Gramicidin A Blocks Tumor Growth and Angiogenesis through Inhibition of Hypoxia-Inducible Factor in Renal Cell Carcinoma

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Abstract

 Ionophores are hydrophobic organic molecules that disrupt cellular transmembrane potential by permeabilizing membranes to specific ions. Gramicidin A is a channel-forming ionophore that forms a hydrophilic membrane pore that permits the rapid passage of monovalent cations. Previously, we found that gramicidin A induces cellular energy stress and cell death in renal cell carcinoma (RCC) cell lines. RCC is a therapy-resistant cancer that is characterized by constitutive activation of the transcription factor hypoxia-inducible factor (HIF). Here, we demonstrate that gramicidin A inhibits HIF in RCC cells. We found that gramicidin A destabilized HIF-1α and HIF-2α proteins in both normoxic and hypoxic conditions, which in turn diminished HIF transcriptional activity and the expression of various hypoxia-response genes. Mechanistic examination revealed that gramicidin A accelerates O2-dependent downregulation of HIF by upregulating the expression of the von Hippel–Lindau (VHL) tumor suppressor protein, which targets hydroxylated HIF for proteasomal degradation. Furthermore, gramicidin A reduced the growth of human RCC xenograft tumors without causing significant toxicity in mice. Gramicidin A–treated tumors also displayed physiologic and molecular features consistent with the inhibition of HIF-dependent angiogenesis. Taken together, these results demonstrate a new role for gramicidin A as a potent inhibitor of HIF that reduces tumor growth and angiogenesis in VHL-expressing RCC.

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Introduction

Kidney cancer is a relatively rare but deadly disease that is among the top 10 causes of cancer-related deaths in men in the United States (1). Most kidney tumors are classified as renal cell carcinomas (RCC) and are highly therapy-resistant (2–4). RCC is actually a histologically heterogeneous group of several distinct tumor subtypes that originate from the epithelial cells of the renal tubule. Each subtype, including clear cell RCC (ccRCC; 70%), papillary RCC (pRCC; 10%–15%), chromophobe (5%), and collecting duct (<1%), is associated with unique morphologic and genetic characteristics (3).

RCC characteristically exhibits molecular and biochemical features associated with chronic responses to low oxygen (hypoxia; ref. 4). Adaptation to hypoxia is mediated by an O2-sensitive transcription factor known as hypoxia-inducible factor (HIF; ref. 4), and accumulated genetic, clinical, and experimental evidence suggests that constitutive (i.e., O2-independent) activation of HIF plays a causal role in the development and progression of RCC (4, 5). In normoxic conditions, the α-subunit of HIF (HIF-α) is rapidly hydroxylated at specific proline residues within the oxygen-dependent degradation domain (ODD) by prolyl-4-hydroxylase domain-containing protein 2 (PHD2; ref. 4). Hydroxylation of HIF-α creates a binding interface for the von Hippel–Lindau tumor suppressor protein (VHL) that serves as the substrate recognition component of an E3 ubiquitin ligase complex that promotes the polyubiquitination and subsequent proteasomal degradation of HIF-α (4). Conversely, reduced O2 in hypoxic conditions prevents the hydroxylation/degradation of HIF-α (4). Stabilized HIF-α dimerizes with its β-subunit (HIF-β) and activates various target genes that collectively govern a wide array of processes relevant to cancer development and progression, most notably angiogenesis and metabolism (6). Targeted therapies that block the action of proangiogenic growth factors and their receptors on endothelial cells (e.g., sunitinib, sorafenib, bevacizumab, etc.) are now routinely used for patients with ccRCC, and have succeeded in increasing progression-free survival and quality of life. However, these agents typically fail to achieve durable remission in most
cases (7), and little is known about their utility for non-ccRCC subtypes as these patients were excluded from clinical trials (8).

Another antiangiogenesis therapeutic strategy is to target HIF directly, and several points of regulation have been exploited to develop novel HIF-inhibiting agents. These drugs include (i) mTOR inhibitors (rapamycin, temsirolimus, and everolimus) that interfere with the translation of HIF-α subunit transcripts; (ii) histone deacetylase inhibitors, HSP90 inhibitors, and nonsteroidal anti-inflammatory drugs that enhance HIF-α subunit protein degradation; (iii) anthracyclines (doxorubicin and daunorubicin) and DNA-intercalating agents (echinomycin) that interfere with the binding of HIF to DNA; and (iv) dimerization inhibitors that block the binding of HIF-α subunits with HIF-β (6, 9–12). All of these agents are in various stages of preclinical development, clinical trials, or clinical use.

Ionophores are lipophilic molecules that render membranes permeable to specific cations and are classified as mobile carriers and channel formers. These drugs are potent antibiotics and are used in veterinary medicine and as feed additives for agriculture (13, 14). Mobile-carrier ionophores are known to exhibit broad-spectrum anticancer activities and are capable of overcoming drug resistance, improving chemo- and radiosensitization, and inhibiting oncogenic signaling (13, 15, 16). Accumulated research has now demonstrated that ionophores are not simply nonspecific cytotoxic chemicals, but are also capable of working at multiple levels to selectively disrupt cancer cell growth and survival (17).

In contrast with the mobile carriers, use of channel formers as antitumor agents has not been widely evaluated. Gramicidin A is a prototypical channel-forming ionophore that forms a 4-Å pore that can accommodate water, protons, and monovalent cations. Channel formation results in Na⁺ influx, K⁺ efflux, osmotic swelling, and cell lysis in biologic systems (18, 19) and confers gramicidin A with potent antibiotic activity against gram-positive bacteria, fungi, and protozoa (20, 21). We have previously demonstrated that gramicidin A is toxic to RCC cells and induces metabolic dysfunction and cellular energy depletion (22). In this study, we investigated whether treatment with gramicidin A specifically affects HIF in RCC cells. We found that gramicidin A destabilizes HIF-1α and HIF-2α in both normoxia and hypoxia leading to reduced HIF transcriptional activity and loss of target gene expression. We determined that gramicidin A accelerates the O₂-dependent degradation of HIF-α subunits via upregulation of the VHL tumor suppressor protein. Furthermore, we show that in vivo administration of gramicidin A reduces the growth and angiogenesis of VHL-expressing RCC cell line tumor xenografts without significant toxicity in mice. To our knowledge, this is the first time that an ionophore has been reported to (i) specifically inhibit HIF-dependent hypoxia responses, and (ii) specifically upregulate a tumor suppressor (VHL). Our results reveal an additional “targeted” role for gramicidin A as a potent inhibitor of HIF and suggest its utility as an antiangiogenic therapeutic agent for RCC tumors that express wild-type VHL.

Materials and Methods

Cell culture

Human ccRCC (A498, 786-O, SN12C, Caki-1, UMRC6, and UMRC6 + VHL), pRCC (ACHN), and embryonic kidney (HEK293T) cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 25 U/mL penicillin, and 25 μg/mL streptomycin. For hypoxia experiments, we cultured the cells in a HERAcell 150 tri-gas cell incubator (Thermo Fisher Scientific) with a regulated environment of 1% O₂, 5% CO₂, and 94% N₂ at 37°C. Of note, 786-O, Caki-1, and HEK293T cells were purchased from the American Type Culture Collection in 1995. A498, SN12C, and ACHN cells were kindly provided by Dr. Charles L. Sawyers (Memorial Sloan-Kettering Cancer Center, New York City, NY) in 2005 (23). UMRC6 and UMRC6 + VHL cells were kindly provided by Dr. Michael I. Lerman (National Cancer Institute, Bethesda, MD) in 2000 (24). All cell lines obtained from investigators have been authenticated before use.

Reagents

The following chemicals were purchased from Sigma-Aldrich; gramicidin A, monensin, valinomycin, calcimycin (A23187), MG132, and cobalt chloride.

Antibodies

We purchased primary antibodies specific for HIF-1α (BD Biosciences), HIF-2α, CA-IX (carbonic anhydrase IX; Novus Biologicals), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), α-tubulin, HA (Cell Signaling Technology), GLUT-1 (glucose transporter 1; Alpha Diagnostic International), β-actin (Sigma-Aldrich), and VHL (EMD Chemicals). Horseradish peroxidase (HRP)–conjugated secondary antibodies were purchased from Cell Signaling Technology.

Plasmids and transfections

Plasmids pGL2-HRE-luciferase (Addgene plasmid 26731; ref. 25), pcDNA3-ODD-luciferase (Addgene plasmid 18965; ref. 26), pcDNA3-HA-HIF1α (Addgene plasmid 18949; ref. 27), and pcDNA3-HA-HIF1α-P402A/P564A (Addgene plasmid 18955; ref. 28) were purchased from Addgene. Plasmid pcDNA3 vector was purchased from Life Technologies and plasmid phRL-renilla was purchased from Promega Corporation. Transient transfections were accomplished using lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Transfection of Caki-1 cells with td-Tomato-N1 (Clontech) was accomplished by electroporation with a Nucleofector II (Lonza) using Kit V according to the manufacturer’s instructions. Cells were examined using a Leica DMI microscope (Leica Microsystems) and single cells expressing red fluorescent protein were picked after 2 weeks of
selection with 800 µg/mL G418 (Geneticin) to establish stable cell lines. These cells were used for in vivo studies.

**Immunoblot analysis**

Cell lysates were prepared in a buffer containing 95 mmol/L NaCl, 25 mmol/L Tris pH 7.4, 0.5 mmol/L EDTA, and 2% SDS. Tumor lysates were prepared by mincing tumor samples and then lysing in a buffer containing 150 mmol/L NaCl, 20 mmol/L Tris pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 1% IGE-PAL (octylphenoxypolyethoxyethanol), 1 mmol/L β-glycerol phosphate, 1 mmol/L Na₃VO₄, 2.5 mmol/L Na₂P₂O₇, 50 mmol/L NaF, and 12 mmol/L deoxycholate. Lysates were sonicated, centrifuged, and the protein concentrations of the supernatants were determined using the detergent-compatible protein assay (Bio-Rad). Equal amounts of protein were then resolved by SDS–PAGE and transferred to nitrocellulose. The membranes were blocked in 5% nonfat milk in TBS with 0.1% Tween 20 and incubated overnight at 4°C with primary antibodies diluted in 5% bovine serum albumin (BSA)/TBS-T. The following day, the membranes were washed and incubated with HRP-conjugated secondary antibodies diluted in 5% nonfat milk/TBS-T at room temperature for 1 hour. The protein bands were visualized using Amersham ECL Prime (GE Healthcare). Images were acquired using Photoshop (Adobe Systems Inc.) and relative quantification was performed using ImageJ (NIH, Bethesda, MD).

**Quantitative real-time PCR**

Total RNA was extracted using TRIzol reagent (Life Technologies) and reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) as per the respective manufacturer’s instructions. The cDNA was amplified via real-time PCR (RT-PCR) using the SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used to measure specific target genes: HIF-1α forward, 5′-CCACAGCACTAGTACATG-3′, reverse 5′-TCAAGTCTCTGGTCTGATAATCC-3′; HIF-2α forward, 5′-GTCTCTCCACCCCATGTCTC-3′, reverse 5′-GGTCTCTCATCCGGTCCTAC-3′; VHL forward, 5′-ATGGGCTCAACTTGGAGGCC-3′, reverse 5′-CAAGAGGCCATCTGTGTC-3′; and GAPDH forward, 5′-GCTCTCCACCATGCTCCTC-3′, reverse 5′-TGGGGGCGAGATCCTGTTTT-3′. Samples were assayed in a 384-well format in triplicate using a 7900HT Fast Real-Time PCR system (Applied Biosystems). Variation in cDNA loading was normalized to GAPDH expression, which remained constant at the 24 hours incubation periods used, and relative expression was determined using the ΔΔCt method of relative quantification. Graphs represent the average relative quantification value with error bars (SE of the relative quantification value) from one representative of three independent experiments. Graphs were generated using the GraphPad Prism Software (GraphPad Software).

**Luciferase activity assay**

HEK293T cells were cotransfected with 100-ng phRL-renilla and 2 µg of pGL2-HRE-luciferase or 1 µg of pcDNA3-ODD-luciferase using lipofectamine 2000 and incubated for 24 hours before drug treatment. Following drug treatment, the Dual-Luciferase Reporter Assay (Promega Corporation) was performed according to the manufacturer’s instructions. Briefly, lysates were prepared using the provided buffer and then diluted 1:10, then 2 µL of diluted sample lysate was added in triplicate to a white-walled 96-well plate, mixed with 100 µL of firefly luciferase assay substrate, and luminescence was immediately recorded using a VictorX4 plate reader (PerkinElmer). Then 100 µL of renilla luciferase substrate was added to each well and luminescence was immediately recorded using the plate reader. Values were corrected for background luminescence using the reading from the media only, and the corrected values were first normalized to renilla luciferase (internal control) and then to the vehicle-treated samples to calculate the relative luciferase activity. Data represent the mean ± SD of one representative of three independent experiments.

**Tumor growth experiment**

Animal experiments were performed according to the NIH guidelines and approved by the Nemours Institutional Animal Care and Use Committee. Female hairless 6- to 8-week-old Nu/J mice were injected subcutaneously with a suspension of Caki-1-td-Tomato cells (1.5 × 10⁶) in a 50% growth factor-reduced Matrigel solution. Caki-1 tumors were allowed to grow for 1 week before randomization into control (vehicle solution only) and drug (gramicidin A) groups of 8 mice each with an average initial tumor volume of approximately 85 mm³ in each group. Gramicidin A (0.22 mg/kg body weight) was diluted in a 1:1 solution of ethanol and saline, and mice were dosed thrice weekly with 50 µL of either vehicle or gramicidin A solutions by intratumoral injection. Mouse body masses and tumor sizes were recorded before each injection. Tumor size was measured using calipers and tumor volume was estimated using the formula (length × width²)/2, where length was the longer of the measurements. Upon completion of the study, mice were euthanized and the tumors were imaged, harvested, and prepared for immunohistochemical and immunoblot analysis. Fluorescence signals from Caki-1 xenografts were acquired at the end of the study using the Kodak In Vivo Multispectral FX PRO imaging system (Carestream) using the following settings: excitation (EX), 550 nm; emission (EM), 600 nm; no binning; f/stop, 2.8; focal plane, 13.1 mm; field-of-view, 119.1 mm.

**Immunohistochemistry**

Formalin-fixed paraffin-embedded samples of vehicle and gramicidin A-treated tumors were prepared using routine procedures. Of note, 5-µm sections were floated onto charged slides and dried for 1 hour at 65°C. Following deparaffinization in xylene and graded alcohols,
tissues underwent heat-induced epitope retrieval using the Decloaking Chamber and Reveal Decloaking Buffer (Biocare Medical) according to the recommended manufacturer’s protocols. The VHL polyclonal antibody (#PA5-17477; Thermo Fisher Scientific) and polyclonal CD31 (#LS-B1932; LifeSpan Biosciences) were diluted in 1% BSA (Sigma-Aldrich) and applied overnight at 4°C. Slides were incubated with PromARK anti-rabbit HRP polymer (Biocare Medical) and stained with diaminobenzidine using the Betazoid DAB Kit (Biocare Medical) according to the recommended manufacturer’s protocols. Nuclei were stained with hematoxylin (EMD Millipore) and Bluing Reagent (Thermo Fisher Scientific), cleared, and mounted for microscopic analysis.

**Statistical analysis**
Quantitative RT-PCR (qRT-PCR) results were analyzed using one-way ANOVA followed by the Dunnett multiple comparison test.
comparison test. All other analyses were performed using the two-tailed unpaired Student t test.

Results

Gramicidin A reduces HIF-1α and HIF-2α protein expression

Because constitutive activation HIF is central to RCC pathogenesis, we investigated whether gramicidin A affects the expression of HIF in RCC cells. Using Caki-1, SN12C, and ACHN cell lines, we found that treatment with gramicidin A for 24 hours provoked a dose-dependent decrease in the expression of both HIF-1α and HIF-2α protein in these cell lines (Fig. 1A, left). Because HIF-α subunits are stabilized by hypoxia (1% O2), we next assessed whether gramicidin A reduces HIF-α expression in hypoxia. Exposure to 1% O2 dramatically increased HIF-1α and HIF-2α as expected, but treatment with gramicidin A prevented this increase in a dose-dependent manner (Fig. 1A, right). Strikingly, 1 μmol/L gramicidin A was sufficient to reduce the hypoxic expression of both isoforms below even their normoxic level (Fig. 1A, lane 8) with the exception of HIF-1α in ACHN cells. Concomitant analysis of HIF mRNA expression revealed that gramicidin A significantly altered transcript expression for only HIF-2α in SN12C cells (P = 0.01 by one-way ANOVA; Fig. 1B), suggesting that gramicidin A primarily affects only HIF protein levels. Finally, we assessed whether mobile-carrier ionophores also reduce hypoxic HIF protein expression. We compared equal doses (0.5 μmol/L) of gramicidin A with monensin (Na+-selective), valinomycin (K+-selective), and calcimycin (Ca2+-selective) in hypoxic SN12C cells. We observed that monensin slightly reduced HIF-1α and HIF-2α at 72 hours, valinomycin moderately reduced both proteins from 24 to 72 hours, and calcimycin had no effect on either protein (Fig. 1C). Only gramicidin A elicited a profound decrease in both isoforms that persisted from 24 to 72 hours (Fig. 1C). These data reveal that only gramicidin A is a potent inhibitor of HIF-1α and HIF-2α protein expression.

Gramicidin A reduces HIF transcriptional activity and target gene expression

Next, we analyzed the effect of gramicidin A upon the transcriptional activity of HIF. We used HEK293T cells transfected with a HIF-responsive luciferase reporter plasmid that contains three hypoxia-response elements (HRE) from the PGK1 (phosphoglycerate kinase 1) gene upstream of firefly luciferase (25). HIF-dependent luciferase activity was significantly stimulated by hypoxia, but treatment with gramicidin A diminished this activity to nearly zero (Fig. 2A). Next, we measured the expression of various HIF targets in RCC cells. We found that hypoxic expression of CA-IX, GLUT-1, and GAPDH were all...
decreased by gramicidin A in a dose-dependent manner in SN12C cells (Fig. 2B, left). Similar results were obtained using Caki-1 and ACHN cells (with the exception of GAPDH in Caki-1 cells; Fig. 2B, right). Collectively, these results demonstrate that gramicidin A attenuates hypoxia responses by preventing the transcriptional activation of HIF-responsive genes.

**Gramicidin A destabilizes HIF through proline hydroxylation**

\(O_{2}\)-dependent downregulation of HIF-\(\alpha\) depends upon the proteasome to degrade ubiquitylated HIF. To elucidate whether gramicidin A uses this mechanism, we first measured HIF expression in HEK293T cells treated with increasing doses of gramicidin A in the absence or presence of 10 \(\mu\)mol/L MG-132 (left) or 1 mmol/L CoCl\(_2\) (right) and protein expression was measured by immunoblot analysis. B, HEK293T cells were cotransfected with ODD-luciferase and renilla-luciferase plasmids and treated with vehicle or GA for 24 hours before luciferase activity was measured. 

\(^*\) \(P < 0.05\). NS, not significant. C, HEK293T cells were transfected with empty vector (pcDNA3), HA-HIF-1\(\alpha\)-wt, or HA-HIF-1\(\alpha\)-mut and treated with vehicle or 1 \(\mu\)mol/L GA for 24 hours before protein expression was measured by immunoblot analysis.

Figure 3. GA destabilizes HIF protein through proline hydroxylation. A, HEK293T cells were treated with vehicle or GA in the absence or presence of 10 \(\mu\)mol/L MG-132 (left) or 1 mmol/L CoCl\(_2\) (right) and protein expression was measured by immunoblot analysis. B, HEK293T cells were cotransfected with ODD-luciferase and renilla-luciferase plasmids and treated with vehicle or GA for 24 hours before luciferase activity was measured. 

\(^*\) \(P < 0.05\). NS, not significant. C, HEK293T cells were transfected with empty vector (pcDNA3), HA-HIF-1\(\alpha\)-wt, or HA-HIF-1\(\alpha\)-mut and treated with vehicle or 1 \(\mu\)mol/L GA for 24 hours before protein expression was measured by immunoblot analysis.

that treatment with gramicidin A significantly reduced ODD-luciferase activity (\(P < 0.05\) by \(t\) test; Fig. 3B). In a related experiment, we transfected HEK293T cells with either HA-tagged wild-type HIF-1\(\alpha\) (HA-HIF-1\(\alpha\); ref. 27) or ODD-mutant HIF-1\(\alpha\) (HA-HIF-1\(\alpha\)-P402A/P564A; ref. 28). Treatment of these cells revealed that wild-type HIF-1\(\alpha\) but not ODD-mutant HIF-1\(\alpha\) was reduced by gramicidin A (Fig. 3C). Altogether, these results demonstrate that gramicidin A uses the \(O_{2}\)-dependent regulatory mechanism to destabilize HIF protein via PHD-dependent hydroxylation of its ODD.

**Gramicidin A upregulates VHL protein expression**

Mutational inactivation of VHL occurs extensively in sporadic ccRCC (up to 80%), and a remaining proportion of tumors (<10%) silence the VHL gene through DNA methylation (31, 32). Loss of VHL cripples the ability of the cell to degrade HIF in normoxia yielding chronic activation of the HIF transcriptional program (33). In our aforementioned experiments, we used VHL-expressing RCC cells to establish that gramicidin A destabilizes HIF through proline hydroxylation and proteasomal degradation. To ascertain whether VHL is involved in gramicidin A-mediated degradation of HIF, we used the naturally VHL-deficient ccRCC cell line UMRC6 and found that gramicidin A failed to reduce HIF-1\(\alpha\) or HIF-2\(\alpha\) expression (Fig. 4A, left). In contrast, treatment of VHL-reconstituted UMRC6 + VHL cells did yield a reduction in HIF-2\(\alpha\) protein expression (Fig. 4A, right). HIF-1\(\alpha\) expression was undetectable in this cell line. These data demonstrate that VHL is essential for gramicidin A-mediated destabilization of HIF.
Although hypoxia reduces proline hydroxylation of HIF, it does not completely abolish it (34). Because gramicidin A treatment reduced HIF expression even in hypoxic conditions (Fig. 1) and uses the O2-dependent degradation mechanism (Fig. 3), we speculated that gramicidin A enhances a component of this pathway to accelerate HIF destabilization. We investigated this possibility and observed that treatment with gramicidin A dramatically increased the expression of VHL protein in a dose-dependent manner in HEK293T cells as well as Caki-1, SN12C, and ACHN RCC cells (Fig. 4B). This increase was not reflected at the mRNA level as transcript expression was significantly elevated in only SN12C cells (\(P < 0.001\) by one-way ANOVA; Fig. 4C). These results demonstrate that gramicidin A inhibits HIF by enhancing VHL expression.

**Gramicidin A inhibits the growth and angiogenesis of VHL-expressing RCC tumor xenografts**

Tumor growth and development beyond a microscopic mass depends on the recruitment of new blood vessels (35). Our *in vitro* data suggested that gramicidin A may reduce tumor growth *in vivo* by disrupting tumor angiogenesis. We previously found that gramicidin A reduced the *in vivo* growth SN12C tumor xenografts in mice (22). To evaluate the antiangiogenic efficacy of gramicidin A, we performed a similar experiment in which we engrafted human Caki-1 RCC cells that express the red fluorescent protein td-Tomato and can be visualized *in vivo*. Once the average tumor volume reached approximately 85 mm³, the mice were randomized into two groups (each \(n = 8\)) and administered 50 \(\mu\)L of either vehicle solution or gramicidin A (0.22 mg/kg) by intratumoral injection thrice weekly for 26 days. As shown in Fig. 5A and B, the control tumors were noticeably larger than the gramicidin A–treated tumors. We found that the average mass of the gramicidin A–treated tumors was 52% less than that of the control tumors (Fig. 5C; \(P = 0.017\) by t test). Analysis of tumor growth revealed that the tumors of the gramicidin A group essentially failed to grow once treatment with gramicidin A was initiated (Fig. 5D). The difference in mean tumor volume achieved significance at day 5 and continued throughout the duration of the study (\(P < 0.05\)).
Significantly, the increased dose, frequency, and duration of gramicidin A treatment did not elicit significant toxicity as no changes in average body mass (Fig. 5E) or activity levels were observed in the mice. Taken together, these data demonstrate that gramicidin A inhibits the growth of VHL-expressing RCC tumors.

To confirm that reduced tumor growth was due to a blockade of tumor angiogenesis, we histologically examined the tumor tissue. Gramicidin A–treated tumors recapitulated our in vitro findings as we observed an overall increase in VHL immunostaining (Fig. 6A) and a 57% reduction in the average number of CD31-positive microvessels in the gramicidin A–treated tumors (vehicle = 7.13 ± 0.18 vs. gramicidin A = 3.04 ± 0.54; \( P = 0.0004 \); Fig. 6A and B). In agreement with these data, immunoblot analysis revealed that HIF-2\( \alpha \) and GAPDH protein expression was also substantially reduced in the gramicidin A–treated tumors (Fig. 6C). HIF-1\( \alpha \) was not detectable by immunoblot analysis but this result was not surprising as it has been reported that RCC growth in vivo is driven by HIF-2\( \alpha \) but repressed by HIF-1\( \alpha \) (5). Taken together, these results are consistent with our in vitro data and indicate that gramicidin A inhibits tumor growth through the suppression of HIF-dependent angiogenesis.

Discussion

Here, we report for the first time that gramicidin A is a novel inhibitor of tumor angiogenesis. We have demonstrated that treatment with gramicidin A enhances VHL expression, which destabilizes HIF-1\( \alpha \) and HIF-2\( \alpha \) protein to suppress the transcription of various HIF targets. Loss of the HIF transcriptional program leads to reduced tumor angiogenesis, which curtails tumor growth in vivo. These novel findings suggest that gramicidin A has therapeutic potential as an angiogenesis inhibitor for VHL-positive RCCs and possibly for other cancers that express VHL.

Gramicidin A–mediated destabilization of HIF-\( \alpha \) subunits required both proline hydroxylation and VHL expression indicating that gramicidin A used the \( \text{O}_2^- \)-dependent degradation mechanism to target HIF. Strikingly, gramicidin A reduced HIF expression even in
hypoxic conditions. Although hypoxia (1% O₂) limits PHD-mediated hydroxylation by depleting molecular oxygen, it does not completely abolish it (34). We determined that gramicidin A increases the expression of VHL protein to accelerate O₂-dependent degradation of HIF. Because upregulation of VHL was sufficient to compensate for the inhibitory effects of hypoxia, we suggest that VHL levels are another important limiting factor in the regulation of HIF in hypoxic conditions. However, whether gramicidin A also increases PHD expression and/or activity is an additional possibility that remains for further investigation.

To our knowledge, this is the first time that an ionophore has shown to specifically upregulate a tumor suppressor protein, yet precisely how gramicidin A increases VHL expression remains to be elucidated. We previously reported that treatment of RCC cells with gramicidin A activates the AMPK pathway (22), but whether AMPK-mediated stress responses are directly related to VHL upregulation is not known. Our results show that VHL protein, but not mRNA, increases in gramicidin A–treated cells indicating that either the translation of VHL transcripts or the stability of VHL protein is increased by gramicidin A. VHL is known to be targeted for degradation by the ubiquitin–proteasome pathway, and VHL is stabilized by association with ubiquitin ligase components (elongin B, elongin C, RBX1, and cullin 2; ref. 36). Our results clearly show that VHL was active in mediating the degradation of HIF in gramicidin A–treated cells, so it is possible that gramicidin A enhances complex formation to stabilize and upregulate VHL protein. In addition, signaling by Src was recently identified as a therapeutic target in RCC (37), and phosphorylation of tyrosine 185 by Src destabilizes VHL (38). Several other proteins are also known to specifically target and destabilize VHL, including E2-EPF ubiquitin-carrier protein.

Figure 6. GA reduces tumor microvasculature and HIF expression in vivo. A, immunohistochemical staining of representative sections from the control and GA-treated Caki-1 tumors. Magnification, ×20. Arrows, CD31⁺ microvessels. B, quantification of CD31⁺ microvessels from 10 random fields of each tumor at ×40 magnification. Graph, mean ± SD of four tumors from each group. *: P < 0.05. C, immunoblot analysis of HIF-2α and GAPDH expression from the Caki-1 tumors.
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(39), casein kinase 2 (40), and transglutaminase 2 (41). Whether inhibition of any of these proteins is involved in the gramicidin A-mediated increase in VHL expression remains to be investigated.

The plausibility of VHL overexpression as a therapeutic strategy has been demonstrated in various reports; Sun and colleagues first showed that VHL gene delivery using liposomes in vivo reduced HIF-1α and VEGF expression, reduced tumor angiogenesis, and induced the regression of murine lymphoma tumor xenografts (42) and rat glioma tumor xenografts (43). More recently, VHL overexpression by adenovirus infection was found to synergize with doxorubicin to suppress the growth of murine hepatocellular carcinoma xenografts (44), and a novel small molecule inhibitor of HIF-1α was shown to reduce the growth and vascularization of human colorectal carcinoma tumor xenografts via VHL overexpression (34). These studies demonstrate the effectiveness of enhancing VHL expression to block tumor growth as well as combining VHL overexpression with other treatments to augment therapeutic efficacy.

Constitutive activation of HIF is regarded as a hallmark of RCC pathology. This is most prominent in ccRCC in which the overwhelming majority of tumors feature inactivating mutation of the VHL gene (31, 32). We observed that gramicidin A failed to reduce HIF-1α and HIF-2α expression in VHL-deficient cells implying that gramicidin A may not be effective as an antiangiogenic therapy for patients with ccRCC with functional inactivation of VHL. However, constitutive activation of HIF is also a characteristic of certain nonclear cell RCC subtypes (45–48) even though VHL mutation is exceedingly rare in these tumors (49). Because VHL is functional in these subtypes, gramicidin A is likely to have therapeutic utility in this traditionally underserved patient population (8). Furthermore, gramicidin A may also prove effective in other cancers as upregulation of HIF occurs in the majority of solid tumors and generally correlates with poor survival (6). Toxicity is an essential factor in clinical drug development. Our preliminary investigations confirmed that systemic administration of gramicidin A by either intravenous or intraperitoneal injection was lethal to mice. However, we found that repeated intratumoral injection of gramicidin A blocked tumor growth without causing significant toxicity. Intratumoral administration is by nature localized, and it improves the therapeutic index of drugs by increasing the tumor-to-organ ratio, which greatly reduces systemic toxicity (50). Although systemic administration is commonly regarded as essential for the treatment of invasive cancer, intratumoral injection is now a feasible approach for certain inoperable and/or metastatic tumor sites through the use of X-ray computed tomography. Furthermore, intratumoral administration can actually enhance immune responses against disseminated (noninjected) tumors by enhancing the processing of tumor-associated antigens expressed in cell debris from the injected tumor (51). Nevertheless, systemic administration of gramicidin A may be possible in the near future. Chemical modification of gramicidin A has been shown to change the characteristics of the peptide enough to increase microbial targeting and/or decrease nonspecific toxicity (18, 19, 52, 53), and encapsulation of gramicidin A within a targeted drug carriers such as nanoparticles may be a plausible method to safely deliver gramicidin A to only the tumor (54). Whether these approaches can be effectively applied to the use of gramicidin A as a novel cancer therapy is an essential area of future investigation.

Disclosure of Potential Conflicts of Interest

J.M. David and A.K. Rajasekaran have ownership interest in a patent. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J.M. David, A.K. Rajasekaran

Development of methodology: J.M. David, A.K. Rajasekaran

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. David, T.A. Owens, L.J. Inge, A.K. Rajasekaran

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