Remodelin, an inhibitor of NAT10, could suppress hypoxia-induced or constitutional expression of HIFs in cells

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Abstract
Hypoxia-inducible factors (HIFs) are key mediators expressed under hypoxic condition and involved in many kinds of disease such as cancer and abnormal angiogenesis. Thus, development of their inhibitor has been extensively explored. Here, we describe a finding that Remodelin, a specific inhibitor of NAT10, could also inhibit the expression of HIFs. The presence of Remodelin could suppress the elevated level of HIF-1α protein and its nuclear translocation induced by either treatment of cobalt chloride (CoCl2) or hypoxia in dose or time-dependent way. More importantly, Remodelin could also inhibit the constitutional expression of HIF-1α and HIF-2α in VHL mutant 786-0 cells. With using of cells with depletion of NAT10 by shRNA or Crispr-Cas9 edited, we further demonstrated that inhibition of HIFs by Remodelin should need NAT10 activity. In biological analysis, the treatment of cultured HUVECs with Remodelin could inhibit in vitro cell migration and invasion and tube-formation. Our investigation implied that Remodelin could be a new potential inhibitor of HIFs for using in angiogenesis targeting therapy in either cancers or inflammatory diseases.

Keywords Remodelin · NAT10 · HIF · Hypoxia

Introduction
Hypoxia-inducible factors (HIFs), the major transcription regulators for hypoxic cells, are composed primarily of regulatory alpha-subunits (HIF-1α, HIF-2α, and HIF-3α) [1, 2]. Under well-oxygenated conditions, HIFs rapidly degraded by hydroxylation and bound to the von Hippel-Lindau (VHL) protein, while the hydroxylation of HIFs was inhibited and resulted in its highly expression in hypoxic conditions [3, 4]. The elevated HIF-1α accumulates and makes a translocation to the nucleus, regulating the expression of target genes in the nucleus, which regulate a variety of cellular processes to promote survival. Especially, this allows ATP and VEGF (vascular endothelial growth factor) to be synthesized in an oxygen-independent manner, thereby promoting angiogenesis, cell survival and tumor growth [5]. It has been observed that the expression of HIF-1α and HIF-2α is increased in a variety of human cancer cell types and in many cases, it is associated with poor prognosis. In fact, HIF-1α is a major factor for regulating tumor microenvironment (TME) for tumor cell survival and angiogenesis [6, 7]. Therefore, inhibition of HIFs protein expression may be an attractive way to prevent tumor progression [6]. The expression of HIFs protein should be inhibited because it is great essential for tumor growth. HIFs have been considered a therapeutic target for cancer and development of their inhibitor has been extensively explored.

N-acetyltransferase 10 (NAT10, or hALP, human N-acetyltransferase-like protein) is a nucleolar protein with lysine acetylation activity, which embraces GNAT, RNA helicase and tRNA-binding domain [8]. Previous studies have shown that NAT10 has lysine acetylase activity, and its substrates include histone, tubulin, tRNA and mRNA [8–11]. Owing to those, NAT10 has been identified to involve in variety of cell activities, such as ribosome biogenesis, transcription and translation. Elevated expression of NAT10 has been found in cell stress and various human cancers. The distribution of NAT10 and its role in cell division raised the possibility that this protein play an important role in the proliferation of cancer cells [10]. Remodelin, a small-molecule compound, can specifically target and inhibit the N-acetyltransferase NAT10 [12]. Recent experiments
have determined that Remodelin can inhibit the function of the acetyltransferase protein NAT10 and reduce cancer cell migration and invasion [13–15].

In this study, we demonstrated that Remodelin could not only inhibit the expression of HIF-1α or HIF-2α under hypoxic conditions but also suppress the constitutional expression of HIFs owing to genetic mutation. In addition, the inhibitory effects of Remodelin were dependent on the status of NAT10.

Materials and methods

Antibodies

The rabbit polyclonal antibody against the N-terminus of human NAT10 was used as previously described [8]. Mouse monoclonal antibody to human NAT10 (sc-271770) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-HIF-1α antibody was from Abcam (ab51608, Cambridge, MA, USA), and Rabbit Polyclonal HIF-2α/EPAS1 antibody was from Novus Biologicals (NB100-122PE, Littleton, CO, USA). EDC4/Ge-1 antibody (2548S) was purchased from Cell Signaling Technology (Danvers, MA, USA), mouse polyclonal DCP1A antibody (H00055802-A01) was from Abnova (Taipei, Taiwan), and DAPI (C0060) was purchased from Solarbio (Beijing, China).

Cell culture and treatment

HeLa, MCF-7, LoVo, HCT116, and HUVECs cell lines were purchased from China Infrastructure of Cell Line Resource. 786-0 cell line was obtained from Qilu College of Medicine, Shandong University. HeLa, MCF-7, LoVo, HCT116 cells and HUVECs were maintained in Dulbecco modified Eagle medium (DMEM) with high glucose (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco). 786-0 cells were cultured in RPMI-1640 medium (Gibco) with 10% fetal bovine serum (Gibco). All the above cells were incubated in a humidified atmosphere with 5% CO2 at 37 °C.

For cell treatments, varying concentrations of Remodelin (Selleckchem, S7641, Houston, TX, USA) and CoCl2 (Sigma-Aldrich, St Louis, MO, USA) were added to the cultured cells in DMEM. For hypoxic culture, cells were incubated under hypoxic (1% O2, 5% CO2, 94% N2) conditions.

Lentivirus-mediated short hairpin RNA (shRNA)

Lentivirus-mediated NAT10 (sh-NAT10) and control (sh-C) shRNA were purchased from GenePharma (GenePharma Co., Ltd, Shanghai, China). Cells were transfected with NAT10-shRNA-lentivirus or control-shRNA-lentivirus particles, respectively, and supplemented with 50 μg/ml polybrene (Sigma, St Louis, MO, USA) for 3 days. And then cells were further selected in the presence of puromycin (2 μg/ml) for 3 days, and the resultant stable cells were maintained at a lower concentration of puromycin (0.2 μg/ml). The expression of NAT10 was verified by immunofluorescence and western blotting.

LentiCRISPR v2 mediated deletion of NAT10 gene

Deletion of the NAT10 gene was mediated by LentiCRISPR v2 (Addgene, Cambridge, MA, UK), containing expression cassettes for S. pyogenes CRISPR-Cas9 and chimeric guide RNA. To target exon 5 of the NAT10 gene, a guide RNA sequence of GTGAGTTCATGGTCCTAGG was selected through the https://crispr.mit.edu website. Detailed NAT10-deleted cell line construction was performed as previously described [16]. Plasmid containing the guide RNA sequence was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The expression NAT10 was detected by western blotting, Immunofluorescence and DNA sequencing. Primers flanking exon 5 were designed: Forward: 5′- GTCCCTTTGGTTGCTATT TG -3′; Reverse: 5′- GCTCTTAGCCCCAGGCTGT -3′.

Western blotting

Total cell lysates were obtained by incubating the cells in 2× SDS (5× SDS was purchased from Appygen Technologies Inc) for 5 min on ice. After denatured by boiling for 15 min, the supernatant was collected and stored at −20 °C for subsequent analysis. Western blotting was performed as previously described [17]. Alternatively, proteins were transferred to PVDF membranes (Millipore, Billerica, MA, USA), which were blocked by nonfat dry milk (BD Bioscience, CA, USA) or BSA (Sigma). Anti-NAT10 mouse (1:1000), Anti-NAT10 rabbit (1:5000), anti-HIF-1α rabbit (1:1000) and anti-HIF-2α/EPAS1 rabbit (1:400) antibodies were used as primary antibodies for the assay, and incubated by the second antibody of either peroxidase-conjugated goat anti-rabbit IgG or peroxidase-conjugated goat anti-mouse IgG (Zhongshan Jinqiao Biotechnology Co., Ltd, Beijing, China), respectively. Proteins were visualized using an enhanced chemiluminescence kit (Bio-Rad, Hercules, CA, USA). β-actin (1:2000) was used as internal control. Each of the bands was quantified by optical density using the Lab-Works 4.6 software (Bio-Rad) and represented the average from three independent experiments.
Detailed immunofluorescence was performed as previously described [8]. The primary antibodies used were anti-NAT10 mouse (1:800), anti-HIF-1α rabbit (1:800), anti-EDC4/Ge-1 rabbit (1:400), and anti-DCP1A mouse (1:800). Fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (Zhongshan Jinqiao Biotechnology Co., Ltd) were used as secondary antibodies. Nuclei were counterstained with 1 μg/ml DAPI for 10 min. Images were observed and recorded using fluorescence microscope (Model CX51; Olympus, Tokyo, Japan), and the presentation of multichannel photographs (green/red/DAPI) were merged by Photoshop version 7.0 (Adobe Systems Inc.). The experiments were performed independently at least three times.

Dual-luciferase reporter assay

pGMHIF-1-Luc, containing multiple tandem-repeated HRE (hypoxia response element), was purchased from Genomeditech Inc. (Shanghai, China) and Dual-Glo® Luciferase Assay System (Promega, Madison, WI, USA) was used according to the manufacturer’s instructions. After co-transfection with pGMHIF-1-Luc and pRL-TK control vector (renilla) for 6 h, HeLa, MCF-7 or HUVEC cells were, respectively, incubated in DMEM supplemented with 10% FBS at 37 °C for 1 h. HUVECs (5 × 10^5 cells/well) were transferred to the pre-cooled 96-well tissue culture plate and solidified at 37 °C for 1 h. The firefly and renilla luciferases were measured by Luminoskan™ Microplate Luminometer (ThermoFisher Scientific), respectively. The activity of the HIF-1 reporter gene (firefly luciferase) was normalized with the activity of control reporter gene (renilla luciferase). The experiments were performed independently at least three times.

Cell migration and invasion assays

Transwell assay was assessed using 8-μm inserts (BD Bioscience). A total of 1 × 10^5 cells were suspended in 200 μl serum-free DMEM media and loaded into the upper chambers, while the lower wells were filled with 600 μl of complete medium (DMEM supplemented with 20% FBS). For invasion assay, additionally, each insert was coated with 1 mg/ml Matrigel and incubated at 37 °C for 30 min before loading the suspended cells into the upper chambers. The migration and invasion chambers were incubated for 17 h and 30 h, respectively, in a humidified incubator at 37 °C. The cells were then fixed with 4% formalin for 15 min at r. t. The inner surface of the upper chambers was wiped with cotton swabs to remove retained cells in the migration assay or to scrape the Matrigel in the invasion assay. The chambers were then washed with PBS and stained with 0.1% crystal violet for 15 min at r. t. After washing with PBS, the stained cells were counted in 5 random fields at 200× magnification, and recorded by photography. The experiments were performed independently at least three times.

Statistical analysis

All analyses were performed using Image J and GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Relationships between control and other parameters were analyzed by t tests. A P value of less than 0.05 was considered to be of statistically significance. All the statistical tests and P values were 2-sided, and P < 0.05 was considered to be statistically significant.

Results

Remodelin could inhibit hypoxia-induced expression of HIF-1α

Our previous experiment has demonstrated NAT10 is a response gene in oxidative stress or DNA damage [18]. Recently, further experiments unexpectedly revealed that Remodelin, the inhibitor of NAT10, could reduce the level of HIF-1α in hypoxia. As shown in Fig. 1a, apparently with 200 μM CoCl_2 treatment of HeLa cells, the induced
upregulation of HIF-1α was correspondingly suppressed by increasing concentration of Remodelin. Similarly, HIF-1α expression was also suppressed by Remodelin in HeLa cells cultured under hypoxic conditions (Fig. 1b). Moreover, Remodelin could inhibit the CoCl₂-induced expression of HIF-1α in different treatment ways including adding CoCl₂ and Remodelin when pre-treatment with Remodelin for 3 h, or post-treatment with CoCl₂ for 6 h, or adding both of them at the same time. Furthermore, HeLa cells were treated with 200 μM CoCl₂ or 20 μM Remodelin alone. Control: 20 μM DMSO. **** indicates that compared with 200 μM CoCl₂ group

HIF-1α in different ways of treatment, by either 3 h before, 6 h after or with simultaneous addition of 200 μM CoCl₂ (Fig. 1c).

Since hypoxia-induced over-expression of HIF-1α generally presents its nuclear accumulation, the effect of

Fig. 1 Remodelin inhibited the expression of HIF-1α in hypoxia. All the images were representatives of three times independent experiments. a Remodelin inhibited the CoCl₂ induced expression of HIF-1α in a dose-dependent manner. HeLa cells were treated with indicated concentrations of Remodelin for 36 h in 200 μM CoCl₂. b Remodelin inhibited the expression of HIF-1α in hypoxia. HeLa cells cultured under hypoxia (1% O₂) were treated with 20 μM Remodelin for 36 h. Control: original HeLa cells. **** indicates that compared with Control group or DMSO group.

d Remodelin inhibited nuclear translocation of Hif-1α in hypoxia. HeLa cells were treated with 200 μM CoCl₂ alone, or with addition of 20 μM Remodelin for 36 h and fixed with 4% formaldehyde/TritonX-100 before double staining for NAT10 (green) and HIF-1α (red). Control: 20 μM DMSO. The level of statistical significance was < 0.0001(****)
Remodelin on expression of HIF-1α was also analyzed by immunofluorescence staining. HeLa cells were subjected to treatment of CoCl2 (200 μM) with or without Remodelin (20 μM) for 36 h, and stained by double immunofluorescence. The nuclear distribution of HIF-1α was markedly accumulated in CoCl2-treated cells but not in the presence of Remodelin, even lower than that of control HeLa cells (Fig. 1d).

Therefore, these results indicated that Remodelin could inhibit the hypoxia-induced up-expression and nuclear translocation of HIF-1α protein.

Remodelin could also inhibit the constitutional expression of HIF in VHL mutant cells

HIFs could be upregulated owing to genomic mutation of VHL, which mediates degradation of HIFs in normoxia. Interestingly, some of HIFs inhibitors, such as microtubules interfering agents, could not be able to inhibit constitutional expression of HIFs in VHL-mutant cells [19]. Therefore, the inhibitory effect of Remodelin on the expression of HIF-1α caused by its disrupted degradation machinery was also investigated. 786-0 cells, from VHL-deficient renal cancer cell with high constitutional expression of HIF (especially HIF-2α), was used to the clarification. The results showed that both of HIF-1α and HIF-2α protein were significantly reduced after Remodelin treatment (Fig. 2a, b). Similarly,

Fig. 2 Remodelin inhibited the constitutional expression of HIF-1α in VHL mutant cells. 786-0 cells were treated with 200 μM CoCl2 or/and 20 μM Remodelin for 36 h, respectively. The protein levels of NAT10, HIF-1α (a) and HIF-2α (b) were determined by western blot, and β-Actin was used as internal standards. Control: 20 μM DMSO. c Remodelin reduced nuclear translocation of HIF-1α in 786-0 cells. The treated 786-0 cells were fixed with 4% formaldehyde/TritonX-100 for subsequent double staining of NAT10 (green) and HIF-1α (red). Control: 20 μM DMSO. All the experiments were repeated three times independently. The level of statistical significance was <0.01 (**) or <0.001 (***)
immunofluorescent staining proved that Remodelin could also reduce the nuclear translocation of HIF-1α in 786-0 cells regardless of whether treated with CoCl₂ or not (Fig. 2c).

The results demonstrated that the constitutional over-expression of HIFs in VHL-mutant cells could also be inhibited by Remodelin.

**Remodelin could potentially inhibit transcription activation of HIF-1α target genes in hypoxia**

The up-regulation of HIF-1α under hypoxic conditions usually forms heterodimers with HIF-1β and translocated to the nucleus, where the HIF-1 complex binds to a hypoxia response element (HRE) in up-stream regulatory sequences of target gene, resulting in activation of their transcription [20]. To clarify the effect of Remodelin on target genes of HIF-1α in transcription levels, HRE-driven luciferase assay was performed in the presence of Remodelin. After co-transfection of HRE-driven luciferase reporter and pRL-TK control vector (renilla) for 6 h in HeLa or MCF-7 cells, the transfected cells were incubated with or without 20 μM Remodelin in either CoCl₂ (200 μM) treatment or under normoxic (20% O₂) or hypoxic (1% O₂) culture for 36 h, respectively. The results showed that Remodelin significantly down-regulated HRE-driven luciferase in HeLa or MCF-7 cells under hypoxic conditions. Apparently, in HeLa cells, Remodelin could reduce HRE-driven activity down about threefold in CoCl₂–treatment (Fig. 3a), while about 1.5 fold under hypoxic culturing (Fig. 3b). Similarly, Remodelin could reduce HRE-driven activity down about threefold in MCF-7 cells cultured under hypoxia (Fig. 3c).

The results implied that Remodelin could suppress the transcriptional activity mediated by HRE in hypoxia.

**The inhibition of HIFs by Remodelin possibly through NAT10**

Remodelin is a specific inhibitor of NAT10, and it could be assumed that Remodelin might inhibit HIF expression through modulating NAT10 activity. To prove this possibility, the effects of Remodelin on HIF expression were further analyzed in NAT10 knock-down cells by interfering RNA. With treatment of CoCl₂ (200 μM) for 36 h, both of NAT10 and HIF-1α expression was significantly induced in either original HeLa or sh-control cells (Sh-C), but not in the cells with knockdown of NAT10 (Sh-NAT10) (Fig. 4a). Similarly, immunofluorescent staining also confirmed that CoCl₂ could induce nuclear translocation of HIF-1α in control cells (Sh-C) but not in depleted NAT10 cells (Sh-NAT10) (Fig. 4b).

In another hand, the treatment of CoCl₂ in either HeLa or HCT116 cells, both of NAT10 and HIF-1α expression presented significant increasing in dose-dependent way (Fig. 4c, d). Moreover, hypoxic culture could also induce time-dependently up-regulation of NAT10 and HIF-1α expression in both HeLa and LoVo cells (Fig. 4e, f). These results indicated that the activity of NAT10 could be important for induction of HIF-1α in hypoxia.

**The effects of Remodelin on NAT10 potentially influenced P-body assembly**

The regulation of HIFs has been demonstrated predominantly through their translation and post-translation. Meanwhile, it has been reported that P-body is necessary for mRNA degradation in translation of HIFs [21, 22]. Therefore, the assembly of P-body under treatment of Remodelin were analyzed through staining of DCP1A and EDC4, the key components of P-body in human cells [23, 24]. The results showed that the number of P-body increased under the treatment of Remodelin. (Fig. 5a). And interestingly, when NAT10 was knock-down by shRNA, some portion of NAT10 showed cytoplasmic bodies which co-localized with P-body (Fig. 5b), however, Cas9-edited NAT10 cells presented a loss of P-body (Fig. 5b).

The results indicated that NAT10 could be crucial for assembly of P-body, and down-activity NAT10 could enhance formation of P-body, resulting in elevated degradation of mRNA.

**Remodelin inhibited migration, invasion and tube-formation of HUVECs**

To confirm the effects of Remodelin on HIF-1α regulated cellular biological activity, HUVECs were used as an in vitro model to further explore their biological changes in angiogenesis. Expectedly, Remodelin could inhibit not only CoCl₂ (200 μM) induced up-expression of HIF-1α in HUVECs (Fig. 6a), but also CoCl₂ (200 μM) induced luciferase activity driven by HRE of HIF-1α in HUVECs (Fig. 6b). At the same time, the migration and invasion ability of HUVECs were analyzed in Transwell assays with or without Matrigel. With Remodelin treatment in different concentrations for 36 h, the migration and invasion ability of HUVECs were significantly inhibited in dose-dependent, with compared to untreated cells (Fig. 6c, d). Further, in vitro tube-formation assays showed that under either normoxic or hypoxic condition, Remodelin treatment could significantly reduce the number of meshes in a dose-dependent manner, to more great extent in hypoxic condition (Fig. 6e, f).

The results indicated that Remodelin could inhibit the angiogenetic potential of HUVECs through inhibition of HIF-1α and its target response.
Remodelin could suppress the growth of 786-0 cells

Several investigations have confirmed that Remodelin could suppress growth of cancer cells in vitro and in vivo [13, 15]. To further determine whether Remodelin treatment would suppress the growth of VHL-mutant 786-0 cells, the cells were grown in the presence of Remodelin with increasing concentrations for 76 h. And the results showed that as the dose of Remodelin increases, the growth of 786-0 cells was inhibited markedly, and at 20 μM the growth rate was just about 50% of the control (Fig. 7a). And time course showed that the growth of 786-0 cells was inhibited in the presence of Remodelin but not cytotoxic (Fig. 7b). These indicated that Remodelin could inhibit the growth of VHL-mutant 786-0 cells.

Discussion

It has been well established that HIF-1α is an important transcription factor that specifically activates during hypoxia [6, 7]. The regulation of HIF-1α level seems to be dependent on proteasome degradation machinery. Under well-oxygenated conditions, HIF-1α rapidly degraded by hydroxylation and bound to the von Hippel-Lindau (VHL) protein, which recruits an ubiquitin ligase that targets HIF-1α for
proteasomal degradation. In hypoxia, the degradation is relieved by downregulated enzyme-mediated hydroxylation of HIF-1α, and consequently the HIF-1α subunit becomes stable, accumulates, and translocates to the nucleus, regulating the expression of target genes. Actually, CoCl₂ could mimic hypoxia by preventing the degradation of HIF-alpha through occupying the VHL-binding domain of HIF-alpha [25]. It seems that the expression level of HIFs is largely regulated through post-translation mechanism. NAT10 has been demonstrated to regulate many respects of protein translation: ribosome biogenesis, acetylation of tRNA and mRNA [26–28]. Therefore, Remodelin, an inhibitor of NAT10, its inhibition of HIFs could also be credited to acting at translation level. In fact, our other data from knockdown or inhibition of showed that depletion of NAT10 could not affect the transcription of HIFs (unpublished data). In addition, the described experiments also revealed that both of NAT10 and HIF could be simultaneously induced in oxidative stress and hypoxia, suggesting both could have a close mutual relationship.

It has been considered that Remodelin could inhibit NAT10 activity, which in turn induce redistribution of microtubules through its acetylation activity [12]. Since many agents targeting tubulin could suppress the level of HIFs [29], it could be simple that Remodelin act as a tubulin interfering agent to inhibit the expression of HIFs. However, general tubulin targeting agents could not be able to inhibit up-regulation in VHL-mutant cells [30], while Remodelin could also suppress the constitutional expression of HIF-1α or HIF-2α. Therefore, it seems that Remodelin is not simply inhibit the expression of HIFs through disruption of tubulin dynamics. Tubulin targeting agents could disrupt microtubules and cause mRNA of HIFs traffic to P-body, resulting in translation repression. But, with treatment of Remodelin or knock-down of NAT10 by sh-RNA could increase formation of P-body. Much interestingly, the cells with knock-down of NAT10 by sh-RNA, NAT10 showed localization with P-body. These results indicating that NAT10 could take part in regulation of P-body assembly to influence translation activity. Nevertheless, the specific mechanism of how Remodelin inhibits HIFs is still worthy of further investigation.

Consistent with other researches our described investigation also revealed that Remodelin could inhibit the growth of many kinds of cancer cells, however, the inhibition of expression HIFs could not be credited to its
Fig. 5 The effects of chemical inhibition or genetic knock-down of NAT10 on assembly of P-body. 

(a) HeLa cells were treated with 20 μM Remodelin for 36 h, the treated cells were fixed with 4% formaldehyde/TritonX-100 for double staining of DCP1A (green) and EDC4 (red). 

(b) HeLa cells, or treated with 20 μM Remodelin for 24 h, shRNA-specific knockdown of NAT10 (sh-NAT10), and Cas9-edited NAT10 cells (Cas9-NAT10) were fixed with 4% formaldehyde/TritonX-100 for subsequent double staining of NAT10 (green) and EDC4 (red). All the experiments were repeated three times independently.
Fig. 6 The effects of Remodin on expression of HIF-1α, or its response genes, and biological activities of HUVECs. The experiments were performed as described in Materials and Methods. a Remodin inhibited CoCl₂-induced up-regulation of HIF-1α in HUVECs. HUVECs were treated as indicated ways for 36 h, respectively. b Remodin inhibited hypoxia (1% O₂) induced targets response of HIF-1α in HUVECs. c Remodin inhibited migration of HUVECs in dose-dependent. The treated HUVECs were subjected to non-matrigel migration assays. d Remodin dose-dependently inhibited invasion of HUVECs. The treated HUVECs were subjected to matrigel invasion assays. e Remodin inhibited dose-dependently tube-formation of HUVECs under normoxic conditions. f Remodin inhibited dose-dependently tube-formation of HUVECs under hypoxic (1% O₂) conditions. The treated HUVECs were subjected to in vitro tube-formation assays for 12 h. Images (left panels) show the in vitro tube-formation, and the results were quantitative plotted (right panels). All the experiments were repeated three times independently. NS, *, **, *** represents $P > 0.05$, $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

suppression of cell growth. Since apparently, with treatment Remodin for 24 h at the concentration of 20 μM was not able to significantly reduce cell viability in 24 h (Fig. 7b). In addition, the inhibition of HIFs could be achieved by addition of Remodin in either pre-, post- or simultaneous way (Fig. 1b). Moreover, 10 μM of Remodin could reduce the expression of HIFs as the same extend as 20 μM or beyond (Fig. 1a). All of the results indicated that Remodin could suppress cell growth but not induce cell death in cytotoxic. More worth mentioned, the dual effects of Remodin in inhibition of cell growth and expression of HIFs could be potentially suitable to cancer treatment, not only acting to cancer cells but also to TME, resulting in their less progression.

In summary, the described investigation proved Remodin could reduce the expression of HIFs possibly through inhibition of NAT10 activity associated protein translation. And the results also implied that Remodin would be potentially utilized in clinical to anti-angiogenesis in inflammatory or neoplastic diseases. And especially, Remodin would be a special therapeutic agent for VHL-mutant cancers to suppress the constitutional expression of HIFs. With respect to its nature compound with little cytotoxic effects, Remodin could be potentially suitable for long term or in vivo uses.
Conclusions

The results of this study demonstrated that Remodelin could significantly inhibit HIFs expression induced by hypoxia or constitutional activation, and also inhibit HIFs-associated angiogenesis, indicating that Remodelin should be a potential drug for tumor treatment.

Patent

The patent on Applications of NAT10 inhibitor in the preparation of drugs for inhibiting the expression of HIFs (CN2019104554042) is under substantive examination.

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Compliance with ethical standards

Conflicts of interest All authors declare that they have no competing interests.
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