSA that was used as an attractant media were added into the lower layer. After 24 h, HUVECs, which passed through the membrane, were fixed and stained in 0.1 % crystal violet. Stained filters were photographed under a microscope (ECLIPSE Ti-s, Nikon, Japan).

Tube formation assay

When HUVECs grew to 80 % confluence, different concentrations of RY10-4 (0.1 μM, 0.5 μM, 2.5 μM) were added into the cells, and SU5146 (1 μM) was added to HUVECs as a positive control. HUVECs were seeded into a 96-well plate that was precoated with Matrigel (100 uL/well, 8 mg/mL, BD Biosciences, Bedford, MA) and allowed to gel in 37° for 4 h. After 24 h, the formation of tubes was observed and imaged under an inverted microscope (ECLIPSE Ti-s, Nikon, Japan) at 200× magnification. The tube length was quantified by Adobe Photoshop CS2 software.

Morphological observations of zebrafish embryos

Transgenic zebrafish Tg (VEGFR2: GFP) and wild-type zebrafish were provided by the Biology Institute of Shandong Academy of Sciences. The embryos were maintained in the embryo water and manually dechorionated by forceps before drug treatment. Then, the embryos were placed into the 96-well plate, one embryo per well. The embryos were treated with three concentrations (1 μM, 5 μM, 10 μM) of RY10-4 and treated with SU5416 10 μM as a positive control. After drug administration, the zebrafishs were incubated at 28.5 °C for 24 h with 100 uL of embryo water per well. The embryos were examined for blood vessel development and counted the average number of the inter-segmental blood vessels (ISVs) using an inverted microscope (ECLIPSE Ti-s, Nikon, Japan). Dead and abnormal embryos were discarded.

Western blot analysis

MCF-7 and MDA-MB-231 cells were treated with RY10-4 (0.1 μM, 0.5 μM, 2.5 μM) for 24 h in normoxic and hypoxic conditions, and then, the cells were harvested and lysed on ice for 30 min in a lysis buffer containing 50 mM Tris–HCl, pH 8.0, 20 mM EDTA, 150 mM NaCl, 1 % NP-40, 50 mM NaF, and 0.02 % NaN_3 with protease inhibitors (1 mM phenyl-methanesulfonyl fluoride (PMSF), 1 μg/mL aprotinin and 1 μg/mL leupeptin) to prevent proteolysis and dephosphorylation [16]. Total soluble proteins were collected after further centrifugation at 10,000×*g* at 4 °C for 10 min. After protein content was determined by the Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA), the equal protein samples were resolved on SDS-PAGE and electro-transferred to PVDF membranes (Millipore). The membranes were blocked with 5 % (w/v) non-fat dry milk for 2 h and probed against the following antibodies: mTOR, p-mTOR, AKT, p-AKT, VEGF, HIF-1α and β-actin (1:500) overnight at 4 °C. Blots were washed three times in a TBST buffer, followed by incubation for 1 h at room temperature with the horseradish peroxidase-conjugated specific secondary antibodies. Then, the blots were visualized by using an enhanced chemiluminescence detection system. All the antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The densitometric analysis of the bands was determined by using a gel documentation system (ShineTech, GelAnalyse). The data were normalized using β-actin as the internal control, with the vehicle control as 100 %.

Statistics

All data were done at least three times. Data were analyzed using SPSS 16.0 software (SPSS, USA). Results were presented as mean ± standard deviation (SD) and evaluated by one-way ANOVA followed by the Dunnett post hoc test used for multiple comparisons. $P < 0.05$ was considered to be statistically significant.

Results

The effect on HUVECs, MCF-7 and MDA-MB-231 proliferation

RY10-4 inhibited the proliferation of MCF-7, MDA-MB-231 and HUVECs in an apparent dose-dependent manner. The IC_{50} of the MDA-MB-231 and MCF-7 were respectively 0.52 and 0.94 μM. As seen in Fig. 2, in HUVECs, the inhibition rate of RY10-4 was slightly inferior to SU5416. In HUVECs, MCF-7 and MDA-MB-231, RY10-4 has the potent advantage over RY10-3.

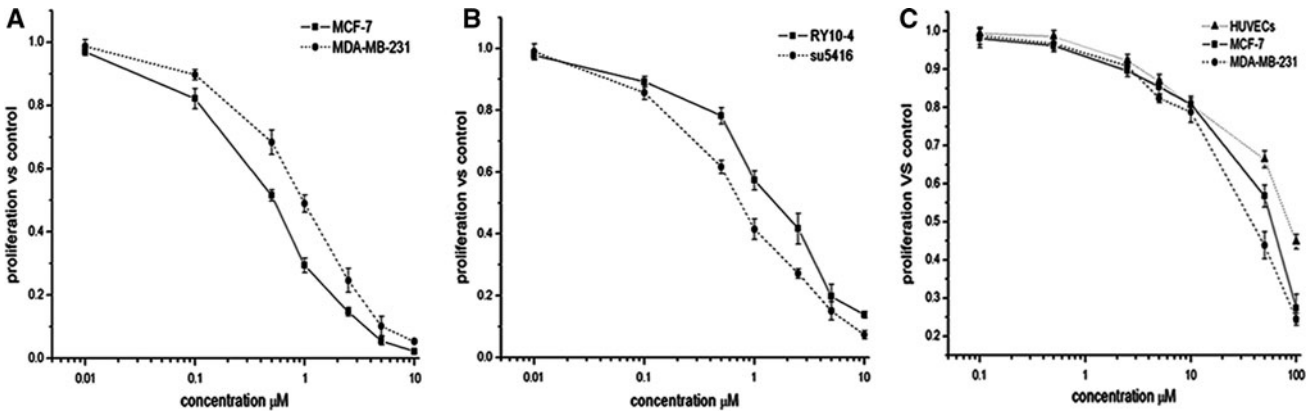

Fig. 2 Anti-proliferation activities of RY10-4, RY10-3 and SU5416 on MCF-7, MDA-MB-231 and HUVECs. **a** Inhibitory effect of RY10-4 on MCF-7 and MDA-MB-231. **b** Inhibitory effect of RY10-4 and SU5416 on HUVECs. **c** Inhibitory effect of RY10-3 on MCF-7, MDA-MB-231 and HUVECs ($P < 0.05$). The cell anti-proliferation rates were demonstrated by MTT assay, and they are presented as mean ± SD ($n = 3$). The viability rate is expressed as a percentage to the vehicle control (0.1 % DMSO)

Inhibition of tube formation and migration

RY10-4 decreased HUVECs migration caused by the higher concentrations of BSA distinctly. The data presented in Fig. 3 demonstrated that there was a dose-dependent inhibition of the chemotactic migration of HUVECs. SU5416, a selective VEGF receptor-2 inhibitor that was used as a positive control, possessed an inhibitory effect with 32 % at 1 μM. While at 2.5 μM, RY10-4 got better performance than SU5416.

HUVECs can elongate and form capillary network mimicking the in vivo angiogenesis process [17]. Cells were seeded on the matrigel for 24 h. The tube network formed completely and changed little in the next 48 h. When the concentration of RY10-4 was 0.5 μM, the inhibition effect was inferior to SU5416, while the concentration was 2.5 μM, as seen in Fig. 4. The effect was equally matched with SU5416. The RY10-4 at 2.5 μM almost totally blocked the formation of the neovessel.

Anti-angiogenesis effect of RY10-4 on zebrafish model

The zebrafishs were treated with different concentrations of RY10-4, and SU5416 (10 μM) was set as a positive control. Then, blood vessel morphologies were photographed under a microscope. The vehicle control containing 0.1 % DMSO showed little difference to the blank control. When the concentration was 1, 5 and 10 μM, vessel florescence image that appeared significantly dose-dependent was shown as Fig. 5. The date and the images were tested in at least three different experiments.

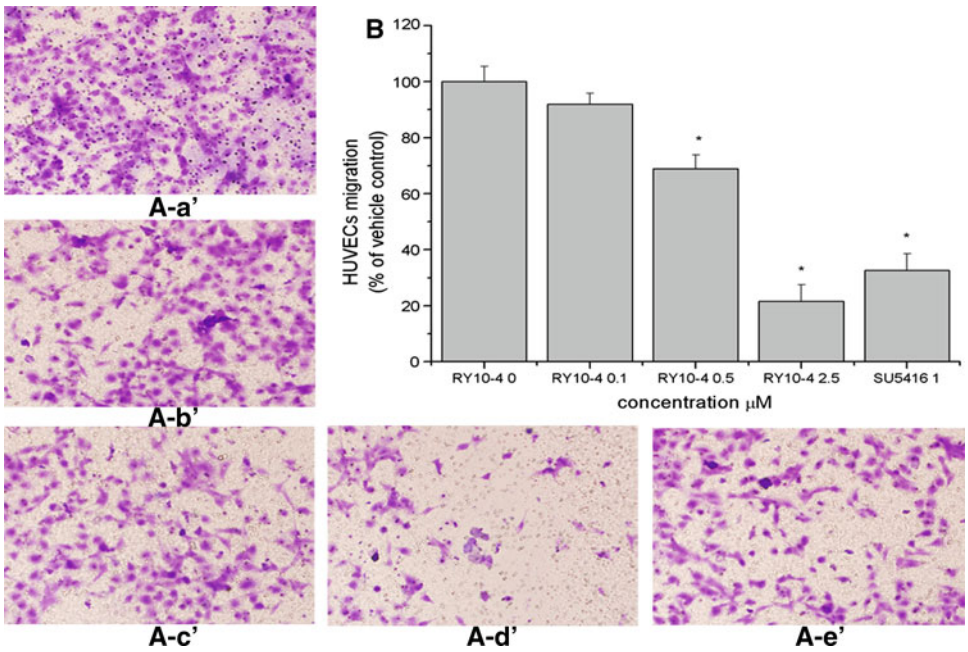

Fig. 3 Effect of RY10-4 on HUVECs chemotaxis. Migrated cells were counted and photographed after 24 h incubation under six microscopic fields at 200× magnification. **a** Images were taken after different concentrations of drug administration. *a* Vehicle control *b* 0.1 μM RY10-4 *c* 0.5 μM RY10-4 *d* 2.5 μM RY10-4 *e* 1 μM SU5416 **b** inhibitory effects of RY10-4 on HUVEC migration, inhibitory percentage were determined using 0.1 % DMSO as a vehicle control. Values are means ± SD ($n = 4$). *Bars* with *different superscripts* are significantly different ($P < 0.05$; $^*P < 0.01$)

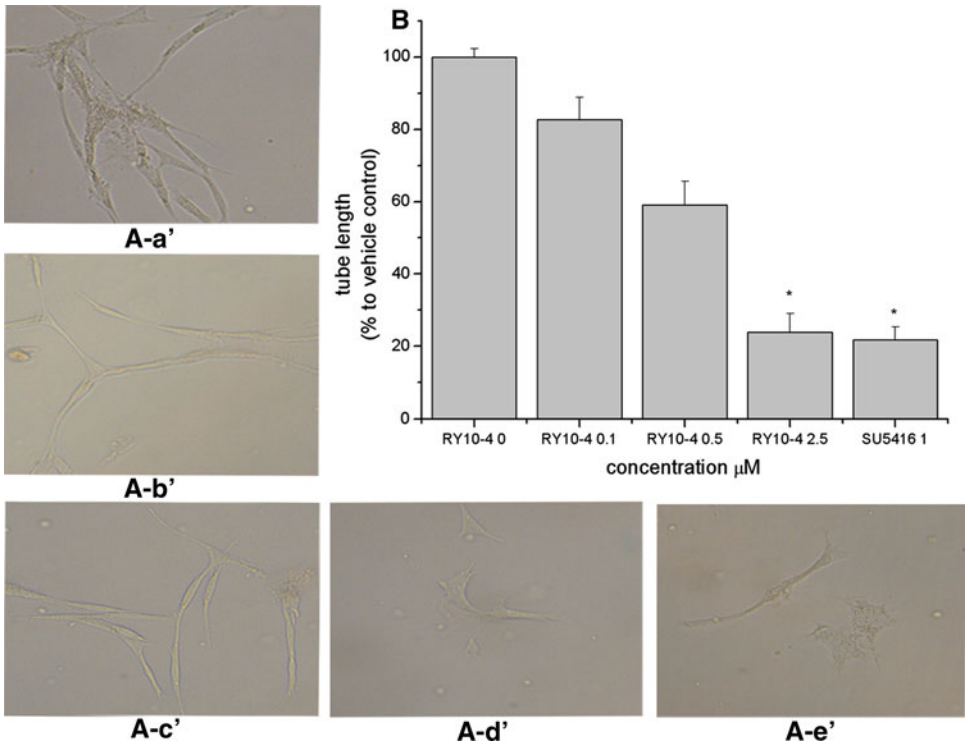

Fig. 4 Inhibition effect of HUVECs network formation in by RY10-4. The cells were seeded into the layer on matrigel, after 24 h incubation, images were taken under inverted microscope. **a** Representative figures of migrating HUVECs are shown, *a* Vehicle control *b* RY10-4 0.1 μM *c* RY10-4 0.5 μM *d* RY10-4 2.5 μM *e* SU5416 1 μM. **b** The network length in each treatment conditions was also quantified. Values are means ± SD ($n = 3$). *Bars* with *different superscripts* are significantly different ($P < 0.05$; $^*P < 0.01$)

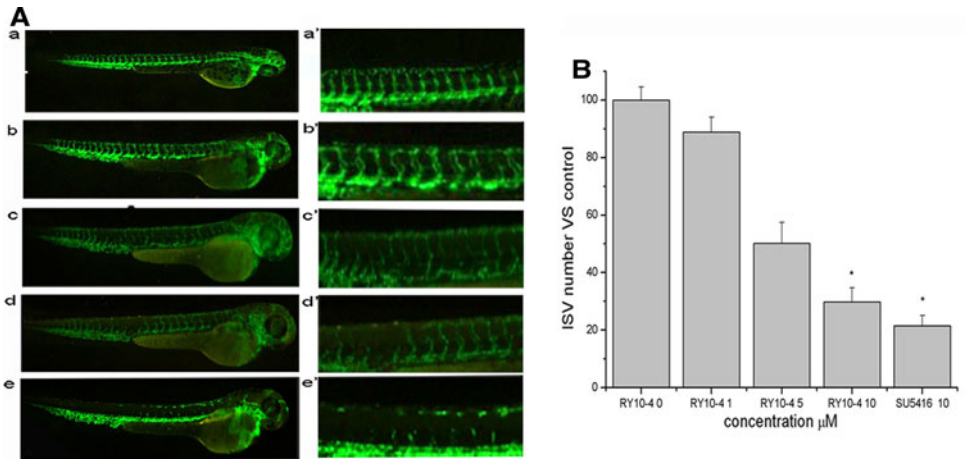

Fig. 5 Lateral view of transgenic zebrafish embryos Tg (VEGFR2:GFP) at 24 hpf immersed in RY10-4 at different concentrations. **a** Fluorescence microscopic images highlight the GFP expressing intersegmental blood vessels (ISVs): *a* and *a′* vehicle control (0.1 % DMSO) *b* and *b′* RY10-4 1 μM *c* and *c′* RY 10-4 5 μM *d* and *d′* RY10-4 10 μM *e* and *e′* SU5416 10 μM. The magnified views of *a–d* are shown in *a′–d′*, respectively. **b** The number of ISVs were measured, and values are means ± SD ($n = 3$). *Bars* with *different superscripts* are significantly different ($P < 0.05$; $^*P < 0.01$)

RY10-4 attenuates the over-expression of HIF-1α and related proteins caused by hypoxia

The over-expression of HIF-1α and VEGF were milestone events to hypoxia and angiogenesis. That hypoxia can induce angiogenesis in tumor cells is very clear today [18]. As seen in Fig. 6, compared to the vehicle control, in the normoxic condition, the expression of VEGF reduced in the dose-dependent manner, meanwhile HIF-1α, which has weak expression in the vehicle control, changed a little after drug administration. But in the hypoxic condition, the expression of HIF-1α, VEGF decreased greatly in a dose-dependent manner. The result indicated that RY10-4 can weaken the expression of HIF-1α in the two breast cancer cells. In addition, the low expression of HIF-1α attenuated the expression of VEGF.

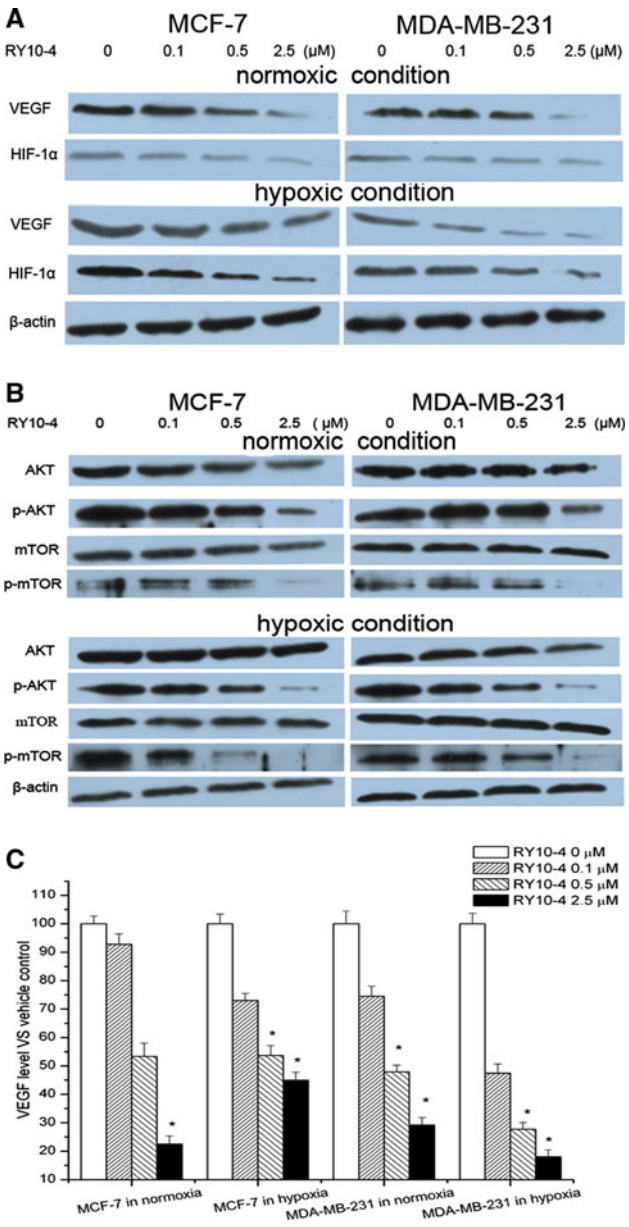

Fig. 6 RY10-4 diminished the expression of VEGF and HIF-1α in the presence of different concentrations of RY10-4 for 24 h, meanwhile effect the AKT/mTOR pathway in both MCF-7 and MDA-MB-231. **a** Down-regulating the expression of VEGF and HIF-1α under both hypoxic condition and normoxic condition. **b** The protein amount of AKT, p-AKT, mTOR, p-mTOR was determined under both normoxic and hypoxic conditions. *β*-Actin was used as internal control. **c** Quantification of VEGF protein level ($P < 0.05$; $^*P < 0.01$)

Down-regulation of p-Akt/p-mTOR levels by RY10-4

It is known that VEGF and HIF-1α are the downstream targets of PI3 K/AKT/mTOR pathway. The phosphorylation of AKT and mTOR is very remarkable events in angiogenesis [19]. They were evaluated by the western blot in hypoxic and normoxic conditions. As Fig. 6 showed, after drug administration, the phosphorylation level of AKT and mTOR was significantly decreased both in the hypoxic and normoxic environment in a dose-dependent manner. While the content of normal AKT/mTOR did not decrease as significantly as p-AKT/p-mTOR did. So the results of the western blot verified that the high phosphorylation levels of AKT and mTOR will cause the expression of HIF-1α, and then, down-stream proteins such as VEGF began to express. So angiogenesis occurs in the tumor tissue.

Discussion

Nowadays quinol compounds that have a 4-hydroxy-2,5-cyclohexadien-1-one structure such as protoapigenone [20, 21], DHEC, DEDC [22, 23] and 4-(benzothiazol-2-yl)-4-hydroxycyclohexa-2,5-dienone [24] had attracted a lot of attentions as a new series of anti-tumor compounds. And the synthesis of the probably minimal pharmacophore of protoapigenone was processed in our laboratory. The article investigated the possible mechanism of how minimal pharmacophore impairs the tumor cells. RY10-3 that has a similar structure to RY10-4 showed little cytotoxicity in the MTT assay against HUVECs and breast cancer cells. A similar conclusion was concluded between protoapigenone and apigenin [25]. This gave us the opinion that the 1-hydroxycyclohexa-2,5-dien was the essential pharmacophore in the series of compounds. So RY10-4 became the central issue in our research, and then, anti-angiogenesis was found as an activity of RY10-4. The anti-tumor compounds that own anti-angiogenesis property were generally regarded as a weak cytotoxicity anti-tumor drug due to their selective effect on tumor vasculature [26]. Although the RY10-4 has a similar IC_{50} like protoapigenone against some tumor cells, the weak normal cytotoxicity that was shown in the primary screening of normal cells was partly attributed to the anti-angiogenesis effect in selectively impairing the tumor cells.

To identify and characterize how RY10-4 inhibited the over-expression of VEGF, we cultured the two breast tumor cells in normoxic and hypoxic conditions to analyze the expression of related proteins such as VEGF and HIF-1α by the western blots. The expression of HIF-1α and VEGF decreased in the dose-dependent manner. Due to the rapid growth rate of tumor cells, tumor cells often face the shortage of oxygen and nutrition. Thus, the expression of VEGF and formation of vessel are central events to supply oxygen and nutrition. HIF-1 that can cause the expression of down-stream protein such as VEGF plays a key role to avoid the tumor cells from suffering the hostile condition. In all, tumor vascularization through VEGF expression was partly relied on the stable expression of HIF-1 [27]. The activation of HIF-1 is the mandatory reaction for several

responses including apoptosis and cell survival, cell adhesion and angiogenesis [28]. HIF-1, a heterodimeric protein, consists of a constitutively expression β-subunit and a highly regulated α-subunit. HIF-1α protein levels in the cells are regulated by a balance between HIF-1α protein generation and degradation. Hypoxia can rapidly induce HIF-1α protein accumulation due to a marked decrease in HIF-1α protein degradation [29, 30]. In our study, HIF-1α has weak expression in the normoxic conditions, so no obvious change was noticed in the normal environment. While under hypoxic conditions, HIF-1 that plays an important role in anti-hypoxic stress has a stable expression. The expression of HIF-1α was obviously suppressed by RY10-4. The expression of VEGF that was mediated by HIF-1 decreased in a dose-dependent manner displayed both in qualitative and quantitative due to the RY10-4 afterward.

Studies showed that the over-activation of PI3K/AKT/mTOR pathway is frequently detected in many cancers [31]. PI3K/Akt/mTOR signal pathways were found to activate abnormally, associate with tumor progression and exert great effects on patient prognosis. Phosphorylation of AKT and mTOR that were the important events in the activation of PI3 K/AKT/mTOR pathway had been reported in a variety of cancers. It can be modulated by the related genes such as HER2, PTEN [32, 33]. mTOR, mammalian target of rapamycin, functions as a sensor of extra-cellular signals and then regulates angiogenesis, cell growth, apoptosis and autophagy, etc. mTOR also can regulate HIF-1 in the abnormal proliferative cells as tumor cells [34]. In addition, mTOR connects the PI3 K/AKT pathway and the down-streaming targets: HIF-1 and VEGF. Our research had confirmed this view in the protein expression way by RY10-4. The route of AKT/mTOR down-regulated by RY10-4 was done by suppressing the phosphorylation of AKT and mTOR. After drug administration in both normoxia and hypoxia, the expression of AKT and mTOR was slightly decreased, and at the same time, the amounts of p-AKT and p-mTOR were dramatically weakened. The HIF-1 was the primary activator of VEGF expression and acted as a bridge between PI3 K/AKT/mTOR pathway and VEGF, and thus, the expression of VEGF was also down-regulated in a similar trend as p-mTOR and p-AKT were in our study.

Conclusion

Taking the results of HUVECs and breast cancer cells proliferation, immigration of HUVECs, tube network formation and the zebrafish assay into consideration, the anti-tumor ability of RY10-4 should be partly mediated through anti-angiogenesis by suppressing the route of AKT/mTOR/HIF-1. The anti-angiogenesis ability of RY10-4 had a comparable effect to the positive control. In view of broad-spectrum and potent anti-tumor activity, there must be the other ways to inhibit the tumor progress. The related research such as in vivo research and the other anti-tumor pathways are undergoing in our laboratory. Based on the study of RY10-4, protoapigenone, DHEC, DEDC and 4-(benzothiazol-2-yl)-4-hydroxycyclohexa-2,5-dienone, the compound that contains the structure of 1-hydroxycyclohexa-2,5-dien will be the new hot-spots in the development of novel anti-tumor drugs.

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Conflict of interest None.

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