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GL331 inhibits HIF-1α expression in a lung cancer model☆

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Abstract

We have studied GL331's anti-cancer mechanisms by studying their effect on the tumor-induced angiogenesis. Human lung adenocarcinoma CL1-5 cells were treated with GL331 and then maintained in serum-reduced, GL331-free medium for the preparation of condition mediums. These condition mediums were tested for their capability to induce in vitro angiogenesis, i.e., HUVEC tube formation and migration. We found that mediums generated from GL331-treated CL1-5 cells presented reduced ability of inducing in vitro angiogenesis. Western blot analyses showed that both VEGF and HIF-1α were down-regulated in GL331-treated CL1-5 cells. Northern blot and EMSA analyses showed that GL331 down-regulated HIF-1α expression without decreasing the stability of HIF-1α mRNA, and that GL331 decreased the binding of CL1-5-derived nuclear components to the promoter of HIF-1α gene. Therefore, our data showed that GL331 is a potent inhibitor of tumor-induced angiogenesis. The underlying mechanisms might involve at least the inhibition of HIF-1α expression, probably through transcriptional repression.

Keywords: GL331; HIF-1α; HUVEC; Angiogenesis

Cancer is a leading cause of death in the world. Traditional cancer treatments include chemotherapy and radiotherapy. Although new therapeutic strategies such as immunotherapy and gene-targeted therapy have been proposed to treat cancers [1], chemotherapy is by far the major fighting force against most of cancers. To improve the efficacy of chemotherapy, numerous compounds have been isolated from nature or synthesized in laboratories. Studies of the anti-cancer capacity of new compounds will reveal the mechanisms that make them effective in treating cancers, which will in turn provide valuable information for further modification of the parental compounds into more potent derivatives.

GL331 is a semisynthetic compound derived from a plant toxin podophyllotoxin [2,3]. GL331 showed strong inhibitory effect on the growth of several cultured cell lines derived from cervical, gastric, hepatocellular, nasopharyngeal, and lung cancer types [4]. GL331 shares many structural and biochemical properties with those of etoposide (VP-16) which is the drug of choice for cancers such as lymphoma, leukemia, testicular carcinoma, small cell lung cancer, non-small cell lung cancer, breast, and other malignancies [5–7]. Interestingly, GL331 was found to be effective in killing cancer cells resistant to VP-16 treatment [8]. The phase I clinical trial of GL331 has defined the maximal tolerated dose as 300 mg/m^2; and a phase II clinical trial protocol has been proposed for lung cancer.

The information regarding the mechanisms underlying GL331's anti-cancer activities has been limited. It was found that GL331 was able to cause DNA damage via the inhibition of topoisomerase II (Topo II); binding of GL331 to Topo II prevented the enzyme from rejoining the transient double-stranded DNA breaks, resulting in DNA damage [7]. It was found that GL331-induced DNA breaks initiated an inappropriate poly (ADP-ribose)polymerase (PARP) and in turn caused an extensive poly(ADP-ribosyl)ation of nuclear proteins

☆ *Abbreviations:* HIF-1α, hypoxia-inducible factor-1α; PARP, poly(ADP-ribose)polymerase; EMSA, electrophoretic mobility-shift assay; HRE, hypoxia-response element; ERK, extracellular signal-regulated kinase; VEGF, vascular endothelial growth factor.

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that eventually lead to cell death [9]. GL331 was also found to facilitate the association of CDC 25A phosphatase with Raf-1, causing the activation of CDC 2 kinase, and resulting in apoptotic death in a human nasopharyngeal cancer cell line, NPC-TW01 [10]. Recently, GL331 was reported to block the extracellular signal-regulated kinase (ERK) signaling pathway, which contributed to the depletion of cyclin D1, hence, the inactivation of the cyclin-dependent kinase 4 (CDK4) and cell growth arrest in a human lung adenocarcinoma cell line [11].

In this report, we continued to elucidate the anti-cancer mechanisms of GL331. We used a human lung cancer cell line, CL1-5 [12], to study GL331's inhibitory effect on the tumor-induced angiogenesis. We observed that GL331-treated CL1-5 cells were not able to induce in vitro angiogenesis, which was accompanied by the finding that GL331 reduced the expression of vascular endothelial growth factor (VEGF) in both normoxic and hypoxic CL1-5 cultures. Finally, we identified the HIF-1α as a target of GL331, which revealed an important part of GL331's anti-cancer mechanisms.

Materials and methods

Cell culture. Human lung adenocarcinoma CL1-5 cells were maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin. HUVECs were isolated from umbilical cord veins as described [13] and maintained in EGM medium (Clonetics). Both CL1-5 and HUV ECs were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. HUVECs grown before the sixth passage were used for studies.

In vitro endothelial capillary tube formation assay. Mediums used in capillary tube formation assays were prepared as described below. After treatments, CL1-5 cells were washed with phosphate-buffered saline (PBS) and then cultured in fresh RPMI 1640 medium (containing 1% FBS) free of GL331, under either normoxic or hypoxic (2% O_2) condition for 24 h, and then the mediums were collected. The tube formation assays were performed using angiogenesis assay kit (Chemicon International, Temecula, California) following the protocol provided by the manufacturer. Briefly, 50 μl of ECMatrix gel solution was placed into each well of a 96-well culture plate on ice and allowed to polymerize by incubation at 37 °C. HUVECs (5×10^4) were seeded on the gel in 150 μl of condition medium. The cells were incubated at 37 °C for 16 h under normoxic condition. Three different phase-contrast microscopic high power fields (HPF, 100×) per well were photographed. The total length of capillary tubes in each photograph was measured using a scale ruler.

HUVEC migration assay. The migration activity of HUVECs was determined using the growth factor-reduced Matrigel invasion system (Becton Dickinson) following the protocol provided by the manufacturer. Briefly, HUVECs (5×10^4) were suspended in 0.5 ml of condition medium and added to the upper chamber. The upper chamber was lodged into the lower chamber containing 0.75 ml of condition medium. After incubating at 37 °C for the time as indicated, the cells in the upper side of the filter membrane were removed with cotton swabs. The membranes were then soaked in the fixation solution containing 4% of paraformaldehyde for 10 min. Cells that migrated to the lower side of the membranes were stained with Liu stain (Handsel Technologies, Taipei, Taiwan). The stained cells were counted in three fields under a 200× high power field. Photographs were taken by a microscope video system.

Western blot analysis. Total cell lysate was prepared by the method as described [14]. Aliquots (40 μg) of cell lysates were separated on 12% SDS–polyacrylamide gels and electrotransferred onto polyvinylidene membranes (Amersham Pharmacia). After blocking with PBST (PBS plus 0.1% Tween 20) plus 5% non-fat milk, the blots were incubated with indicated antibody and the signals were obtained by enhanced chemiluminescene (ECL, Amersham Pharmacia).

Northern blot analysis. Total RNA was isolated from CL1-5 cells using Trizol (Life Technologies) according to the protocol provided by the manufacturer. Twenty μg of total RNA was resolved in a 1% agarose gel containing 6.7% formaldehyde and then transferred to the nylon membrane. DNA probes were labeled by redi prime II random priming system following the manufacturer's protocol (Amersham Pharmacia). Hybridization was performed as described [15]. Signals were visualized and quantitated by PhosphorImager.

Gel-shift analysis. Electrophoretic mobility-shift assays (EMSA) were performed to detect the binding of cellular proteins to the HRE (CCACACTGCATACGTGGGCTCCAACA) derived from the promoter of VEGF gene and to the promoter region of HIF-1α gene. CL1-5 cells were fractionated into nuclear and cytoplasmic fractions as described previously [15]. A 685-bp DNA fragment in the promoter region of HIF-1α gene was prepared by polymerase chain reaction (PCR). The 5′ and 3′ primers were GATCATGGTTAGGTAATC TGG and TCCTCAGGTGGCTTGTCAG, respectively. This DNA fragment was radiolabeled by the redi prime II random priming system following the manufacturer's protocol (Amersham Pharmacia). The HRE DNA was 3′ end-radiolabeled by polynucleotide kinase. Five-μg aliquots of nuclear and cytoplasmic fractions were incubated with 50,000 cpm of DNA probe at room temperature for 20 min and then electrophoresed through 6% non-denaturing polyacrylamide gels containing 0.25× Tris–borate–EDTA (TBE) at 150 V for 1.5 h at 4 °C. Gels were dried and radioactive signals were visualized with a PhosphorImager.

Results

Gl331 decreased CL1-5 cell's capability of inducing capillary tube formation and HUVEC migration

To determine if GL331 affected the tumor-induced angiogenesis, we performed in vitro endothelial capillary tube formation and HUVEC migration assays. CL1-5 cells were either left untreated or treated with GL331 (10 μM) for 24 h under normoxic condition in regular RPMI 1640 medium (containing 10% FBS), and were used to prepare for the condition mediums under normoxic or hypoxic condition as described in Materials and methods. We found that the serum-reduced condition medium generated from untreated CL1-5 cells was able to induce the formation of endothelial capillary tubes (Fig. 1A). Medium generated from hypoxia-treated CL1-5 cells showed a slightly decreased ability of inducing capillary tube formation (Figs. 1B and E). On the other hand, compared with that of untreated cells, the medium generated from GL331-treated CL1-5 cells suffered 88% loss of its ability of inducing capillary tube formation (Figs. 1C and E); and this ability was entirely blocked in the medium from GL331-treated and then hypoxia-treated CL1-5 cells (Figs. 1D and E). These

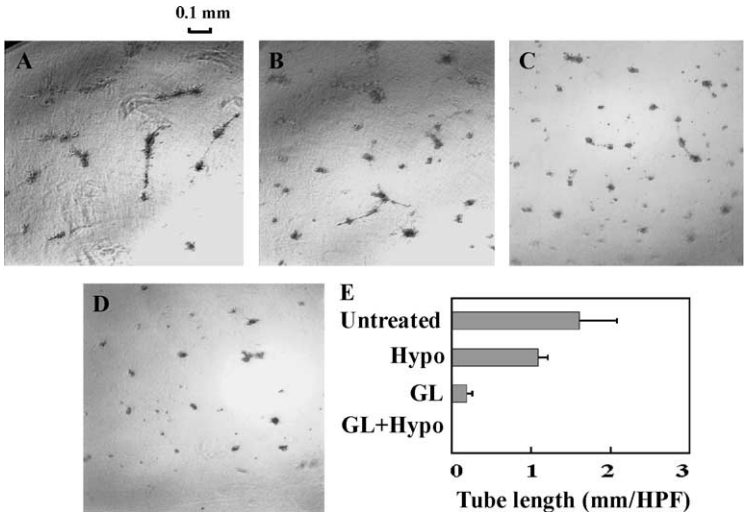

Fig. 1. Effect of GL331 on the CL1-5-induced tube formation by HUVEC. CL1-5 cells were either left untreated or treated with GL331 (10 μM) for 24 h. The procedures for the preparation of condition mediums and the measurement of capillary tubes are as described in Materials and methods. Photographs showed the tube formation by HUVECs cultured in the condition medium generated from (A) untreated cells, (B) hypoxia-treated cells (Hypo), (C) GL331-treated cells (GL), and (D) GL331-treated and then hypoxia-treated cells (GL + Hypo). As shown in (E), the length of capillary tubes was measured as described in Materials and methods. Each bar represents the means ± SD ($n = 3$). HPF, high power field.

data suggested that GL331 is a potent anti-angiogenic agent. Based on these data, we examined the impact of GL331 on the HUVEC migration activity (Fig. 2A). We found that the HUVECs, cultured in the condition mediums generated from untreated CL1-5 cells, efficiently migrated through the filter membrane (33.4 ± 4.5 cells/HPF 200×); however, in the condition medium generated from GL331-treated CL1-5 cells, the HUVEC migration was decreased (9.8 ± 1.6 cells/HPF 200×) (Fig. 2B).

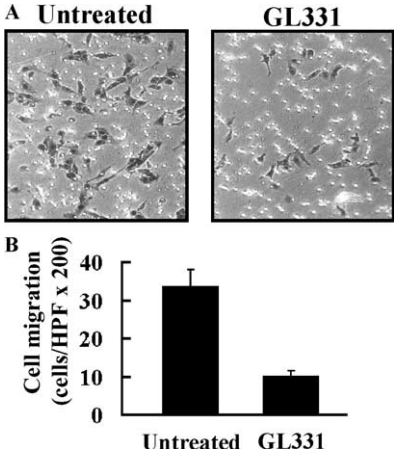

Fig. 2. Effect of GL331 on the CL1-5-induced HUVEC migration through Matrigel-coated filter. CL1-5 cells were either left untreated or treated with 10 μM of GL331 for 24 h. The preparation of condition mediums and the measurement of in vitro HUVEC migration are as described in Materials and methods. (A) HUVECs migrated through filter were stained. (B) The stained HUVECs were counted as described in Materials and methods. Each bar represents the means ± SD ($n = 3$).

Gl331 down-regulated the expression of VEGF in CL1-5 cells

The results of in vitro angiogenesis assays indicated the possibility that the condition mediums from GL331-treated CL1-5 cells might be lack of growth factor(s) required for angiogenic induction. Therefore, we examined if GL331 down-regulated the expression of VEGF in CL1-5 cells. After collecting the condition mediums for capillary tube formation assays, the remaining CL1-5 cells were subjected to Western blot analyses for their cellular levels of VEGF. As shown in Fig. 3, the untreated cells expressed the basal level of VEGF and hypoxia slightly increased VEGF production. On the other hand, GL331 treatment reduced the expression of VEGF and also blocked the stimulatory effect of hypoxia on VEGF expression.

GL331 inhibited the expression of HIF-1α

GL331's inhibitory effect on the hypoxia-induced VEGF expression led us to examine the impact of

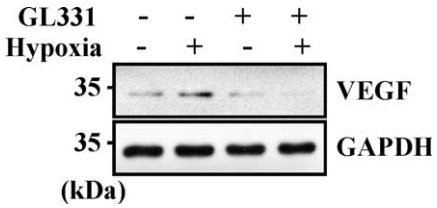

Fig. 3. GL331 decreased VEGF expression. CL1-5 cells were either left untreated or treated with 10 μM of GL331 for 24 h and then maintained in GL331-free, low serum medium (1% FBS) for 24 h under either normoxic or hypoxic condition. Cells were then subjected to Western blot analysis for VEGF.

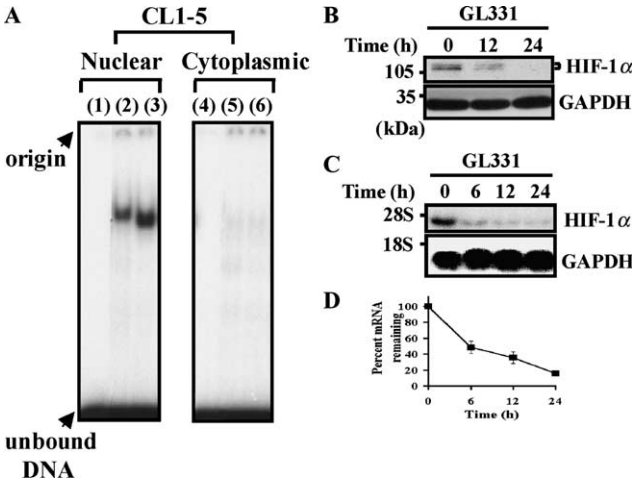

Fig. 4. GL331 affected the binding activity of HIF-1 and the expression of HIF-1α. CL1-5 cells were either left untreated or treated with 10 μM of GL331 for 24 h, and were used to prepare whole-cell lysates, or were further fractionated into nuclear and cytoplasmic components. (A) Binding assays. The nuclear and cytoplasmic fractions were subjected to EMSA analysis to detect their interactions with HRE. Lanes 1 and 4, HRE probe only; lanes 2 and 5, HRE plus lysates of untreated cells; lanes 3 and 6, HRE plus lysates of GL331-treated cells. (B) Protein analysis of HIF-1α. The whole-cell lysates were subjected to Western blot analysis to detect the HIF-1α protein. (C) RNA analysis of HIF-1α. Northern blot analyses were performed to detect the expression of HIF-1α mRNA in CL1-5 cells exposed to GL331. (D) The HIF-1α signals were quantitated and normalized to those of GAPDH. Data represent means ± SD of three separate experiments.

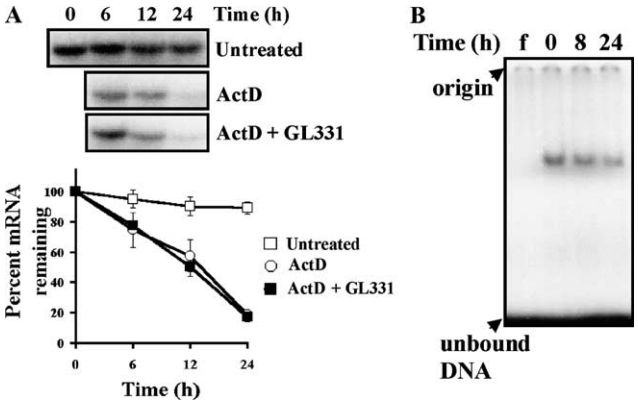

Fig. 5. GL331 did not enhance the degradation of actinomycin D-resistant HIF-1α mRNA, but decreased the interaction of cellular proteins with the promoter of HIF-1α gene. (A) CL1-5 cells were either left untreated or treated with actinomycin D (1 μg/ml) or combined GL331 (10 μM) with actinomycin D for the time periods indicated. Total RNA was then isolated and subjected to Northern blot analysis for the detection of HIF-1α mRNA. The signals were quantitated and normalized to those of GAPDH. Data represent means ± SD of three separate experiments. (B) Cells were either left untreated or treated with GL331 (10 μM) for the time periods indicated and were subjected to the preparation of nuclear components. EMSA analyses were performed to demonstrate the binding activity of these nuclear components to a 685-bp DNA fragment derived from the promoter of HIF-1α gene. In this experiment, after the reaction mixtures were incubated at room temperature for 20 min, DNase I (1000 U/reaction mixture) was added and incubated at 37 °C for another 20 min. f, Radiolabeled DNA digested with DNase I without incubation with cell lysate.

GL331 on the DNA-binding activity of HIF-1, a transcription factor up-regulating VEGF expression in hypoxic culture. We carried out gel-shift analyses to show the complex formation between the cellular components and radiolabeled HRE derived from the promoter of VEGF gene. As shown, only the nuclear component of CL1-5 cells bound HRE, whereas GL331 treatment changed the migration rate of the protein–DNA complexes (Fig. 4A). These data suggested that GL331 might affect the activity of HIF-1 protein. Therefore, we further examined the GL331's effect on the expression of HIF-1α. Western blot analyses showed that 10 μM of GL331 significantly decreased the cellular level of HIF-1α in 12 h. After 24 h of treatment, HIF-1α was not detectable (Fig. 4B). Consistently, Northern blot analyses showed that GL331 caused a 50% loss of HIF-1α mRNA in 6 h and an 80% loss in 24 h (Figs. 4C and D). These data indicated HIF-1α as a target of GL331.

To determine if GL331 enhanced the degradation of HIF-1α mRNA in CL1-5 cells, we used actinomycin D to block RNA synthesis and then measured the half-life of remaining HIF-1α mRNA in cells with or without GL331 treatment. As shown, GL331 did not increase the turnover rate of HIF-1α mRNA (Fig. 5A). In addition, we examined the interaction of cellular proteins with the promoter of HIF-1α gene. EMSA assays showed that GL331 decreased the protein–DNA complex formation, in a time-dependent manner (Fig. 5B). Taken together, our data suggested that GL331 down-regulated HIF-1α expression at least by transcriptional repression.

Discussion

GL331 is a compound that showed strong inhibitory effect on the growth of several cultured cell lines derived from cervical, gastric, hepatocellular, nasopharyngeal, and lung cancer types [4], and is currently under clinical evaluation. For a better understanding of GL331's anti-cancer activity, in the present study, we explored its inhibitory effect on the tumor-induced angiogenesis. Considering that the cytotoxicity of GL331 might directly inhibit the growth and function of endothelial cells, which might decrease the in vitro endothelial capillary tube formation, resulting in false positive results regarding GL331's anti-angiogenesis effect, we took an indirect approach to address GL331's anti-angiogenic capability. In our in vitro angiogenesis experiment, cancer cells, instead of HUVECs, were treated with GL331. Therefore the functions of HUVECs were exempted from the direct impact of GL331's cytotoxicity. Meanwhile, we incubated CL1-5 cells in low serum

medium (for collecting condition mediums) so that the stimulating effects of the medium-born growth factors on CL1-5 cells could be minimized. Under these experimental settings, we showed that the condition medium generated from GL331-treated CL1-5 cells was not able to induce the endothelial capillary tube formation and migration (Figs. 1 and 2), and that GL331 decreased the expression of VEGF in CL1-5 cells (Fig. 3). VEGF has been found as a survival factor for endothelial cells. It binds to its cognate receptors which are located exclusively on endothelial cells and drives angiogenesis [16]. It is noteworthy that 24-h of GL331 treatment caused growth arrest in CL1-5 cells without significant cell death [11, and data not shown]. Taken together, our data indicated that 24-h of GL331 treatment deprived the CL1-5 cells of their ability to induce angiogenesis and that down-regulation of VEGF is another important pharmacological property of GL331. Since angiogenesis is critical for the survival and expansion of a tumor, inhibition of the tumor-induced angiogenesis well represents an important part of GL331's anti-cancer capacity.

Our findings did not exclude the possibility that GL331 might also down-regulate the expression of the other angiogenic factors such as epidermal growth factor (EGF) and its counterpart receptor (EGFR) of CL1-5 cells to achieve its anti-angiogenic effects. Recent study of ZD1839 (Iressa), a potent growth inhibitor of several cancer cell types, showed that blockade of EGFR signaling could inhibit EGF-induced expression of VEGF and interleukin 8 in cancer cells inhibited the migration of HUVECs cocultured with EGF-stimulated cancer cells and blocked tumor angiogenesis [17]. Therefore, GL331 might also affect the EGF–EGFR signaling to modulate the growth of CL1-5 cells as well as CL1-5-induced angiogenesis. On the other hand, our effort to elucidate the mechanisms underlying GL331's inhibitory effect on the tumor-induced angiogenesis, especially the hypoxia-induced VEGF expression, has helped us identify HIF-1α as a target of GL331 (Figs. 4 and 5). HIF-1α1 is the rate-limiting subunit of HIF-1 which is the major transcription factor that controls the cellular hypoxic response. HIF-1 can transactivate genes such as VEGF, erythropoietin, glucose transporters 1 and 3, and hexokinases (for review, see [18]), mediating hypoxia-triggered cellular adaptive responses such as angiogenesis and glycolysis to enhance the survival of cells under hypoxic stress. So then, through the finding of HIF-1α as a target of GL331, we reported that GL331 was able to sabotage cellular adaptive responses to hypoxia, which represented an important mechanism underlying GL331's inhibitory activity on tumor-induced angiogenesis. In advance, we showed that GL331 down-regulated the expression of HIF-1α mRNA without decreasing its stability (Fig. 5) and that GL331 inhibited the binding of cellular components to the promoter of HIF-1α gene. Taken together with the findings that GL331 could selectively inhibit ERK activity [11], and that ERK pathway could regulate the transcriptional activity of HIF-1α, our data suggested that GL331 might inhibit the expression of HIF-1α by transcriptional repression, at least through the inhibition of ERK pathway.

In summary, through studying the upstream regulator(s) mediating GL331's inhibitory effect on the tumor-induced angiogenesis in vitro, we expanded the spectrum of GL331's action to include the expression of VEGF and HIF-1α. Our study revealed that by down-regulating HIF-1α expression, GL331 could decrease the transcriptional activity of HIF-1, and consequently, deprived the tumor cells of adaptive mechanisms that would otherwise enable them to survive hypoxic stress. Therefore, blockade of tumor-induced angiogenesis represents an important part of GL331's anti-cancer capacity. On the other hand, little is known about the transcriptional regulation of HIF-1α. We demonstrated that GL331 might also serve as a tool for us to study the transcription of HIF-1α in response to extracellular stress.

Acknowledgments

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