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Stabilization of VEGF G-quadruplex and inhibition of angiogenesis by quindoline derivatives

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ABSTRACT

Background: Angiogenesis is thought to be important in tumorigenesis and tumor progress. Vascular endothelial growth factor (VEGF) is a pluripotent cytokine and angiogenic growth factor that plays crucial roles in embryonic development and tumor progression. In many types of cancer, VEGF is overexpressed and is generally associated with tumor progression and survival rate. The polypurine/polypyrimidine sequence located upstream of the promoter region in the human VEGF gene can form specific parallel G-quadruplex structures, raising the possibility for transcriptional control of VEGF through G-quadruplex ligands.
Methods: PCR stop assay, circular dichroism (CD) spectra, RNA extraction and RT-PCR, enzyme-linked immunosorbent assay (ELISA), luciferase Assays, cell scrape test, xCELLigence real-time cell analysis (RTCA), and chick embryo chorioallantoic membrane (CAM) assay.
Results and conclusions: We found that quindoline derivatives can interact with the G-rich DNA sequences of the VEGF promoter to stabilize this G-quadruplex and suppress the transcription and expression of the VEGF protein. We also demonstrated that these derivatives exhibit potential anti-angiogenic activity in chick embryos and antitumor activity, including the inhibition of cell proliferation and migration.
General significance: Our new findings have significances not only for understanding the mechanism of the G-quadruplex ligands mediating the VEGF transcription inhibition, but also for exploring a new anti-tumor strategy to blocking the transcription of VEGF to inhibit the angiogenesis in cancer cells.

1. Introduction

Angiogenesis is fundamental to successful wound healing and blood flow restoration in the impaired tissues of embryos and healthy adults. Starving tumor cells must form new blood vessels to supply the required oxygen and nutrients, and these newly formed blood vessels serve as escape routes for disseminating tumor cells. This procedure is regulated by many angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and nitric oxide synthase (NOS). Among these angiogenic regulators, VEGF appears to play a key role as a potent mediator of tumor angiogenesis by stimulating the proliferation, migration, survival and permeability of endothelial cells [1,2]. Several anti-VEGF medicines with various mechanisms have found clinical use in cancer treatment including bevacizumab [3–5], sorafenib [6] and sunitinib [7,8]. As a monoclonal antibody against VEGF, bevacizumab binds directly to VEGF and is clinically beneficial against a variety of tumor types [1].

Guanine-rich (G-rich) DNA segments have a high propensity for self-association into planar guanine quartets (G-quartets) to form unusual structures called G-quadruplexes. Guanine-rich sequences exist in important regions of the eukaryote genome such as the telomere and promoter regions of many genes; thus, such structures may play important roles in regulating biological events. Therefore, for the past few decades, G-quadruplexes have been targets for new anti-cancer drugs [9]. The polypurine/polypyrimidine sequence, consisting of five runs of at least three contiguous guanines, is located upstream (−85 to −50) of the promoter region in human *VEGF* gene and serves as a multiple binding site for transcription factors such as Sp1 and Egr-1. This tract can form specific G-quadruplex structures and potentially allows transcriptional control of the *VEGF* promoter through G-quadruplex ligands [10–12]. Because human VEGF expression is primarily regulated at the transcriptional level [13,14], designing drugs that targets VEGF by stabilizing the G-quadruplex formation in its promoter region should prove to be an effective strategy.

Quindoline derivatives are valid G-quadruplex ligands that can interact with human telomeres and with the oncogene *c-myc*. These compounds have demonstrated effects on the biological functions of these two genes, including the extension of telomeres; the proliferation,

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senescence and apoptosis of cells; and the down-regulation of *c-myc* transcription [15–18]. Previous studies have indicated that the 11-alkylamino group of SYUIQ-05 can protonate the 5-*N* atom in situ. However, the positive charge on 5-*N* was unstable under various buffering conditions. Thus, *N*-methylation at the 5-position was used to introduce a stable positive charge to participate in an electrostatic interaction and to increase π-stacking interactions due to the reduced electron density of the aromatic core of the ligand [19]. In addition, one fluorine substituent was introduced at the 7-position to enhance stacking onto the G-quadruplex.

We found that quindoline derivatives can interact with the G-rich DNA of the *VEGF* promoter to stabilize G-quadruplex formation, thus down-regulating the transcription and expression of the VEGF protein. In addition, further studies have indicated that these derivatives exhibit strong anti-angiogenic activity in chick embryos and antitumor activity, including the inhibition of proliferation, migration and induction of apoptosis. Taken together, the aforementioned results indicate that the new G-quadruplex ligands may inhibit angiogenesis by interacting directly with the specific *VEGF* promoter sequence.

2. Results

2.1. Quindoline derivatives bind and stabilize the G-quadruplex in the VEGF promoter region in vitro

The G-rich region located upstream (−85 to −50) of the promoter region in human *VEGF* gene can form specific G-quadruplex structures and contains multiple binding sites for transcription factors such as Sp1 and Egr-1 (Fig. 1). A PCR stop assay was utilized to quantitatively evaluate the interaction between the G-rich sequence in the *VEGF* promoter and the quindoline derivatives by measuring the ability of these derivatives to block DNA amplification [20]. These results indicate that the derivatives can bind to the DNA and are able to stabilize the G-quadruplex. SYUIQ-05, SYUIQ-F05 and four quindoline derivatives (Fig. 2) were used in this assay. As indicated in Fig. 3 (A to E), the quantity of double-stranded PCR products decreased as the concentration of all derivatives increased, indicating that these ligands can block the formation of PCR products under certain concentrations. The sequences of the DNA oligomers used were listed in Table 1. To further demonstrate the inhibitory effects of the derivatives on the G-quadruplex stabilization of PuT, a control oligomer, PuTmu, which cannot form the G-quadruplex structure, was included in the assay. Under the same conditions, the control oligomer demonstrated no inhibition, even at 50 μM, which was the highest concentration used (Fig. 3F).

For comparison, the ligand concentrations capable of decreasing the PCR product by 50% (the IC_{50} values) were calculated using the optical densities from the gel images. All derivatives demonstrated higher inhibitory effects on the amplification of the PuT oligomer, with IC_{50} values of 4.9 ± 1.2 μM (SYUIQ-FM04), 1.0 ± 0.4 μM (SYUIQ-FM05), 6.3 ± 1.0 μM (SYUIQ-FM06) and 7.2 ± 0.6 μM (SYUIQ-FM07). The IC_{50} value for SYUIQ-05 was 16.6 ± 0.7 μM, which implies that the introduction of 5-*N*-methyl and 7-fluoro groups onto quindoline can enhance its interaction with the *VEGF* G-quadruplex DNA. Of the derivatives, SYUIQ-FM05, which contains an *N*,*N*-dimethylpropane-1,3-diamine side chain, demonstrated the strongest interaction with PuT; thus, it was chosen as the lead derivative for subsequent studies.

To further investigate the effects of the derivatives on the G-quadruplex in the *VEGF* promoter region, circular dichroism (CD) spectra were obtained to identify the conformation of the G-quadruplex DNA PuT. No significant change was observed in the conformation of the G-quadruplex at room temperature, while its thermal stability was dramatically enhanced by SYUIQ-FM05: its melting temperature increased from 40 to 55 °C (Fig. 4).

2.2. SYUIQ-FM05 can bind and stabilize the G-quadruplex in the VEGF promoter region and down-regulate the VEGF transcription and expression levels in vivo

To directly evaluate the association between the observed regulation effect and in vivo G-quadruplex stabilization, a luciferase assay was conducted. We transfected the dual-luciferase reporter plasmids with wild-type (PuT) and mutant (PuTmu) G-quadruplex-forming sequences of the *VEGF* promoter (Fig. 5A) to the MCF-7 cells. As indicated in Fig. 5B, adding various concentrations of SYUIQ-FM05 to the MCF-7 cells decreased the luciferase activity up to ~30% (with 0.5 μM ligand) in cells with the wild-type *VEGF* promoter, whereas little difference was observed with the mutant (<10%). This result indicates that the down-regulation effect of the luciferase was likely due to the interaction between the ligand and G-quadruplex structure in the *VEGF* promoter.

The transcription of the *VEGF* gene may be regulated by forming the G-quadruplex structures in the G-rich sequence of its promoter region, thus blocking the interaction of the transcription factors with their cognate sites, such as Sp1 and Egr-1 [11,12]. The effects of SYUIQ-FM05 on the VEGF transcription and expression were evaluated via RT-PCR and ELISA assays. Meanwhile we utilized SYUIQ-F05 as a control quindoline derivative due to its higher bioavailability than SYUIQ-05. The VEGF expression decreased dramatically in both the mRNA and protein levels after treating the MCF-7 cells with SYUIQ-FM05 for 48 h (Fig. 6).

2.3. Inhibition of tumor cell proliferation, migration and angiogenesis in chick embryo chorioallantoic membranes by SYUIQ-FM05

VEGF acts as a potent mediator for tumor angiogenesis by stimulating the proliferation, migration, survival and permeability of endothelial cells [1,2]. It has been demonstrated that cell proliferation could be inhibited by quindoline derivative SYUIQ-05 through its preferential

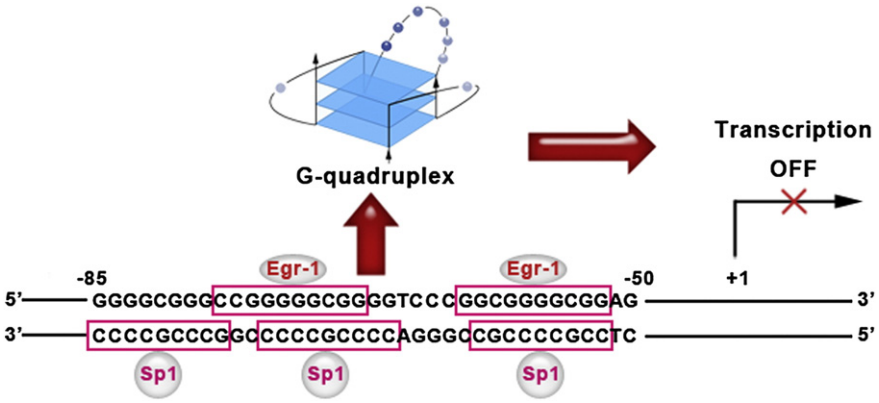

Fig. 1. The G-rich sequences in the VEGF promoter and its proposed role in transcription.

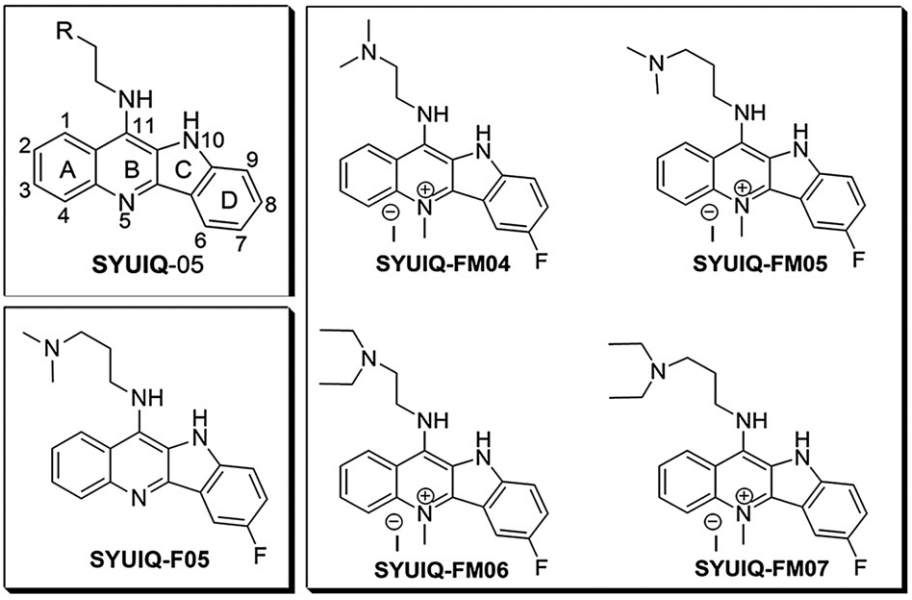

Fig. 2. Structures of the 11-amino-7-fluoro-quindoline derivative (SYUIQ-F05) and 11-amino-5-*N*-methyl-7-fluoro-quindoline derivatives (SYUIQ-FM04, SYUIQ-FM05, SYUIQ-FM06 and SYUIQ-FM07).

interaction with G-quadruplex within c-myc promoter [21]. Here we proved SYUIQ-FM05 could also show significant inhibitory effect on the proliferation of MCF-7 cells, either in the short-term proliferation (with an IC_{50} of 0.96 μM) and in the long-term proliferation assay (Fig. 7).

In addition, the inhibitory effect of SYUIQ-FM05 on cell migration was evaluated using a cell scrape test and real time cellular analysis (RTCA). To increase the VEGF-A expression, the cells were treated with 100 μM $CoCl_2$ for creating a cellular chemical hypoxia environment [22–24]. As indicated in Fig. 8A–B, the inhibitory effect of SYUIQ-FM05 on cell migration was enhanced at high concentrations, which was in correspondence with the decreased expression of VEGF-A protein. The cell migration rate of 1 μM SYUIQ-FM05 treated group (56%) was lower than 1 μM control quindoline derivative SYUIQ-F05 treated group (65%). Furthermore, RTCA was employed to real time monitor the migration rate of MDA-MB-231 cells without or with various concentrations of SYUIQ-F05 or SYUIQ-F05 over 24 h. The cell index indicates the migrated cells in different medium conditions (OptiMEM represented serum-free medium, DMEM + 10%FBS contained 10% fatal bovine serum). The fitted curves in Fig. 8C showed that the cell index dropped from 3.5 (DMEM + 10%FBS control without compound) to less than 1.5 when the cells were treated with 1 μM SYUIQ-FM05, and decreased from 3.5 to 2.5 when 1 μM SYUIQ-F05 treated as it showed in Fig. 8C. This result indicated that the inhibitory effect of SYUIQ-F05 was better than SYUIQ-F05 on MDA-MB-231 cell migration.

VEGF is a well-known vascular permeability factor, and VEGF-related antibodies have demonstrated the ability to block angiogenesis in CAM [25,26]. The anti-angiogenic potential of the quindoline derivatives on CAM was studied by adding SYUIQ-FM05 directly into the chorioallantoic membrane to determine their inhibitory effects on blood vessel growth. Fig. 9 showed the microscopy images of the new blood vessels in the chorioallantoic membrane and the inhibitory effects of the drugs. Compared with the blank sample (0.9% NaCl), SYUIQ-FM05 showed significant reduction of the blood vessels, with an inhibitory response of 62.7%, which is stronger than that of the control drug Thalidomide (53.3%) and the control quindoline derivative SYUIQ-F05 (54.6%).

3. Discussion

VEGF is a pluripotent cytokine and angiogenic growth factor [27]. When VEGF binds with its cognate receptor, the complex can stimulate the proliferation, migration, survival and permeability of endothelial cells, thus forming new blood vessels [28]. The formation of new blood vessels provides oxygen and nutrients to the adjacent primary tumors and promotes the growth of tumor cells. VEGF can initiate angiogenesis to aid in tumor cell survival, and its expression is typically

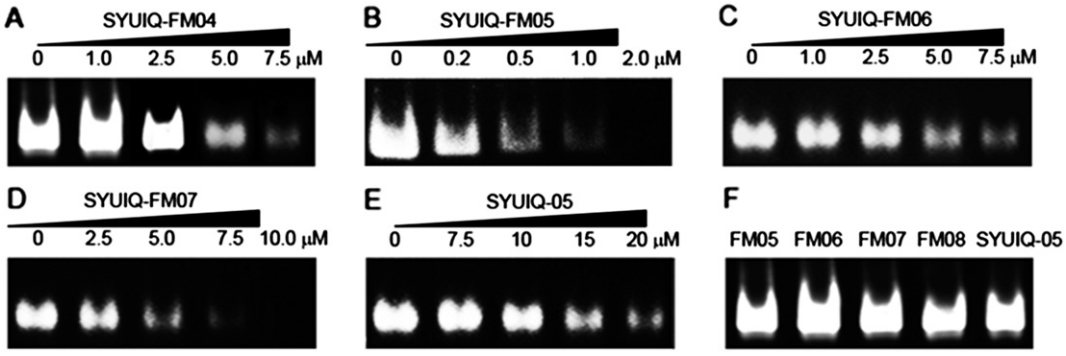

Fig. 3. Effects of the 11-amino-5-N-methyl-7-fluoro-quindoline derivatives on DNA amplification of the G-quadruplex-forming oligomer PuT in the PCR stop assay. This assay also utilized the control oligomer PuTmu (F) with all derivatives. The derivatives were added to the G-quadruplex to form the PuT oligomer over a range of concentrations (0–20.0 μM), or to the mutated PuTmu oligomer at 50 μM. The 40-bp double-stranded PCR products were separated on 15% non-denaturing polyacrylamide gels in 1× TBE and were EtBr stained.

Table 1
Sequences of oligomers or primers used in this study.

Oligomer	Sequence (from 5′ to 3′)
PuT	TTTTTGGGCGGGCCGGGGGCGGGTTTTT
PuTmu	TTTTTGGGCGAACCGGGGG CGGGTTTTT
PuTrev	ATCGCTTCTCGTAAAAACCC
Primer	**Sequence (from 5′ to 3′)**
VEGF-Mut A	AACATATGGCACCCATGGCAGAAGGAGG
VEGF-Mut S	AAGGATCCGCGGCCGCTATCACCGCCTCGGCTTGTC
$VEGF_{165}$ A	ACCGGTGGTTTCGGAGGCCCGAC
$VEGF_{165}$ S	CCCAAGCTTGGGGCTAGCTCGGGCCGGG
β-actin A	GTTGCTATCCAGGCTGTGC
β-actin S	GCATCCTGTCGCCAATGC

elevated in many cancers [13]. VEGF-dependent actions include increased vascular permeability and paracrine/autocrine growth factor release, resulting in enhanced cell motility and inhibition of apoptosis [29]. These phenomena occurred not only in endothelial cells but also in hematopoietic cells, stromal cells and malignant cells in certain human cancers [30–33], such as the MCF-7 cells used in the present work.

G-quadruplexes, which involve a stacked tetrameric arrangement of guanines, have been considered to be targets for antitumor drugs in recent years [34]. This special DNA secondary structure exists in certain gene promoter regions [11,12,35,36] and acts as a transcription regulator [12,36]. The transition from duplex to quadruplex in the VEGF promoter region can be driven by G-quadruplex ligands, such as TMPyP4, Se2SAP and telomestatin, thus modulating enzyme binding to this region [11] and suppressing transcription [12]. The stronger G-quadruplex ligands, such as TMPyP4 and Se2SAP, demonstrated a more significant effect on the transcriptional inhibition of VEGF than the weak G-quadruplex ligand TMPyP2 [12]. Thus, the formation of G-quadruplexes may play a role in regulating VEGF expression. Moreover, G-quadruplex ligands, such as TMPyP4, BMVC and polycyclic acridines, inhibit the growth of tumor cells [37–39] decreasing the cells observed in the G1 phase, increasing those observed in the S and G2/M phases [38] and down-regulating related oncogenes.

Quindoline derivatives demonstrated strong interactions with G-rich DNA in the VEGF promoter. The interactions primarily stabilized the G-quadruplex structure of the VEGF promoter DNA (according to the CD melting data) and blocked the amplification of the G-rich DNA catalyzed by *Taq* polymerase (per the PCR stop assay data). Subsequently, the down-regulation of transcription and expression by SYUIQ-FM05 was evaluated via RT-PCR and ELISA assays. SYUIQ-FM05 exhibited significant inhibitory activity against both the transcription and expression

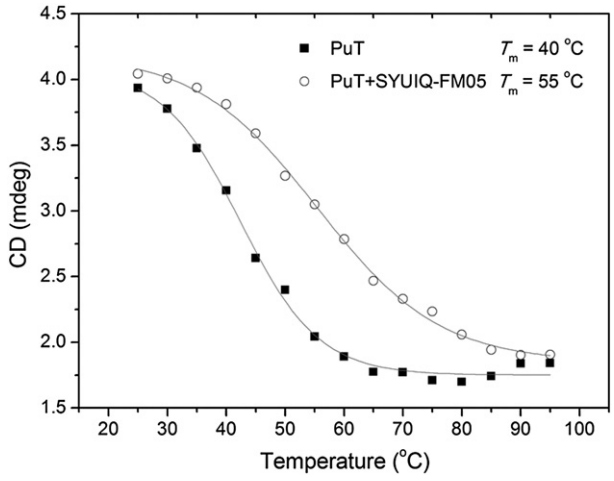

Fig. 4. CD melting profiles of PuT without the ligand or in the presence of 15 μM SYUIQ-FM05.

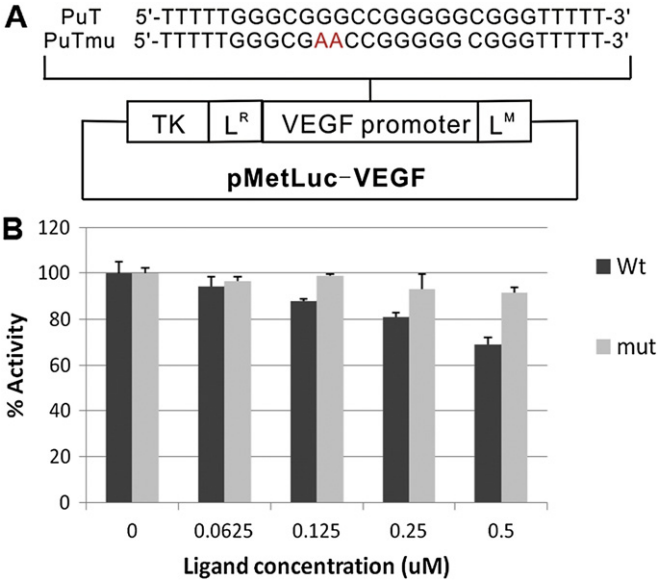

Fig. 5. Effects of SYUIQ-FM05 on VEGF promoter activity obtained via a dual-luciferase reporter assay. (A) Schematic diagram of dual-luciferase bicistronic constructs with wild-type (PuT) and mutant (PuTmu) G-quadruplex-forming sequences of the VEGF promoter. (B) Histogram presenting the percent activity of the wild-type and mutant constructs in the presence of various ligand concentrations normalized to the group with no ligand. All experiments were performed in triplicate to obtain standard deviations.

of VEGF, indicating that this G-quadruplex ligand may down-regulate gene transcription by stabilizing G-quadruplex formation.

The quindoline derivatives demonstrated significant effects on related VEGF biological functions, including embryonic angiogenesis, tumor cell proliferation and cell migration. The CAM assay data indicated that

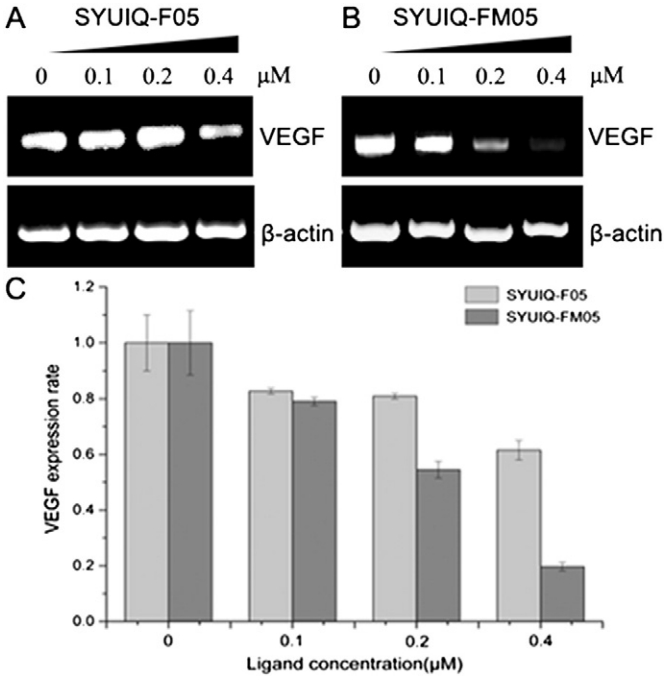

Fig. 6. The transcription/expression of VEGF in MCF-7 cells incubated with SYUIQ-FM05. SYUIQ-F05 was selected as a control quindoline derivative. The transcription of the *VEGF* gene was determined using RNA extracted from the treated MCF-7 cells via RT-PCR in the presence of various concentrations of SYUIQ-F05 (A) or SYUIQ-FM05 (B). The expression of the *VEGF* gene was determined by obtaining the secreted proteins from the medium of the MCF-7 cells (for VEGF) in the presence of various concentrations of SYUIQ-F05 or SYUIQ-FM05 with ELISA assays (C).

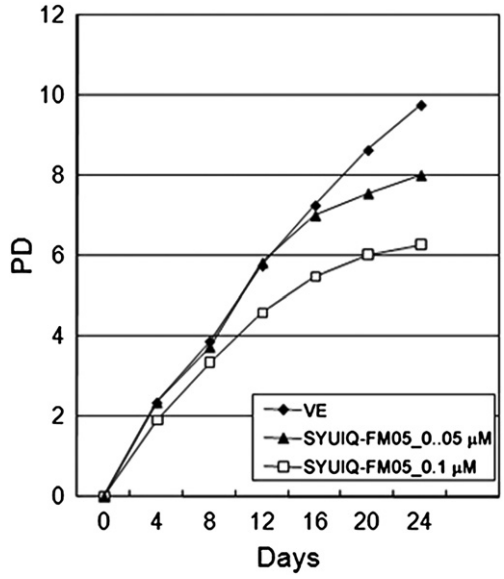

Fig. 7. Long-term exposure of MCF-7 cells with 0.1% DMSO (VE), 0.05 μM and 0.1 μM SYUIQ-FM05 for 24 days. Every 4 days, the cells in control and drug-exposed flasks were counted and flasks reseeded with cells. Results showed a representative of three experiments.

SYUIQ-FM05 strongly inhibits blood vessel growth, an effect that may arise from the down-regulation of VEGF expression by SYUIQ-FM05. These results support the hypothesis that quindoline derivatives can stabilize the G-quadruplex structures formed in vitro in the promoter region of the *VEGF* gene and thus inhibit embryonic angiogenesis in tumor cells through the VEGF pathway. In addition, previous data has already proved that quindoline derivatives, such as SYUIQ-F05 and SYUIQ-FM05 could induce apoptosis of tumor cells by targeting G-quadruplex structure in bcl-2 promoter [40,41] or c-kit promoter [40, 41], respectively. Moreover, angiogenesis and apoptosis are complicated intracellular processes controlled by many intrinsic and extrinsic factors. Thus, ascertaining whether the VEGF G-quadruplex is the only factor involved in inhibiting blood vessel growth remains difficult, but could prove important.

As most of the G-quadruplex ligands on one hand quindoline derivatives has the ability to bind to and stabilize G-quadruplex structures within telomeric DNA [19], *c-myc* promoter [21], *bcl-2* promoter [40] and *c-kit 1* promoter [41] on the other hand these quindoline derivatives or any other small molecular ligands are all difficult in targeting specific G-quadruplex within certain promoter region or telomere region. From recent data about SYUIQ-FM05, the ligand is able to target different kinds of G-quadruplex and the inhibitory effects are similar either from cell toxicity or stabilization ability, but each gene with G-quadruplex target has its own bio-function in different tumor cells. Thus quindoline derivative stabilizing G-quadruplexes provides us a valid background to find its potential target in genome, and play different roles by interacting with different genes.

In conclusion, *VEGF* is a critical gene in angiogenesis and tumorigenesis. The formation or stabilization of the G-quadruplex structure in the promoter region of VEGF may play a role in regulating this gene. Quindoline derivatives, especially SYUIQ-FM05, demonstrated strong interactions with the G-quadruplex DNA of VEGF, resulting in the down-regulated transcription and expression of this gene and potentially reducing VEGF-simulated angiogenesis. It has been given evidences by Qi-Biao Su et al. that SYUIQ-5 (one of a series of novel quindoline derivatives possessing anti-tumor activity due to inducing the G-rich telomeric DNA sequence to fold into quadruplex and stabilize the G-quadruplex [15–17]) exhibited a nonlinear, dose-dependent pharmacokinetics in rats and it was mainly metabolized by Cyp3A1/2. Cyp3A is the primary enzyme involved in SYUIQ-5 metabolism in rat liver microsomes. SYUIQ-5 induces the mRNA and protein of rat Cyp1A1 and 1A2 in a dose-dependent manner, while SYUIQ-5 does not affect the expression and activity of Cyp2B1/2 and 2E1 [42]. SYUIQ-5 has also demonstrated substantial antitumor activity in nude mouse studies (unpublished data, Huang et al.). Therefore, anti-angiogenic effects of quindoline derivatives could be exploited therapeutically and further studies were in progress.

4. Materials and methods

4.1. Materials

The oligomers/primers used in this study were purchased from Invitrogen (China). Anti-VEGF, anti-caspase-3, anti-PARP and anti-β-actin primary antibodies and horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies were purchased from Chemicon International, Inc., USA.

To prevent the mixing of intra- and intermolecular structures caused by large DNA fragments, a truncated sequence (named PuT) from the *VEGF* promoter region containing only those guanine regions involved in forming the intramolecular G-quadruplexes was used in the assays [12]. The sequence is provided in Table 1.

Plasmid construction: The wild-type pMetLuc-VEGF promoter (wt) luciferase plasmid was generated by inserting the VEGF promoter sequence into the pMetLuc reporter vector (Clontech) using the *Hind*III and *Eco*RI restriction sites. The corresponding G-rich mutant (mut) luciferase plasmid was introduced via PCR using the following primers: VEGF-Mut A and VEGF-Mut S (Table 1).

4.2. Preparation of compounds

The synthesis and structural characterization of the derivatives was performed as reported [19]. All derivatives were prepared as 10 mM stock solutions in DMSO. The positive control drug thalidomide was purchased as a standard substance from the National Institute for the Control of Pharmaceutical and Biological Products and used as a 10 mM stock solution in DMSO. Further dilutions to working concentrations were performed using double-distilled water.

4.3. Cell culture

The MCF-7 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in 25-cm^2 culture flasks in a 37 °C humidified atmosphere with 5% CO_2. The cells from passages 3–6 were used.

4.4. PCR stop assay

The reactions were performed in 1× PCR buffer containing 2 μmol of a pair of oligomers, PuT or PuTmu (Table 1), and the corresponding complementary sequence PuTrev (Table 1), 0.16 mM dNTP, 2.5 U *Taq* polymerase and varying derivative concentrations. The reaction mixtures were incubated in a Mastercycler Personal® (Eppendorf) with the following cycling conditions: 94 °C for 3 min, followed by 10 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The amplified products were resolved on 15% non-denaturing polyacrylamide gels in 1× TBE and were EtBr stained and the gel images were obtained by a Gel Doc 2000 Imager System (Alpha Innotech, CA, USA).

4.5. Circular dichroism (CD) spectra

The PuT oligomer at a final concentration of 5 μM was resuspended in Tris–HCl buffer (10 mM, pH 7.4) containing the derivatives to be tested. The samples were heated to 95 °C, then gradually cooled to room temperature and incubated at 4 °C for at least 6 h. The CD spectra were recorded on a Chirascan spectrometer (Applied Photophysics),

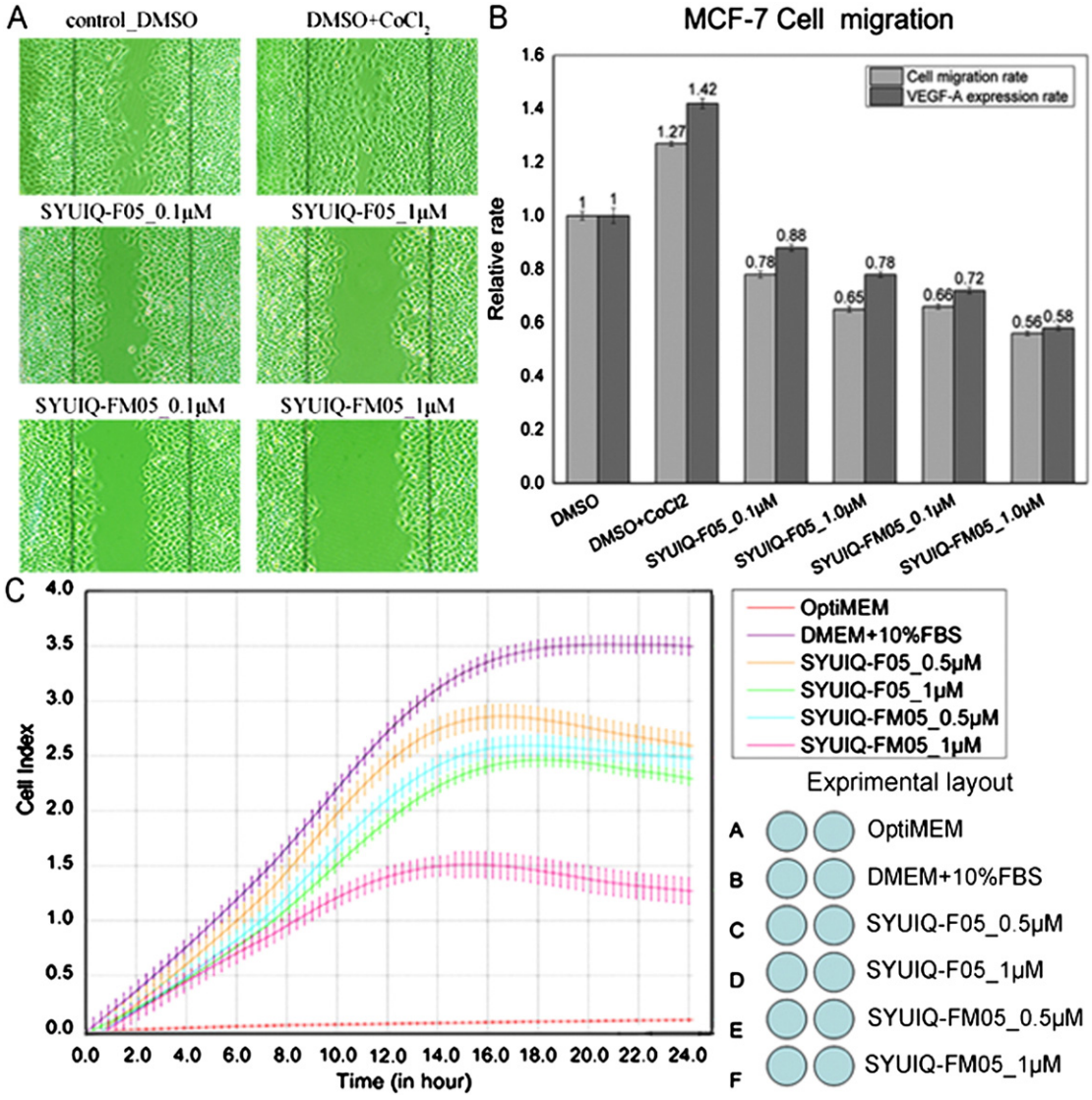

Fig. 8. SYUIQ-FM05 inhibited cell migration. The cells were cultured to confluence in normal growth medium. Then, a cross-shaped scrape was made through the confluent monolayer using a plastic pipette tip. The cells were treated with 0.1% DMSO or 0.1% DMSO with 100 μM $CoCl_2$, or in the presence of SYUIQ-F05 or SYUIQ-FM05 for 1 h. Several wounded areas were observed and photographed via phase-contact microscopy at 24 h after the scratch. The photos above are representative of six experiments (A). Then the cell migration rate of each well was quantified by using ImageJ software and the corresponding VEGF protein expression rate was measured by ELISA assay (B). Real-time cell analysis (RTCA) was employed to monitor the migration rate of MDA-MB-231 cells without or with various concentrations of SYUIQ-F05 or SYUIQ-F05. The cell index indicates the migrated cells in different medium conditions (OptiMEM represented serum-free medium, DMEM + 10%FBS contained 10% fatal bovine serum) (C).

at a rate of 0.5 s per point from 200 to 400 nm with a 1-nm bandwidth. In the melting studies, the sample temperature was maintained using a Pelter temperature controller. The melting curves of the G-quadruplex were measured at 280 nm. Prior to measuring the CD spectra, all samples were thermally treated as previously described.

4.6. RNA extraction and RT-PCR

The MCF-7 cells were cultured in 10-cm Petri dishes. After reaching subconfluence, the cells were treated with various concentrations of SYUIQ-FM05 and incubated in a humidified incubator at 37 °C for 48 h. After incubation, the cells were washed with PBS (pH 7.4), and the cell pellets were lysed in a TRIzol solution. The total RNA was extracted according to the protocol supplied by the Takara Company and eluted in distilled, deionized water with 0.1% diethyl pyrocarbonate (DEPC) to a final volume of 50 μl. RNA was quantified spectrophotometrically.

The total RNA was used as a template for reverse transcription according to the following protocol: each 20-μl reaction contained 1× M-MLV buffer, 500 μM dNTP, 100 pmol oligo dT_{18} primer, 100 unit of M-MLV reverse transcriptase, DEPC-H_2O, and 1 μg total RNA. The mixtures were incubated at 42 °C for 60 min for reverse transcription, then at 92 °C for 10 min. PCR was performed according to the following protocol: each 20-μl reaction contained 1× PCR buffer, 500 μM dNTPs, 1.5 μM primer pairs, 1 unit *Taq* polymerase, 0.1% DEPC-H_2O and 3 μl cDNA template. The reactions were incubated in a Mastercycler Personal (Eppendorf) according to the following protocol: 95 °C for 5 min, 36 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min. The amplified products were separated on a 1.5% agarose gel, and the gel was photographed using a Gel Doc 2000 Imager System. The primers used for PCR were presented in Table 1.

4.7. Protein extraction

The MCF-7 cells were cultured in 10-cm Petri dishes. After reaching subconfluence, the cells were treated with various concentrations of SYUIQ-F05 or SYUIQ-FM05 and incubated in a humidified incubator at 37 °C for 48 h. After incubation, the cells were washed with PBS (pH 7.4), incubated with extraction buffer (50 mM glucose, 25 mM

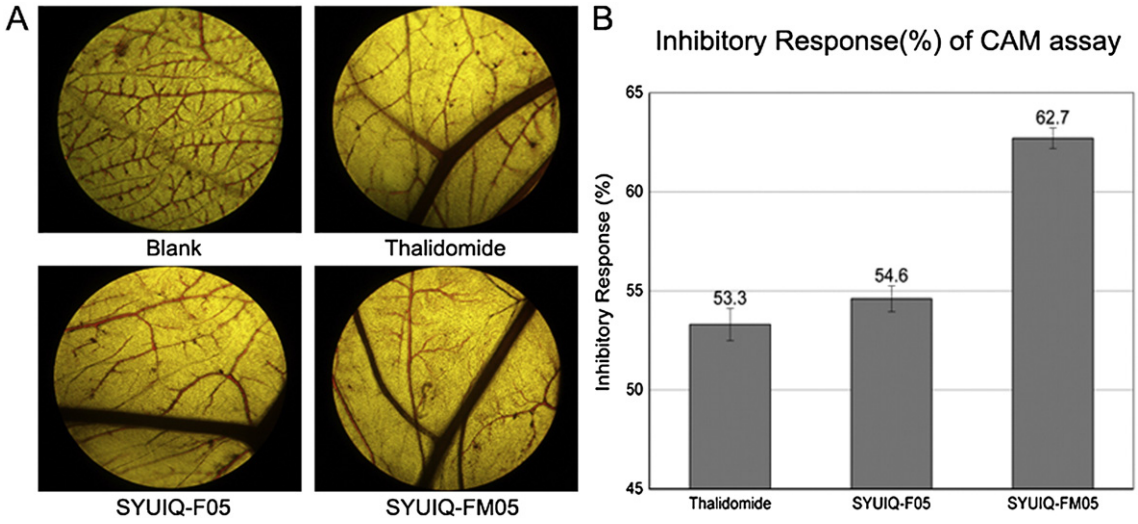

Fig. 9. Results of the CAM assay. SYUIQ-FM05 (750 ng/10 μl) was added directly into the chorioallantoic membrane and incubated for 3 days. The blood vessels were counted under a microscope (4×) (A), and the corresponding inhibitory response was quantified by counting the number of blood vessel branches using ImageJ software (B). The negative control was 0.9% NaCl, thalidomide (at 1500 ng/10 μl) served as a positive control and SYUIQ-F05 (750 ng/10 μl) served as a quindoline derivative control. Each assay was repeated in 8–10 samples.

Tris–HCl, pH 8, 10 mM EDTA, 1 mM PMSF) on ice for 30 min and centrifuged at 12,000 *g*. The protein concentration was quantitated according to the BCA quantitation protocol.

The secreted protein was extracted by collecting the cell culture medium and, after centrifugation and freeze-drying, dissolving the protein in PBS (pH 7.4). The protein concentration was measured according to the BCA quantitation protocol.

4.8. Enzyme-linked immunosorbent assay (ELISA)

MCF-7 cells were seeded at a density of 5×10^5 cells in 10-cm Petri dishes in Opti-MEM Reduced Serum Medium (Gibco) and cultured for 12 h. Then the medium was changed for new Opti-MEM medium and the cells were treated without or with various concentrations of compound SYUIQ-F05 or SYUIQ-FM05. After 48 h, the supernatants were collected. VEGF-A levels in supernatants were determined with the Human VEGF-A ELISA kit (IBL) according to the manufacturer's protocol. The absorbance at 450 nm was measured by a micro-plate reader (BioTek).

4.9. Transfection and luciferase assays

Plasmid transfection was performed with MCF-7 cells in the log phase. First, 200 ng pMetLuc plasmid containing wild-type VEGF promoter (Wt) or Mutant VEGF promoter (mut) and 200 ng pRL-TK (Promega) were co-transfected into MCF-7 cells using Lipofectamine 2000 (Invitrogen). After another 48 h, the transfected cells were washed with ice-cold PBS to reduce the background signal from the medium, and luciferase assays were performed according to manufacturer's instructions using a Ready-To-Glow Secreted Luciferase Reporter System (Clontech) and Renilla Luciferase Assay System (Promega). After a 3-s delay, the secreted luciferase signals were collected for 10 s using a luminometer in a microplate reader (Molecular Devices, Flex Station 3, USA). The Renilla signals were collected for 10 s as an internal control.

4.10. Cell scrape test

The cells were grown to confluence in 24-well plates for 24 h, and a cross-shaped scrape was made through the confluent monolayers using a plastic pipette tip. The cells were treated with SYUIQ-FM05 for 1 h. Several wounded areas were noted for orientation, observed and photographed using phase-contrast microscopy 24 h after scratching.

4.11. xCELLigence real-time cell analysis (RTCA)

Cell migration experiments were performed by using modified 16-well plates (CIM-16, Roche Diagnostics GmbH, Mannheim, Germany) with each well consisting of a top chamber and a bottom chamber separated by a microporous membrane containing randomly distributed 8 μm-pores. This setup corresponds to conventional Transwell plates with microelectrodes attached to the underside of the membrane for impedance-based detection of migrated cells. Prior to each experiment, the cells were deprived of FBS during 24 h. Initially, 165 μl (various concentrations of compound SYUIQ-F05 or SYUIQ-FM05 containing medium) and 30 μl of media were added to the lower and upper chambers respectively and the CIM-16 plate was locked in the RTCA DP device at 37 °C and 5% CO_2 during 60 min to obtain equilibrium according to the manufacturer's guidelines. Lower chambers contained media (DMEM basic 1×, Gibco) with or without FBS (Biological Industries, BI) in order to assess cell migration when exposed to FBS and background migration to serum-free (OptiMEM medium, Gibco) medium as a negative control accordingly. After this incubation period, a measurement step was performed as a background signal, generated by cell-free media. To initiate an experiment, the cells were detached using 0.25% Trypsin & 0.02% EDTA (Genom) and resuspended in OptiMEM medium, counted and seeded in the upper chamber applying 5×10^4 cells in 100 μl without or with various concentrations of compound SYUIQ-F05 or SYUIQ-FM05 that is in accordance with corresponding lower chambers. After cell addition, CIM-16 plates were incubated during 30 min at room temperature in the laminar flow hood to allow the cells to settle onto the membrane according to the manufacturer's guidelines. Each condition was performed in quadruplicate with a programmed signal detection schedule of each 15 min for 24 h.

4.12. The chick embryo chorioallantoic membrane (CAM) assay

CAM construction and blood vessel counting were performed according to published protocols [43]. The fertilized chicken eggs were placed in an incubator upon embryogenesis and were maintained under constant humidity at 37 °C. On day 8, a square window was opened in the shell after removing 2–3 ml albumen to detach the CAM from the shell. Substances containing the test compounds or protein extracts from the cells treated with the testing compounds were added to the detached CAM. The window was sealed with parafilm and incubated for 3 days. The embryo and its extraembryonic membranes were transferred to a Petri dish

and fixed with formalin. The fixed sections were photographed under a microscope, the vessel number and density were determined by two independent observers, and the data were statistically analyzed.

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