**Gramicidin A Induces Metabolic Dysfunction and Energy Depletion Leading to Cell Death in Renal Cell Carcinoma Cells**

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**Abstract**

Ionophores are lipid-soluble organic molecules that disrupt cellular transmembrane potential by rendering biologic membranes permeable to specific ions. They include mobile-carriers that complex with metal cations and channel-formers that insert into the membrane to form hydrophilic pores. Although mobile-carriers possess anticancer properties, investigations on channel-formers are limited. Here, we used the channel-forming ionophore gramicidin A to study its effects on the growth and survival of renal cell carcinoma (RCC) cells. RCC is a histologically heterogeneous malignancy that is highly resistant to conventional treatments. We found that gramicidin A reduced the in vitro viability of several RCC cell lines at submicromolar concentrations (all IC50 < 1.0 μmol/L). Gramicidin A exhibited similar toxicity in RCC cells regardless of histologic subtype or the expression of either the von Hippel-Lindau tumor suppressor gene or its downstream target, hypoxia-inducible factor-1α. Gramicidin A decreased cell viability equal to or greater than the mobile-carrier monensin depending on the cell line. Mechanistic examination revealed that gramicidin A blocks ATP generation by inhibiting oxidative phosphorylation and glycolysis, leading to cellular energy depletion and nonapoptotic cell death. Finally, gramicidin A effectively reduced the growth of RCC tumor xenografts in vivo. These results show a novel application of gramicidin A as a potential therapeutic agent for RCC therapy. *Mol Cancer Ther;* 12(11); 2296–307. ©2013 AACR.

**Introduction**

Ionophores are highly hydrophobic molecules that permeabilize membranes to various cations. They differ based on cation specificity and mechanism of transport. Mobile-carriers physically associate with individual cations to form a complex that diffuses across lipid bilayers, whereas channel-formers incorporate into the membrane to form hydrophilic transmembrane nanopores that permit rapid diffusion of cations through the membrane. Several ionophores are naturally produced and exhibit potent antibiotic activity (1). Research over the past decade has described a role for mobile-carrier ionophores as anticancer therapeutics; monensin induces cell-cycle arrest and apoptosis in various cancer cell lines (2–7) and acts as a radiosensitizing agent (8), and salinomycin acts as a radiosensitizing agent (9). Many groups have now reported selective targeting of cancer stem cells (CSC) and reduces in vivo growth and metastasis formation (9). Many groups have now reported selective targeting of CSCs by salinomycin in a wide range of additional malignancies (10). Moreover, salinomycin can overcome multiple-drug resistance, sensitize cells to chemo-/radiotherapy, and even interfere with oncogenic signaling (10).

Gramicidin A is the simplest and best-characterized channel-forming ionophore. Produced by the bacterial species *Bacillus brevis* and discovered in 1939 by Dr. René Dubos (11, 12), gramicidin A was the very first antibiotic tested in a clinical setting (13). It is a linear 15-residue peptide of alternating L- and D-amino acids (Fig. 1A) and adopts a β-helix conformation within the lipid bilayer in which two gramicidin A monomers dimerize end-to-end to form a functional nanopore of 4 Å that spans the membrane (Fig. 1B; ref. 14). The gramicidin A channel is wide enough to permit the diffusion of water and inorganic monovalent cations, resulting in Na+ influx, K+ efflux, osmotic swelling, and cell lysis (14, 15). This confers gramicidin A with potent antibiotic activity against bacteria, fungi, and protozoa (11, 12, 16, 17). However, whether gramicidin A also possesses anticancer properties akin to the mobile-carrier ionophores has not been established.

Kidney cancer is a devastating disease that is among the top 10 causes of deaths related to cancer in men in the United States (18). The majority (80%–85%) of kidney cancers are classified as various histologic subtypes of renal cell carcinoma (RCC; ref. 19). RCC is characteristically resistant to both chemo- and radiotherapy, and the 5-year disease-specific survival rate for invasive RCC is only 10% (19, 20). RCC development and progression is primarily due to chronic activation of the cellular response to low oxygen (hypoxia; refs. 21, 22). Hypoxia-inducible
factor (HIF) is the master transcriptional regulator of hypoxia responses, and its expression is regulated by cellular oxygen levels. In normoxic conditions, HIF is hydroxylated and then bound by the von Hippel-Lindau (VHL) tumor suppressor protein that promotes its polyubiquitylation and subsequent degradation (23). However, HIF becomes stabilized in hypoxia as low oxygen prevents protein hydroxylation. Constitutive (i.e., oxygen-insensitive) activation of the HIF transcriptional program occurs in RCC via the functional inactivation of VHL in the clear cell RCC subtype, or through various VHL-independent means in the other RCC subtypes (19).

Here, we have conducted the first systematic study of the cytotoxic effects of gramicidin A in RCC cell lines. We report that disruption of cationic homeostasis by gramicidin A is toxic to RCC cells regardless of histologic subtype or HIF expression/activity. We found that gramicidin A impaired cellular metabolism by disrupting both oxidative phosphorylation and glycolysis, leading to profound energy depletion and subsequent nonapoptotic cell death. Gramicidin A also reduced the in vivo growth of RCC tumor xenografts without causing significant toxicity in mice. These findings identify gramicidin A as a new potential anticancer therapeutic for RCC.

Materials and Methods

**Cell culture**

Human clear cell RCC (A498, 786-O, Caki-1, SN12C, UMRC6, and UMRC6+VHL), papillary RCC (ACHN), embryonic kidney (HEK293T), and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mmol/L L-glutamine, 25 U/mL penicillin, and 25 μg/mL streptomycin. 786-O, Caki-1, HEK293T, and MDCK 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 (24). UMRC6 and UMRC6+VHL cells were kindly provided by Dr. Michael I. Lerman (National Cancer Institute, Bethesda, MD) in 2000 (25). All cell lines obtained from investigators have been authenticated before use.

**Reagents**

Gramicidin A, monensin, ouabain, and rhodamine 123 were purchased from Sigma-Aldrich.

**Plasmids and transfections**

pcDNA3-HA-HIF-1α (Addgene plasmid 18949; William Kaelin, Howard Hughes Medical Institute, Dana-Farber Cancer Institute and Brigham and Women’s Hospital, Harvard Medical School, Boston, MA; ref. 26), pcDNA3-HA-HIF-1α-P402A/P564A (Addgene plasmid 18955; William Kaelin; ref. 27) were purchased from Addgene. pcDNA3 vector was purchased from Life Technologies. Transfections were accomplished using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions.

**Antibodies**

We purchased primary antibodies specific for HIF-1α (BD Biosciences), HIF-2α (Novus Biologicals), hemagglutinin (HA)-tag, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), cleaved-PARP, α-tubulin, AMP-activated protein kinase (AMPK), phospho-AMPK (T172), acetyl-coA carboxylase (ACC), phospho-ACC (S79; Cell Signaling Technology), and β-actin (Sigma-Aldrich). Horseradish peroxidase (HRP)–conjugated secondary antibodies were purchased from Cell Signaling Technology.

**Cell viability assay**

The CellTiter-Blue cell viability assay was conducted according to the manufacturer’s instructions (Promega Corporation). Cells were seeded in triplicate into 96-well plates and allowed to attach overnight. Cells were then treated with vehicle (ethanol) or drug and incubated for 72 hours. Next, 20 μL of CellTiter-Blue reagent was added to each well and incubated for additional 4 hours. Fluorescence was then read at 560 nm using a VictorX4 plate reader.
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. Lysates were sonicated, centrifuged, and the protein concentrations of the supernatants were determined using the detergent compatible (DC) protein assay (Bio-Rad). Equal amounts of protein were then resolved by SDS-PAGE and transferred to nitrocellulose. Immunoblots were performed using primary antibodies conjugated with horseradish peroxidase (HRP) and secondary antibodies conjugated with alkaline phosphatase. Following visualization, the blots were stripped from the nitrocellulose membrane by washing with buffer containing 50 mmol/L Tris pH 8.0, 2% SDS, and 0.7% 2-mercaptoethanol. Immunoblotting was performed with goat anti-antibodies to the following proteins: p38, JNK, ERK1/2, NF-κB, and catalase (Catalase). Immunoblots of phospho-p38, phospho-JNK, phospho-ERK1/2, and phospho-NF-κB were detected using goat antiphospho antibodies and horseradish peroxidase-conjugated secondary antibodies. Immunoblots of phospho-catalase were detected using an anti-catalase antibody and horseradish peroxidase-conjugated secondary antibodies.

Cell death assay

The CellTiter-Glo luminescent viability assay was used to quantify cellular ATP and was conducted according to the manufacturer’s instructions (Promega). Briefly, cells were seeded in triplicate into white-walled 96-well plates and treated with thymidine (1 mmol/L) overnight to block cell proliferation and maintain a constant cell number. Cells were then treated with vehicle (ethanol) or drug in culture media containing 1 mmol/L thymidine and incubated for 24 hours. Plates were then equilibrated to room temperature for 30 minutes and then 100 µL of CellTiter-Glo reagent was added to each well. Plate contents were mixed for 2 minutes using an orbital shaker and then incubated an additional 10 minutes at room temperature before luminescence was recorded using a VictorX4 plate reader (PerkinElmer, Inc.). Luminescence values were corrected for background luminescence using the reading from the media only, and corrected values were normalized to vehicle-treated samples to calculate the relative ATP levels. Data represent the mean ± SE of three independent experiments.

Redox activity assay

Cells were seeded in triplicate into 96-well plates and treated with thymidine (1 mmol/L) overnight to block cell proliferation and maintain a constant cell number. Cells were then treated with vehicle (ethanol) or drug in culture media containing 1 mmol/L thymidine and incubated for...
24 hours. Cellular redox activity was measured using the CellTiter-Blue assay as described earlier.

**Mitochondrial transmembrane potential assay**

Cells were seeded into 60-mm dishes and treated with thymidine (1 mmol/L) overnight to block cell proliferation and maintain a constant cell number. Cells were then treated with vehicle (ethanol) or drug in culture media containing 1 mmol/L thymidine and incubated for 24 hours. Cells were then washed twice with 1× PBS and then incubated with 10 μg/mL rhodamine 123 in 1× PBS supplemented with 100 μmol/L CaCl₂, 1 mmol/L MgCl₂, 0.2% BSA, and drug (gramicidin A or monensin) for 30 minutes at 37°C in the dark. After incubation, the cells were washed twice with 1× PBS and then lysed in 200 μL of 1× PBS supplemented with 0.02% SDS for 15 minutes. Lysates were collected by scraping, added to the wells of a 96-well plate, and fluorescence was read at 535 nm using a VictorX4 plate reader (PerkinElmer, Inc.). Fluorescence values were corrected for background fluorescence using the measurement from the media only, and corrected values were normalized to vehicle-treated samples to calculate the relative fluorescence. Data represent the mean ± SE of three independent experiments.

**Glycolysis assay**

The Glycolysis Cell-Based Assay Kit was used to quantify extracellular lactate and was conducted according to the manufacturer’s instructions (Cayman Chemical Company). Cells were seeded in duplicate into 96-well plates, allowed to attach for 4 hours, and then treated with thymidine (1 mmol/L) overnight to block cell proliferation and maintain a constant cell number. Cells were then treated with vehicle (ethanol) or drug in phenol-free DMEM supplemented with 0.5% FBS and 1 mmol/L thymidine. The cells were divided into two treatment groups of either 0 to 12 or 12 to 24 hours. For the 12- to 24-hour samples, the media was removed after the initial 12 hours of incubation and replaced with fresh media to incubate for an additional 12 hours. Following incubation, 10 μL of conditioned media was mixed with the kit reagents in a 96-well plate and absorbance values at 490 nm were obtained using a VictorX4 plate reader (PerkinElmer, Inc.). Readings were corrected for background absorbance using the absorbance value from the culture medium only and the corrected values were applied to a standard curve to calculate extracellular lactate levels. Data represent the mean ± SE of three independent experiments.

**Tumor growth assay**

Animal experiments were carried out according to the NIH guidelines and approved by the Nemours Institutional Animal Care and Use Committee and Institutional Biosafety Committee. Female hairless Nu/J mice (Charles River Laboratories) that were 6- to 8-week old were injected subcutaneously with a suspension of SN12C cells (1.0 × 10⁶) in a 50% growth factor–reduced Matrigel (BD Biosciences) solution. SN12C tumors were allowed to grow for 1 week before randomization into control (vehicle solution only) and drug (gramicidin A) groups of 5 mice each with an average initial tumor volume of approximately 100 mm³, as determined by caliper measurement using the formula [(length × width²)/2] where length was the longest of the two measurements. Gramicidin A (0.11 mg/kg body weight) was diluted in a 1:1 solution of ethanol and saline (0.9% NaCl), and mice were dosed twice weekly with 50 μL of either vehicle or gramicidin A solutions by intratumoral injection. Mouse body masses were recorded before each injection. Upon completion of the study, mice were euthanized, the tumors were surgically excised, and masses were recorded.

**Statistical analysis**

Dose-response viability curves were conducted by ANOVA, all other analyses were conducted using unpaired two-tailed Student t test.

**Results**

**Treatment with gramicidin A reduces the viability of RCC cells in vitro**

The viability of several different cancer cell lines has been shown to be sensitive to treatment with mobile-ionophores. However, the effect of channel-forming ionophores upon RCC cell viability is not known. We measured the in vitro viability of four ccRCC cell lines (A498, 786-O, Caki-1, and SN12C) and one papillary RCC cell line (ACHN; ref. 28) treated with gramicidin A, and found that it caused a dose-dependent decrease in cell viability (Fig. 2A). These cell lines responded similarly to gramicidin A as the absolute IC₅₀ values for all cell lines were less than 1 μmol/L (Table 1). This result shows that the viability of RCC cells is sensitive to treatment with gramicidin A.

**Neither VHL nor HIF-1α expression significantly alters cellular sensitivity to gramicidin A**

Our analysis of cell viability revealed that the IC₅₀ values of the VHL-deficient ccRCC cell lines (A498, 786-O) were higher than those of the VHL-expressing ccRCC cell lines (Caki-1 and SN12C; Table 1). Given the significance of VHL in renal cancer pathology, we sought to ascertain whether its expression affects cellular sensitivity to gramicidin A in vitro. We first compared the viability of gramicidin A–treated isogenic UMRC6 cells (VHL-deficient) with that of VHL-reconstituted UMRC6+VHL cells. We found that these cell lines responded very similarly to gramicidin A (Fig. 2B) yet the dose–response curves just barely achieved statistical significance ($P = 0.0403$ by two-way ANOVA). Further analysis revealed that although the UMRC6+VHL cells had a slightly higher IC₅₀ value than the parental UMRC6 cells (Table 1), this difference was not statistically significantly different ($P = 0.190$ by t test). In addition, because VHL deficiency stabilizes HIF, we also examined whether overexpression of HIF-1α affects the cellular response to gramicidin A. HEK293T
cells were transiently transfected with either empty vector (pcDNA3), HA-tagged wild-type HIF-1α (HA-HIF-1α; 26), or HA-tagged constitutively active mutant HIF-1α (HA-HIF-1α-mut) in which prolines 402 and 564 had been mutated to alanines to abrogate O2-dependent downregulation by VHL (Fig. 2C; ref. 27). No differences in the dose-response viability curves of these transfected cell lines were found in response to treatment with gramicidin A (Fig. 2D; $P = 0.988$ by one-way ANOVA). Analysis of the IC50 values (Table 1) also failed to yield any statistically significant differences ($P > 0.5$ for all possible combinations by $t$ test). Altogether, our results imply that gramicidin A reduces the viability of RCC cells in vitro in a manner that is independent of both VHL and HIF-1α expression.

**Gramicidin A affects cell viability comparable to monensin**

Next, we sought to compare the viability of RCC cells treated with either gramicidin A or the mobile-carrier ionophore monensin. We selected monensin for this comparison because (i) monensin has been shown to inhibit the growth of RCC cells (2) and (ii) both monensin and gramicidin A exhibit selectivity for Na+ and K+ ions (29). Using both VHL-deficient (786-O) and VHL-expressing (SN12C) cell lines, we found that gramicidin A and monensin caused a dose-dependent decrease in cell viability as expected (Fig. 3A and B). Although the absolute IC50 value for gramicidin A–treated 786-O cells was slightly less than monensin (0.430 vs. 0.622 μmol/L), the difference in the dose-response was not found to be statistically significant ($P = 0.528$ by two-way ANOVA). Conversely, we found that the viability of SN12C cells was 13.9-fold more sensitive to treatment with gramicidin A than with monensin (IC50 = 0.104 vs. 1.443 μmol/L), which was highly statistically significant ($P < 0.0001$ by two-way ANOVA). These data suggest that channel-forming ionophores reduce the viability of RCC cells comparable to or greater than mobile-carrier ionophores in a cell-type–specific manner.

**Gramicidin A induces nonapoptotic cell death in RCC cells**

Several investigations have reported that mobile-carrier ionophores (i.e., monensin and salinomycin) induce apoptotic cell death in cancer cells. Microscopic analysis of monensin-treated 786-O cells confirmed the presence of typical apoptotic morphologic features such as cell rounding, membrane blebbing, and vacuolization (Fig. 4A, B).
Gramicidin A was more pronounced in SN12C cells in both 786-O and SN12C cell lines. ATP reduction by gramicidin A for 24 hours decreased cellular ATP as depicted in Fig. 5A. We observed that treatment increasing doses of either gramicidin A or monensin. The ATP content of 786-O and SN12C cells treated with gramicidin A depletes cellular energy, we measured the impairment with both gramicidin A and ouabain decreased cellular ATP levels to a greater extent than monensin in SN12C cells at 1 μmol/L (gramicidin A = 18% ± 2% vs. monensin = 41% ± 14%) and at 10 μmol/L in both cell lines (786-O = 45% ± 10% vs. 70% ± 2%; SN12C = 10% ± 1% vs. 24% ± 0.3%). These observations show that gramicidin A depletes cellular energy in RCC cells.

Cellular energy homeostasis is regulated by AMPK. This kinase is activated by an elevation of the intracellular ratio of AMP–ATP, which occurs in stressful conditions (31). We investigated the activation of the AMPK pathway in gramicidin A–treated 786-O and SN12C cells as a marker of cellular energy stress. Immunoblot analysis revealed that treatment with gramicidin A increased the phosphorylation of AMPKα and its substrate ACC at both 24 and 48 hours (Fig. 5B). AMPKα phosphorylation was more pronounced in SN12C cells than 786-O cells at 24 hours, which is consistent with the previously observed greater sensitivity of this cell line to ATP depletion (Fig. 5A). This observed activation of the AMPK pathway confirms that gramicidin A induces cellular energy stress in RCC cells.

Next, we investigated whether cellular energy depletion caused by gramicidin A is due to the disruption of Na⁺ and K⁺ homeostasis. The Na,K-ATPase pump is a key regulator of intracellular ionic homeostasis that transports 3Na⁺ out of the cell and 2K⁺ into the cell against their respective electrochemical gradients (32). Inhibition of the Na,K-ATPase blocks the active transport of these cations and permits the influx of Na⁺ and efflux of K⁺ similar to gramicidin A. Using the Na,K-ATPase inhibitor ouabain (0.5 μmol/L), we measured cellular ATP and observed that ouabain treatment mimicked gramicidin A and produced a marked reduction in cellular ATP in both 786-O (6% ± 2%) and SN12C (30% ± 15%) cell lines (Fig. 5C). Furthermore, combination treatment with both gramicidin A and ouabain decreased cellular ATP levels more than either drug alone (Fig. 5C). These results indicate that disruption of Na⁺ and K⁺ balance is involved in gramicidin A–induced energy depletion in RCC cells.
Gramicidin A induces metabolic dysfunction in RCC cells

The profound loss of cellular energy that resulted from the treatment with gramicidin A may indicate that ATP production was impaired. Because cellular metabolism depends upon oxidation–reduction (redox) reactions, we first measured whether gramicidin A interferes with redox activity in RCC cells. We used the redox-sensitive dye resazurin (CellTiter-Blue), which is reduced into highly fluorescent resorufin in the presence of metabolically active cells. Treatment of 786-O and SN12C cells with gramicidin A for 24 hours resulted in a significant 30% to 60% reduction in the cellular redox activity at all doses ($P < 0.05$; Fig. 6A). In contrast, monensin failed to significantly reduce the activity in SN12C cells and produced only minimal inhibition in 786-O cells (gramicidin A = 36% ± 3% vs. monensin = 75% ± 6% at 10 μmol/L). These observations indicate that gramicidin A interferes with general cellular metabolism in RCC cells.

Proliferating cells produce ATP almost exclusively from glucose via oxidative phosphorylation and glycolysis. Oxidative phosphorylation occurs within mitochondria and depends upon the establishment and maintenance of a proton gradient between the inner and outer mitochondrial membranes to energetically couple electron transport with ATP synthesis. Gramicidin A is well known to conduct protons and depolarize mitochondrial transmembrane potential, thereby blocking ATP synthesis (34). To determine whether gramicidin A interferes...
with mitochondrial function, we measured the uptake of the cell-permeable dye rhodamine 123 (Rh123), which accumulates within polarized mitochondria (35). As shown in Fig. 6B, treatment with gramicidin A for 24 hours resulted in a 40% to 60% reduction in Rh123 uptake in 786-O and SN12C cells. In contrast, monensin did not significantly alter the Rh123 uptake in either cell line. This result indicates that gramicidin A depolarizes mitochondrial membrane potential and decreases oxidative phosphorylation-dependent ATP synthesis.

Next, we sought to determine whether gramicidin A impairs glycolysis in RCC cells by measuring the production of extracellular L-lactate, the end product of glycolysis. Surprisingly, at 24 hours, the gramicidin A–treated 786-O cells showed a significant increase (+92% ± 40.3%) in lactate production (Fig. 6C), whereas no change was observed in SN12C cells (Fig. 6D). To further probe this effect, we divided the 24-hour incubation into two 12-hour periods and found that gramicidin A induced an initial increase in lactate levels in both cell lines during the first 0- to 12-hour period (786-O = 236% ± 17.7%; SN12C = 136% ± 14.8%; Fig. 6C and D). However, this elevation was not sustained as lactate levels fell significantly during the subsequent 12- to 24-hour period (786-O = 83% ± 13.4%; SN12C = 23% ± 3.3%; Fig. 6C and D). The increased accumulation of lactate at 12 hours suggests that glycolysis was initially stimulated but later inhibited by gramicidin A.
Gramicidin A inhibits the growth of RCC tumor xenografts

To evaluate the in vivo antitumor efficacy of gramicidin A, we engrafted human SN12C RCC cells by subcutaneous injection into female Nu/ν athymic nude mice. When the tumors reached an average size of approximately 100 mm³, the mice were randomized into two groups (n = 5) and administered 50 μL of either vehicle solution (50% ethanol in saline) or gramicidin A (0.11 mg/kg) by intratumoral injection twice weekly for 14 days. As shown in Fig. 7A, at necropsy, the average tumor mass was reduced by approximately 40% with gramicidin A treatment (230 ± 24 mg vs. 140 ± 20 mg; P = 0.0228 by t test). Importantly, intratumoral injection of gramicidin A did

Figure 7. Gramicidin A (GA) reduces the growth of SN12C tumor xenografts. A, xenograft tumors were excised from euthanized mice and the mass of each tumor was measured. B, measurement of the average body masses of the mice. Graphs depict the mean ± SE of n = 5 in each group.
not elicit significant toxicity as no changes in the activity levels or the average body masses of the mice were observed (Fig. 7B).

Discussion

In this study, we provide the first evidence that treatment with the channel-forming ionophore gramicidin A is toxic to RCC cells in vitro and in vivo. This finding is significant because RCC is a highly therapy-resistant malignancy. Mechanistically, we showed that gramicidin A impairs oxidative phosphorylation and glycolysis leading to cellular ATP depletion and nonapoptotic cell death. Disruption of Na⁺ and K⁺ homeostasis by gramicidin A is likely involved in this depletion of energy. Our findings suggest that gramicidin A may have therapeutic potential for the treatment of RCC and possibly other solid tumors.

Constitutive activation of HIF plays a prominent role in RCC pathophysiology. Using a panel of various VHL-positive and -negative RCC cell lines, we found that gramicidin A reduced the in vitro viability of all of these cell lines similarly. We also used stably and transiently transfected cell lines to manipulate VHL and HIF-1α expression, respectively, and failed to find any significant differences in the response of these cells to gramicidin A. Furthermore, we observed that gramicidin A reduced cellular energy and metabolism similarly in VHL-deficient (786-O) and VHL-expressing (SN12C) cells. These results show that histologic subtype, VHL status, and HIF-1α expression/activity are not major factors that determine the cellular response to gramicidin A. However, given the prominent role of VHL/HIF in regulating tumor angiogenesis, it is possible that the in vivo response of RCC cells to gramicidin A will differ based on these factors. Experiments to test this possibility are currently underway in our laboratory.

Monensin has been reported to induce cell-cycle arrest and apoptosis in RCC cells (2) and we also observed morphologic and molecular features consistent with apoptotic cell death in monensin-treated RCC cells. However, gramicidin A seems to induce a different cell death mechanism. Despite obvious cytotoxicity, we did not observe apoptotic features (membrane blebbing, PARP cleavage, and DNA laddering) in gramicidin A–treated cells. In addition, marked cellular energy depletion and inhibition of glycolysis are both features of necrotic cell death (30), and both of these were observed in gramicidin A–treated cells. Furthermore, treatment of RCC cells with necrotatin-1, an inhibitor of necroptosis (36), failed to rescue gramicidin A–induced cell death (data not shown). Collectively, our observations signify that gramicidin A induces a necrotic form of cell death in RCC cells. Necrosis is viewed negatively because it provokes a robust inflammatory response, yet necrotic inflammation may also attract host leukocytes, thereby enhancing tumor antigen presentation and antitumor immunity (30, 37). Thus, gramicidin A–induced necrotic cell death might also facilitate antitumor immune responses in RCC tumors and must be addressed by future studies.

Cellular energy depletion can occur if the plasma membrane is damaged and ATP leaks out of the cell (38). We investigated this possibility but failed to detect ATP in the culture supernatant from gramicidin A–treated cells (data not shown). Enhanced ATP consumption can also contribute to the depletion of energy. The Na,K-ATPase is a very energy-intensive enzyme that uses more than 50% of the cellular energy in the kidney (39) and it is stimulated by the imbalance of Na⁺ and K⁺ provoked by gramicidin A (29). However, we found that the inhibition of Na,K-ATPase with ouabain failed to rescue gramicidin A–induced ATP depletion but actually enhanced it. We finally assessed whether gramicidin A blocks ATP generation as the sheer multitude of ATP-consuming processes makes cells vulnerable to energy depletion once ATP synthesis is halted. We found that gramicidin A reduced cellular redox activity, disrupted mitochondrial transmembrane potential, and decreased extracellular lactate production, indicating that gramicidin A impairs glucose catabolism. The inhibition of glycolysis likely accounts for the bulk of energy depletion by gramicidin A, because RCC cells are known to have a distinct bioenergetic organization in which ATP production is shifted heavily in favor of glycolysis (23, 40). However, we cannot at this time rule out a loss of energy from other sources (amino acids, fatty acids, etc.) as contributing to gramicidin A–induced energy stress.

Although gramicidin A disrupts oxidative phosphorylation directly through energetic uncoupling (34), the precise mechanism whereby gramicidin A initially stimulates and then subsequently inhibits glycolysis is currently not understood. Glycolysis is likely stimulated to compensate for the loss of energy production by oxidative phosphorylation. AMPK is known to increase glycolysis to replenish cellular energy in stressed cells (31). It is possible that the observed activation of AMPK might be involved in the stimulation of glycolysis. In addition, glycolysis depends upon the reduction of NAD⁺ to NADH, and we observed that gramicidin A produced a marked decrease in cellular redox activity. It is possible that gramicidin A reduces glycolysis by preventing NAD⁺/NADH cycling or, alternatively, by activating enzymes that catalyze NAD⁺-consuming ADP-ribosylation reactions such as PARP (which remained intact in gramicidin A–treated cells).

Toxicity is an essential factor to consider in clinical drug development, yet most of the chemotherapeutics used clinically have substantial nonspecific toxicity toward normal cells. Gramicidin A is known to cause hemolysis and is toxic to the liver, kidney, meninges, and olfactory apparatus (14, 41). We tested the toxicity of gramicidin A using nontumorigenic kidney cell lines from various species [LLC-PK1 (porcine), HK-2 (human), and MDCK (canine)] and observed IC₅₀ values that were comparable to those of the RCC cell lines (data not shown). However, we have shown here that gramicidin A was both safe and effective via intratumoral injection in mice. Intratumoral administration improves the therapeutic index of drugs
by increasing the tumor-to-organ ratio, and advances in the use of X-ray–computed tomography have made this route of administration possible for even metastatic and/or inoperable tumors (42). Alternatively, mutation and/or chemical modification of the gramicidin A peptide may be used to decrease the nonspecific toxicity (14), or encapsulation of gramicidin A within an amphiphilic drug carrier may also be used to simultaneously increase solubility, decrease toxicity, and improve tumor targeting (43). Careful evaluation of these options will allow gramicidin A to reach its full therapeutic potential.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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

Development of methodology: J.M. David

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. David, T.A. Owens, S.P. Barwe

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. David, T.A. Owens, S.P. Barwe, A.K. Rajasekaran

Writing, review, and/or revision of the manuscript: J.M. David, T.A. Owens, A.K. Rajasekaran

Administrative, technical, or material support: J.M. David, A.K. Rajasekaran

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