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

Justin M. David,1,2 Tori A. Owens,2 Sonali P. Barwe,2 and Ayyappan K. Rajasekaran2

1Department of Biological Sciences, University of Delaware, Newark, DE 19716, 2Nemours Center for Childhood Cancer Research, Alfred I. duPont Hospital for Children, Wilmington, DE 19803.

Corresponding Author:
Ayyappan K. Rajasekaran, Ph.D.
Nemours Center for Childhood Cancer Research, Alfred I. duPont Hospital for Children
1701 Rockland Road, Wilmington, DE 19803
Telephone: (302) 651-6593 Fax: (302) 651-4827 Email: araj@medsci.udel.edu

Running Title: Cytotoxicity of gramicidin A in renal cell carcinoma

Keywords: gramicidin A, renal cell carcinoma, cell death, metabolism, energy depletion

Abbreviations: ACC (acetyl-coA carboxylase), AMPK (AMP-activated protein kinase), GA (gramicidin A), HIF (hypoxia-inducible factor), MON (monensin), PARP (poly (ADP-ribose) polymerase), RCC (renal cell carcinoma), ccRCC (clear cell renal cell carcinoma), VHL (von Hippel-Lindau)

Financial Support: NIH R01 DK56216 (A.K. Rajasekaran), Nemours Foundation (A.K. Rajasekaran)

Conflicts of Interest: None

Word Count: abstract = 207, manuscript = 5,061

References: 43

Figures: 7

Tables: 1
Abstract:

Ionophores are lipid soluble organic molecules that disrupt cellular transmembrane potential by rendering biological membranes permeable to specific ions. They include mobile-carriers which 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 utilized the channel-forming ionophore gramicidin A (GA) to study its effects upon 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 GA reduced the in vitro viability of several RCC cell lines at sub-micromolar concentrations (all IC₅₀ < 1.0μM). GA exhibited similar toxicity in RCC cells regardless of histological subtype or the expression of either the von Hippel-Lindau (VHL) tumor suppressor gene or its downstream target hypoxia-inducible factor (HIF)-1α. GA decreased cell viability equal to or greater than the mobile-carrier monensin depending on the cell line. Mechanistic examination revealed that GA blocks ATP generation by inhibiting oxidative phosphorylation and glycolysis leading to cellular energy depletion and non-apoptotic cell death. Finally, GA effectively reduced the growth of RCC tumor xenografts in vivo. These results demonstrate a novel application of GA as a potential therapeutic agent for renal cell carcinoma therapy.
Introduction:

Ionophores are highly hydrophobic molecules that permeabilize membranes to various cations. They differ based upon 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 (MON) induces cell cycle arrest and apoptosis in various cancer cell lines (2-7) and acts as a radiosensitizing agent (8), and salinomycin (SAL) preferentially targets breast cancer stem cells (CSCs) and reduces in vivo growth and metastasis formation (9). Many groups have now reported selective targeting of CSCs by SAL in a wide range of additional malignancies (10). Moreover, SAL can overcome multiple-drug resistance, sensitize cells to chemotherapy/radiation, and even interfere with oncogenic signaling (10).

Gramicidin A (GA) 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), GA 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 GA monomers dimerize end-to-end to form a functional nanopore of 4Å that spans the membrane (Fig. 1B) (14). The GA 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 GA with potent antibiotic activity against bacteria,
fungi, and protozoa (11, 12, 16, 17). However, whether GA 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 ten causes of cancer-related deaths in men in the USA (18). The majority (80-85%) of kidney cancers are classified as various histological subtypes of renal cell carcinoma (RCC) (19). RCC is characteristically resistant to both chemotherapy and radiation, 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) (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 tumor suppressor protein (VHL) which 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 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 GA in RCC cell lines. We report that disruption of cationic homeostasis by GA is toxic to RCC cells regardless of histological subtype or HIF expression/activity. We found that GA impaired cellular metabolism by disrupting both oxidative phosphorylation and glycolysis, leading to profound energy depletion and subsequent non-apoptotic cell death. GA also reduced the in vivo growth of RCC tumor xenografts without causing significant toxicity in mice. These findings identify GA 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 canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM 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 (Manassas, VA) 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 prior to use.

Reagents:

Gramicidin A, monensin, ouabain, and rhodamine 123 were purchased from Sigma-Aldrich (St. Louis, MO).

Plasmids and Transfections:

pcDNA3-HA-HIF1α (Addgene plasmid 18949, William Kaelin) (26), pcDNA3-HA-HIF1α-P402A/P564A (Addgene plasmid 18955, William Kaelin) (27) were purchased from Addgene (Cambridge, MA). pcDNA3 vector was purchased from Life Technologies (Grand

Downloaded from mct.aacrjournals.org on June 20, 2017. © 2013 American Association for Cancer Research.
Island, NY). Transfections were accomplished using Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to manufacturer's instructions.

**Antibodies:**

We purchased primary antibodies specific for HIF-1α (BD Biosciences, San Jose, CA), HIF-2α (Novus Biologicals, Littleton, CO), HA-tag, GAPDH, cleaved-PARP, α-tubulin, AMPK, phospho-AMPK (T172), ACC, phospho-ACC (S79), (Cell Signaling, Danvers, MA), and β-actin (Sigma-Aldrich, St. Louis, MO). Horseradish peroxidase (HRP)-conjugates secondary antibodies were purchased from Cell Signaling (Danvers, MA).

**Cell Viability Assay:**

The CellTiter-Blue cell viability assay was performed according to manufacturer's instructions (Promega Corporation, Madison, WI). 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 72hr. Next, 20µL of CellTiter-Blue reagent was added to each well and incubated for an additional 4hr. Fluorescence was then read at 560nm using a VictorX4 plate reader (Perkin-Elmer, Waltham, MA). Readings were corrected for background fluorescence using the fluorescence value from the media only and relative cell viability was normalized to vehicle-treated samples. Data represent the mean±SE of three independent experiments. The data obtained were further analyzed using Prism nonlinear regression software (GraphPad Software Inc., La Jolla, CA) for curve fitting and determination of absolute IC\textsubscript{50} values.

**Immunoblot Analysis:**
Cell lysates were prepared in a buffer containing containing 95 mM NaCl, 25 mM Tris pH 7.4, 0.5 mM EDTA, and 2% SDS. Lysates were sonicated, centrifuged, and the protein concentration of the supernatants were determined using the DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein were then resolved by SDS-PAGE and transferred to nitrocellulose. The membranes were blocked in 5% non-fat milk in tris-buffered saline with 0.1% Tween 20 (TBS-T) and then 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% non-fat milk/TBS-T at room temperature for 1hr. The protein bands were visualized using Amersham ECL Prime (GE Healthcare, Piscataway, NJ). Following visualization, immunoblots of phosphorylated proteins were stripped from the nitrocellulose membrane by washing with buffer containing 50mM Tris pH 8.0, 2% SDS, and 0.7% 2-mercaptoethanol at 55°C and then immunoblottedted for unphosphorylated total proteins as described above. Images were generated using Photoshop (Adobe Systems Inc., San Jose, CA) and quantified using ImageJ (NIH, Bethesda, MD).

**Phase-Contrast Microscopy:**

Phase-contrast images were acquired using a Leica DM IL microscope equipped with a DCF 420 C camera and Leica Application Suite Software (Leica Microsystems GmbH, Wetzlar, Germany). Images were processed using Photoshop (Adobe Systems Inc., San Jose, CA).

**Cell Death Assay:**

The Cytotox96 nonradioactive assay was performed according to manufacturer’s instructions (Promega, Madison, WI). Briefly, cells were seeded in triplicate into 12-well plates
in phenol-free DMEM supplemented with 0.1 - 0.5% FBS and allowed to attach overnight. Cells were then treated with vehicle (ethanol) or GA for 72hr. Conditioned medium was collected to assay for lactate dehydrogenase (LDH) released from the dead cells (A_{490} from medium). An equivalent volume of medium was replaced in the plate and incubated at -80°C for 60min to lyse cells. This medium was then thawed and collected to be assayed for LDH released from the viable cells. Samples were clarified by centrifugation and 50μL aliquots were added to 50μL LDH substrate in a 96-well plate. The reaction was terminated after 30min using 50μL stop solution and absorbance values at 490nm were obtained using a VictorX4 plate reader (Perkin-Elmer Inc., Waltham, MA). Readings were corrected for background absorbance using the absorbance value from the media only. Percentage cell death was calculated using the equation %LDH released = (A_{490} from medium)/(A_{490} from plate + A_{490} from medium) X 100 and normalized to vehicle-treated samples. Data represent the mean±SE of three independent experiments.

**DNA Fragmentation Assay:**

Cells were seeded into 100mm dishes, incubated overnight, and then treated with vehicle or drug for 48hr. Following treatment the cells were removed by scraping the dishes, washed in 1X PBS, and lysed in lysis buffer (100mM Tris-HCl pH 7.5, 10mM EDTA, 0.06% SDS). DNA was precipitated overnight at 4°C by adding NaCl to a final concentration of 1M. The lysate was then thawed, centrifuged to spin down the high molecular weight DNA, and the supernatant was subjected to phenol/chloroform extraction. The low molecular weight DNA contained in the aqueous phase was precipitated with 100% ethanol, pelleted, washed with 70% ethanol, and resuspended in 20μL TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 7.4) containing RNase A at
20µg/ml for 30min at room temperature. Samples were electrophoresed on a 1.2% agarose gel and ethidium bromide staining of the samples were visualized under ultraviolet light and photographed using a Geliance 600 Imaging System (Perkin-Elmer Inc., Waltham, MA).

**Cellular ATP Measurement:**

The CellTiter-Glo luminescent viability assay was used to quantify cellular ATP and was performed according to manufacturer's instructions (Promega, Madison, WI). Briefly, cells were seeded in triplicate into white-walled 96-well plates and treated with thymidine (1mM) overnight to block cell proliferation and maintain a constant cell number. Cells were then treated with vehicle (ethanol) or drug in culture media containing 1mM thymidine and incubated for 24hr. Plates were then equilibrated to room temperature for 30min and then 100µL of CellTiter-Glo reagent was added to each well. Plate contents were mixed for 2min using an orbital shaker and then incubated an additional 10min at room temperature before luminescence was recorded using a VictorX4 plate reader (Perkin-Elmer, Waltham, MA). 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 (1mM) overnight to block cell proliferation and maintain a constant cell number. Cells were then treated with vehicle (ethanol) or drug in culture media containing 1mM thymidine and incubated for 24hr. Cellular redox activity was measured using the CellTiter-Blue assay as described above.
Mitochondrial Transmembrane Potential Assay:

Cells were seeded into 60mm dishes and treated with thymidine (1mM) overnight to block cell proliferation and maintain a constant cell number. Cells were then treated with vehicle (ethanol) or drug in culture media containing 1mM thymidine and incubated for 24hr. Cells were then washed twice with 1X PBS and then incubated with 10μg/mL rhodamine 123 in 1X PBS supplemented with 100μM CaCl$_2$, 1mM MgCl$_2$, 0.2% BSA, and drug (GA or MON) for 30min at 37°C in the dark. After incubation the cells were washed twice with 1X PBS and then lysed in 200μL of 1X PBS supplemented with 0.02% SDS for 15min. Lysates were collected by scraping, added to the wells of a 96-well plate, and fluorescence was read at 535nm using a VictorX4 plate reader (Perkin-Elmer, Waltham, MA). 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 L-lactate and was performed according to manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). Cells were seeded in duplicate into 96-well plates, allowed to attach 4hr, and then treated with thymidine (1mM) 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 1mM thymidine. The cells were divided into two treatment groups of either 0-12hr or 12-24hr. For the 12-24hr samples the media was removed after the
initial 12hr incubation and replaced with fresh media to incubate an additional 12hr. Following incubation, 10µL of conditioned media was mixed with the kit reactants in a 96-well plate and absorbance values at 490nm were obtained using a VictorX4 plate reader (Perkin-Elmer Inc., Waltham, MA). 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 performed 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, Wilmington, MA) that were 6-8 weeks old were injected subcutaneously with a suspension of SN12C cells (1.0 x 10⁶) in a 50% growth factor-reduced matrigel (BD Biosciences, San Jose, CA) solution. SN12C tumors were allowed to grow for 1 week before randomization into control (vehicle solution only) and drug (GA) groups of 5 mice each with an average initial tumor volume of ~100mm³ as determined by caliper measurement using the formula \([(\text{length} \times \text{width}^2) / 2]\) where length was the longest of the two measurements. GA (0.11mg/kg body weight) was diluted in a 1:1 solution of ethanol and saline (0.9% NaCl), and mice were dosed twice weekly with 50µL of either vehicle or GA solutions by intratumoral injection. Mouse body masses were recorded before each injection. Upon completion of the study, mice were euthanized and the tumors were surgically excised and masses were recorded.
Statistics

Dose-response viability curves was performed by ANOVA, all other analyses were performed using unpaired two-tailed Student's T-test.
Results:

Treating with GA 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-carrier 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) (28) treated with GA and found that it caused a dose-dependent decrease in cell viability (Fig. 2A). These cell lines responded similarly to GA as the absolute IC₅₀ values for all cell lines were < 1μM (Table 1). This result shows that the viability of RCC cells is sensitive to treatment with GA.

Neither VHL nor HIF-1α expression significantly alters cellular sensitivity to GA:

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, SN12C) (Table 1). Given the significance of VHL in renal cancer pathology, we sought to ascertain whether its expression affects cellular sensitivity to GA in vitro. We first compared the viability of GA-treated isogenic UMRC6 cells (VHL-deficient) with that of VHL-reconstituted UMRC6+VHL cells. We found that these cell lines responded very similarly to GA (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). Additionally, because VHL-deficiency stabilizes HIF we also examined whether overexpression of HIF-1α affects cellular response to GA. 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) (27). No differences in the dose-response viability curves of these transfected cell lines were found in response to treatment with GA (Fig. 2D, \( P = 0.988 \) by one-way ANOVA). Analysis of the IC\(_{50}\) 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 GA reduces the viability of RCC cells in vitro in a manner that is independent of both VHL and HIF-1α expression.

**GA effects cell viability comparable to monensin:**

Next, we sought to compare the viability of RCC cells treated with either GA or the mobile-carrier ionophore monensin (MON). We selected MON for this comparison because 1) MON has been shown to inhibit the growth of RCC cells (2) and 2) both MON and GA exhibit selectivity for Na\(^+\) and K\(^+\) ions (29). Using both VHL-deficient (786-O) and VHL-expressing (SN12C) cell lines we found that GA and MON caused a dose-dependent decrease in cell viability as expected (Fig. 3A, B). Although the absolute IC\(_{50}\) value for GA-treated 786-O cells was slightly less than MON (0.430\(\mu\)M vs. 0.622\(\mu\)M), 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 GA than with MON (IC\(_{50} = 0.104\mu\)M vs. 1.443\(\mu\)M) 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.
GA induces non-apoptotic cell death in RCC cells:

Several investigations have reported that mobile-carrier ionophores (i.e. MON and SAL) induce apoptotic cell death in cancer cells. Microscopic analysis of MON-treated 786-O cells confirmed the presence of typical apoptotic morphological features such as cell rounding, membrane blebbing, and vacuolization (Fig. 4A, middle panel). However, GA-treated 786-O cells did not display these characteristics but instead featured prominent lamellipodia (arrows) and rounding of the nuclei (Fig. 4A, right panel). In addition, these cells showed floating cellular debris (data not shown). Similar characteristics were also observed in SN12C cells (data not shown). To further analyze the cytotoxicity of GA we measured the cell death-associated release of the stable cytosolic enzyme lactate dehydrogenase (LDH). Treatment of 786-O, SN12C, and Caki-1 cells (data not shown) with GA resulted in a dose-dependent increase in extracellular LDH (Fig. 4B) confirming that GA does induce cell death in RCC cells. Next, we sought to verify our observations in Fig. 4A that GA appears to elicit a non-apoptotic form of cell death. A common marker of apoptosis is the proteolytic cleavage of poly (ADP-ribose) polymerase (PARP). Immunoblot analysis detected PARP cleavage in MON-treated 786-O and SN12C cells but not in GA-treated cells (Fig. 4C). Another indicator of apoptosis is the degradation of cellular chromatin into nucleosome-length fragments that appear as a DNA "ladder" when separated by electrophoresis. Consistent with Fig. 4C, we observed electrophoretic DNA laddering in only MON-treated 786-O and SN12C cells (Fig. 4D). Altogether, these data signify that GA induces a non-apoptotic form of cell death in RCC cells.

GA depletes cellular energy in RCC cells:
Although GA and MON enhance permeability of cells to Na\(^{+}\) and K\(^{+}\), we were surprised to find that these drugs induced differing forms of cell death. A potential determinant of cell death mechanism is cellular energy status; apoptosis is an ATP-dependent process of self-degradation whereas necrosis is typically associated with rapid bioenergetic compromise and energy insufficiency (30). To evaluate whether GA depletes cellular energy we measured the ATP content of 786-O and SN12C cells treated with increasing doses of either GA or MON. As depicted in Fig. 5A, we observed that treatment with GA for 24hr decreased cellular ATP in both 786-O and SN12C cell lines. ATP reduction by GA was more pronounced in SN12C cells than in 786-O cells, which is consistent with the higher sensitivity of this cell line to GA (Fig. 5A, Table 1). Importantly, GA decreased cellular ATP levels to a greater extent than MON in SN12C cells at 1\(\mu\)M (GA = 18±2\% vs. MON = 41±14\%) and at 10\(\mu\)M in both cell lines (786-O = 45±10\% vs. 70±2\%, SN12C = 10±1\% vs. 24±0.3\%). These observations demonstrate that GA depletes cellular energy in RCC cells.

Cellular energy homeostasis is regulated by AMP-activated protein kinase (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 GA-treated 786-O and SN12C cells as a marker of cellular energy stress. Immunoblot analysis revealed that treatment with GA increased the phosphorylation of AMPK\(\alpha\) and its substrate acetyl-coA carboxylase (ACC) at both 24hr and 48hr (Fig. 5B). AMPK\(\alpha\) phosphorylation was more pronounced in SN12C cells than 786-O cells at 24hr 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 GA induces cellular energy stress in RCC cells.
Next we investigated whether cellular energy depletion caused by GA is due to disruption of \( \text{Na}^+ \) and \( \text{K}^+ \) homeostasis. The \( \text{Na,K-ATPase} \) pump is a key regulator of intracellular ionic homeostasis that transports \( 3\text{Na}^+ \) out of the cell and \( 2\text{K}^+ \) into the cell against their respective electrochemical gradients (32). Inhibition of the \( \text{Na,K-ATPase} \) blocks the active transport of these cations and permits the influx of \( \text{Na}^+ \) and efflux of \( \text{K}^+ \) similar to GA (33). Using the \( \text{Na,K-ATPase} \) inhibitor ouabain (0.5\( \mu \)M), we measured cellular ATP and observed that ouabain treatment mimicked GA 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 GA and ouabain decreased cellular ATP levels more than either drug alone (Fig. 5C). These results indicate that disruption of \( \text{Na}^+ \) and \( \text{K}^+ \) balance is involved in GA-induced energy depletion in RCC cells.

**GA induces metabolic dysfunction in RCC cells:**

The profound loss of cellular energy that resulted from treatment with GA may indicate that ATP production was impaired. Because cellular metabolism depends upon oxidation-reduction (redox) reactions, we first measured whether GA 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 GA for 24hr resulted in a significant 30-60% reduction in cellular redox activity at all doses (\( P < 0.05, \) Fig. 6A). In contrast, MON failed to significantly reduce activity in SN12C cells and produced only minimal inhibition in 786-O cells (GA = 36±3% vs. MON = 75±6% at 10\( \mu \)M). These observations indicate that GA 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. GA is well-known to conduct protons and depolarizes mitochondrial transmembrane potential thereby blocking ATP synthesis (34). In order to determine whether GA 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 GA for 24hr resulted in a 40-60% reduction in Rh123 uptake in 786-O and SN12C cells. In contrast, MON did not significantly alter Rh123 uptake in either cell line. This result indicates that GA depolarizes mitochondrial membrane potential and decreases oxidative phosphorylation-dependent ATP synthesis.

Next, we sought to determine whether GA impairs glycolysis in RCC cells by measuring the production of extracellular L-lactate, the end-product of glycolysis. Surprisingly, at 24hr the GA-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 24hr incubation into two 12hr periods and found that GA induced an initial increase in lactate levels in both cell lines during the first 0-12hr period (786-O = 236±17.7%, SN12C = 136±14.8%) (Fig. 6C, D). However, this elevation was not sustained as lactate levels fell significantly during the subsequent 12-24hr period (786-O = 83±13.4%, SN12C = 23±3.3%) (Fig. 6C, D). The increased accumulation of lactate at 12hr suggests that glycolysis was initially stimulated but later inhibited by GA.
GA inhibits the growth of RCC tumor xenografts:

To evaluate the *in vivo* anti-tumor efficacy of GA, we engrafted human SN12C RCC cells by subcutaneous injection into female Nu/J athymic nude mice. When the tumors reached an average size of ~100mm$^3$, the mice were randomized into two groups (n = 5) and administered 50μL of either vehicle solution (50% ethanol in saline) or GA (0.11mg/kg) by intratumoral injection twice weekly for 14 days. As shown in Fig. 7A, at necropsy the average tumor mass was reduced by ~40% with GA treatment (230±24mg vs. 140±20mg, $P = 0.0228$ by T-test). Importantly, intratumoral injection of GA did 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 GA is toxic to RCC cells *in vitro* and *in vivo*. This finding is significant because RCC is highly therapy-resistant malignancy. Mechanistically, we demonstrated that GA impairs oxidative phosphorylation and glycolysis leading to cellular ATP depletion and non-apoptotic cell death. Disruption of Na\(^+\) and K\(^+\) homeostasis by GA is likely involved in this depletion of energy. Our findings suggest that GA 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 VHL-negative RCC cell lines we found that GA reduced the *in vitro* viability of all of these cell lines similarly. We also employed 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 GA. Furthermore, we observed that GA reduced cellular energy and metabolism similarly in VHL-deficient (786-O) and VHL-expressing (SN12C) cells. These results show that histological subtype, VHL status, and HIF-1α expression/activity are not major factors that determine cellular response to GA. However, given the prominent role of VHL/HIF in regulating tumor angiogenesis, it is possible that the *in vivo* response of RCC cells to GA will differ based upon these factors. Experiments to test this possibility are currently underway in our laboratory.

MON has been reported to induce cell cycle arrest and apoptosis in RCC cells (2) and we also observed morphological and molecular features consistent with apoptotic cell death in MON-treated RCC cells. However, GA appears to induce a different cell death mechanism. Despite obvious cytotoxicity, we did not observe apoptotic features (membrane blebbing, PARP
cleavage and DNA laddering) in GA-treated cells. Additionally, marked cellular energy depletion and inhibition of glycolysis are both features of necrotic cell death (30) and both of these were observed in GA-treated cells. Furthermore, treatment of RCC cells with necrostatin-1, an inhibitor of necroptosis (36), failed to rescue GA-induced cell death (data not shown). Collectively, our observations signify that GA 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 anti-tumor immunity (30, 37). Thus, GA-induced necrotic cell death might also facilitate anti-tumor 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 GA-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 utilizes >50% of the cellular energy in the kidney (39) and it is stimulated by the imbalance of Na$^+$ and K$^+$ provoked by GA (29). However, we found that inhibition of the Na,K-ATPase with ouabain failed to rescue GA-induced ATP depletion but actually enhanced it. We finally assessed whether GA 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 GA reduced cellular redox activity, disrupted mitochondrial transmembrane potential, and decreased extracellular lactate production indicating that GA impairs glucose catabolism. The inhibition of glycolysis likely accounts for the bulk of energy depletion by GA since 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) as contributing to GA-induced energy stress.

Although GA disrupts oxidative phosphorylation directly through energetic uncoupling (34), the precise mechanism whereby GA initially stimulates and then subsequently inhibits glycolysis is currently not understood. Glycolysis is likely stimulated in order 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. Additionally, glycolysis depends upon the reduction of NAD\(^+\) to NADH, and we observed that GA produced a marked decrease in cellular redox activity. It is possible that GA 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 GA-treated cells).

Toxicity is an essential factor to consider in clinical drug development, yet most of the chemotherapeutics used clinically have substantial non-specific toxicity towards normal cells. GA is known to cause hemolysis and is toxic to the liver, kidney, meninges, and olfactory apparatus (14, 41). We tested the toxicity of GA using non-tumorigenic kidney cell lines from various species (LLC-PK1 (porcine), HK-2 (human), and MDCK (canine)) and observed IC\(_{50}\) values that were comparable to those of the RCC cell lines (data not shown). However, we have demonstrated here that GA 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 GA peptide may be used to decrease non-specific
toxicity (14), or encapsulation of GA 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 GA to reach its full therapeutic potential.

Acknowledgements:

We thank Dr. Andrew Napper for providing the CellTiter-Glo reagent and Dr. Landon Inge for providing the p-ACC and total ACC antibodies.
References:


Table 1: Calculated IC<sub>50</sub> values for GA-treated cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A498</td>
<td>0.420μM</td>
</tr>
<tr>
<td>786-O</td>
<td>0.430μM</td>
</tr>
<tr>
<td>Caki-1</td>
<td>0.228μM</td>
</tr>
<tr>
<td>SN12C</td>
<td>0.104μM</td>
</tr>
<tr>
<td>ACHN</td>
<td>0.783μM</td>
</tr>
<tr>
<td>UMRC6</td>
<td>0.253μM</td>
</tr>
<tr>
<td>UMRC6+VHL</td>
<td>0.425μM</td>
</tr>
<tr>
<td>HEK293T+pcDNA3</td>
<td>0.057μM</td>
</tr>
<tr>
<td>HEK293T+HA-HIF-1α</td>
<td>0.058μM</td>
</tr>
<tr>
<td>HEK293T+HA-HIF-1α-mut</td>
<td>0.067μM</td>
</tr>
</tbody>
</table>
Figure Legend:

Figure 1: GA structure and function. (A) Chemical structure of the GA peptide. (B) Dimerization of two GA monomers produces the functional transmembrane channel.

Figure 2: GA reduces the viability of RCC cell lines. (A) RCC cell lines were treated with increasing doses of GA for 72hr and cell viability was measured. (B) Stable isogenic UMRC6 and UMRC6+VHL cells were treated with increasing doses of GA for 72hr and cell viability was measured. (C) Immunoblot of transfected HEK293T cells used in (D) to confirm transgene expression. (D) HEK293T cells were transiently transfected with the indicated plasmids and treated with increasing doses of GA for 72hr and cell viability was measured.

Figure 3: GA reduces viability comparable to MON. (A) 786-O and (B) SN12C cells were treated with increasing doses of either GA or MON for 72hr and cell viability was measured. Graphs depict mean±SE of three independent experiments.

Figure 4: GA induces non-apoptotic cell death. (A) Phase-contrast images of 786-O cells treated with vehicle (ethanol) or 10μM of either MON or GA for 48hr, scale bar = 100μm. (B) RCC cell lines were treated with increasing doses of GA for 72hr and cell death was measured by LDH release assay. Graph depicts mean±SE of three independent experiments, * indicates P < 0.05 by T-test. (C) 786-O and SN12C cells were treated with either GA or MON for 48hr and immunoblotted for the presence of the 89kDa cleavage product of PARP. (D) 786-O and SN12C cells were treated with either GA or MON for 48hr and low molecular weight DNA was isolated.
and analyzed for electrophoretic laddering. Positive control = DNA from serum-starved Madin-Darby canine kidney (MDCK) cells.

Figure 5: GA depletes cellular energy. (A) 786-O and SN12C cells were treated with increasing doses of either GA or MON for 24hr and cellular ATP was measured. (B) 786-O and SN12C cells were treated with GA or MON and AMPK pathway activation was assessed by immunoblot. (C) Cells were treated with either 5μM GA (786-O) or 0.5μM GA (SN12C), 0.5μM ouabain (oua, both cell lines), or combination for 24hr and cellular ATP was measured. Graphs depict mean±SE of three independent experiments, * indicates *P* < 0.05, ** indicates *P* < 0.005 by T-test.

Figure 6: GA impairs cellular metabolism. (A) 786-O and SN12C cells were treated with GA or MON for 24hr and cellular redox activity was measured. (B) 786-O and SN12C cells were treated with GA or MON for 24hr and mitochondrial transmembrane potential was measured. Graphs depict mean±SE of three independent experiments. (C and D) 786-O (C) and SN12C (D) cells were treated with 5μM or 1.0μM GA, respectively, for the indicated time periods and the production of extracellular lactate was measured. Graph depicts mean±SD of one representative of three independent experiments. * indicates *P* < 0.05, ** indicates *P* < 0.005 by T-test.

Figure 7: 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.
Figure 1:
Figure 2:

A

B

C

D
Figure 3:
Figure 5:

**A**

![Graph showing relative ATP (%) vs. drug dose (μM) over 24hr for 786-O and SN12C cell lines.](image)

**B**

<table>
<thead>
<tr>
<th>786-O</th>
<th>SN12C</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-AMPKα (T172)</td>
<td>1.00</td>
</tr>
<tr>
<td>AMPKα</td>
<td>1.00</td>
</tr>
<tr>
<td>p-ACC (S79)</td>
<td>1.00</td>
</tr>
<tr>
<td>ACC</td>
<td>1.00</td>
</tr>
<tr>
<td>β-actin</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**C**

![Graph showing relative ATP (%) vs. drug concentration over 24hr for 786-O and SN12C cell lines.](image)
Figure 6:
Figure 7:

A

B

Downloaded from mct.aacrjournals.org on June 20, 2017. © 2013 American Association for Cancer Research.
Molecular Cancer Therapeutics

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

Justin M. David, Tori A Owens, Sonali P Barwe, et al.

Mol Cancer Ther Published OnlineFirst September 4, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0445

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.