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1,2,3,4,6-penta-O-galloyl- β -D-glucopyranose (PGG) Inhibits Angiogenesis via Inhibition of CMG2

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Abstract

CMG2 is a transmembrane extracellular matrix binding protein that is also an anthrax toxin receptor. We have shown that high affinity CMG2 binders can inhibit angiogenesis and tumor growth. We recently described a high throughput FRET assay to identify CMG2 inhibitors. We now report the serendipitous discovery that PGG (1,2,3,4,6-penta-O-galloyl- β -D-glucopyranose) is a CMG2 inhibitor with anti-angiogenic activity. PGG is a gallotannin produced by a variety of medicinal plants that exhibits a wide variety of anti-tumor and other activities. We find that PGG inhibits CMG2 with a submicromolar IC₅₀ and it also inhibits the migration of human dermal microvascular endothelial cells at similar concentrations *in vitro*. Finally, oral or intraperitoneal administration of PGG inhibits angiogenesis in the mouse corneal micropocket assay *in vivo*. Together, these results suggest that a portion of the *in vivo* anti-tumor activity of PGG may be the result of antiangiogenic activity mediated by inhibition of CMG2.

Keywords

pentagalloyl glucose; 5GG; angiogenesis; corneal neovascularization; cancer; polyphenol

INTRODUCTION

Angiogenesis is the process by which new blood vessels are generated from existing vessels. It is necessary whenever more than a few mm of additional tissue are generated and often accompanies tissue remodeling. As a result, a wide variety of diseases are angiogenesis-dependent. These include cancer, psoriasis, macular degeneration, and diabetic retinopathy¹. Angiogenesis is initiated by pro-angiogenic growth factors such as bFGF and VEGF and begins when endothelial cells partially degrade their basement membrane and migrate into the surrounding stroma using a provisional matrix composed of collagen I, fibrin, vitronectin

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Author Contributions

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Supporting Information

Figures S1 and S2, NMR spectra of purified compounds and characterization by NMR and LC-MS together with UV of purchased PGG. This material is available free of charge via the internet at <http://pubs.acs.org>.

and fibronectin. Migration through the stroma is dependent on MMPs including MMP2 and MMP14 and is led by endothelial “tip cells”. Following the formation of the nascent vessel, a new basement membrane composed of laminin, collagen IV, nidogen and perlecan is laid down cooperatively by pericytes and endothelial cells and this stabilizes the vessel both mechanically and against additional angiogenic stimulus².

Capillary morphogenesis gene 2 (CMG2) was originally identified as a gene up-regulated in endothelial cells undergoing tube formation in collagen gels³. It is composed of an amino-terminal, metal binding von Willibrandt factor (vWF) domain, an Ig-like domain, a transmembrane domain, and a hydrophilic intracellular domain³. It is the primary receptor for anthrax toxin, which binds via the vWF domain, and crystal structures of the complex have been generated^{4–6}. In the context of anthrax intoxication, CMG2 has been well studied⁷, however its natural function in the absence of toxin is less well established. Its vWF domain has homology with integrins and like integrins binds to extracellular matrix molecules including collagen IV, laminin, and fibronectin⁸. Certain mutations in CMG2 result in Hyaline Fibromatosis Syndrome which is characterized by the accumulation of hyaline material in the skin and other organs⁹. In contrast, mouse knock-outs do not display this phenotype and are fertile, but cannot perform MMP14-dependent uterine remodelling essential to parturition. CMG2 is expressed in tumor vasculature¹⁰ and modification of CMG2 expression affects the ability of endothelial cells to proliferate and form networks in extracellular matrix¹⁰. Modulation of binding also appears to affect the ability of endothelial cells to migrate *in vitro*¹¹. *In vivo*, we have observed that anthrax protective antigen and mutants with increased half-life exhibit antiangiogenic activity in the corneal micropocket assay in a CMG2-binding dependent manner¹¹. As expected, these agents also inhibit tumor growth in mice¹¹.

PGG (1,2,3,4,6-penta-O-galloyl- β -D-glucopyranose) is a very common polyphenol plant metabolite. It has been studied in a number of diseases including several models of cancer¹², where oral administration leads to a decrease in tumor growth¹³. It inhibits a wide range of biological processes, including PTP1B¹⁴, angiogenesis¹⁵, VEGF receptor binding¹⁶, and DNA polymerase activity¹⁷. However, most of these processes are only inhibited at PGG concentrations in the tens of micromolar. In contrast, recent pharmacokinetic studies demonstrate that intraperitoneal administration of PGG results in single-digit micromolar plasma concentrations and oral administration results in submicromolar plasma concentrations¹⁸. These contrasting results indicate that the pharmacologic target responsible for inhibition of tumor growth by PGG may not yet have been identified. Here we report the serendipitous discovery of CMG2 as additional target for PGG action with the potential to explain a portion of the antitumor activity of the molecule.

RESULTS

As previously reported¹⁹, the screening of a “known bioactives” library for CMG2 inhibitors resulted in a “hit” on tannic acid. Further characterization of this hit demonstrated a complex inhibition curve and toxic effects upon administration in mice¹⁹, damping our enthusiasm for further study in the context of angiogenesis. However, during follow-up experiments at Clemson University we discovered that there were dramatic differences in the anti-CMG2 activity of separate lots of tannic acid, even though they were from the same supplier. In order to ascertain why one sample was active, as determined by the CMG2 FRET assay, while another was not, HPLC analysis was carried out. In the HPLC chromatogram of the CMG2 inactive “tannic acid” sample, one broad peak was observed, while many peaks were exhibited in the chromatogram of the CMG2 active “tannic acid” sample (Figure 1A and B). Clearly, the active “tannic acid” sample is a mixture of many compounds, and we

hypothesized that one of the contaminants was responsible for the significant anti-CMG2 activity observed with the impure sample.

To test this hypothesis, an equivalent dose study was carried out. Forty fractions were collected after a gradient HPLC and tested in the FRET assay. This resulted in the identification of two active peaks. Based on the UV and the polarities of these peaks, the HPLC program was modified, and these two peaks were well separated from the others. One, SC1-129-26 (Retention Time: 22.5 min; Figures 1B), was identified as 1,2,3,4,6-penta-O-galloyl- β -D-glucopyranose (PGG), and another, SC1-129-18 (Retention Time: 18.5 min; Figures 1B), digallic acid (see Supplementary Figures). Both PGG and digallic acid were active against CMG2 with an IC_{50} of $\sim 50 \mu M$ for digallic acid and a complex inhibition curve for PGG that exhibited 50% of maximal CMG2 inhibition at ~ 300 nM (Figure 1C). We have also previously observed that CMG2 interaction with PA is inhibited by phenolic natural products²⁰. Given these results, we also assessed methyl gallate, propyl gallate, and a number of additional polyphenols (Table 1) at concentrations up to $300 \mu M$. None of these compounds exhibited any inhibitory activity.

Since PGG was the more potent of the compounds that had been identified, we decided to assess its capacity to inhibit angiogenesis *ex vivo* and *in vivo*. We have observed that both native Anthrax Protective Antigen (PA) and PA^{SSSR}, a mutated form of PA that spends a prolonged period of time at the receptor surface without being internalized, inhibit the migration of human dermal microvascular endothelial cells (HMVEC-d)¹¹. We assessed PGG's effect in this same *ex vivo* assay. As is true of PA^{SSSR}, PGG inhibited endothelial cell migration at doses where it exhibited no cytotoxic or cytostatic effects (Figure 3).

We went on to assess the effect of PGG on angiogenesis in the corneal micropocket assay. When delivered at a dose of 10 mg/kg IP, or 20 mg/kg/day by oral gavage, PGG significantly inhibited bFGF-driven angiogenesis. Importantly we observed no obvious signs of toxicity (weight loss, hunched posture, behavioral abnormalities) indicating that the reduction in angiogenic response is a result of a pharmacologic effect of PGG, rather than general animal toxicity. These results are consistent with previous results demonstrating that PGG can be delivered both IP and orally at doses up to 30 mg/kg/day without ill effects¹².

DISCUSSION AND CONCLUSION

Here we demonstrate that PGG has anti-CMG2 activity *in vitro* in a FRET-based binding assay, *ex vivo* in an endothelial cell migration assay, and that it has antiangiogenic activity *in vivo* in the corneal micropocket assay. The biphasic inhibition curve that we observe in the FRET assay is unusual and remains to be explained.

In addition to these results, PGG has previously been shown to have a wide variety of effects in both *in vitro* and *in vivo* assays¹². *In vivo*, these effects include inhibition of VEGF binding to VEGF receptor¹⁶, insulin mimetic activity²¹, inhibition of tumor growth^{13, 15}, and inhibition of angiogenesis¹⁵. Importantly the antiangiogenic activity of PGG has been evoked at doses of 4 mg/kg¹⁵. Recent studies demonstrate that even a relatively high oral PGG dose of 80 mg/kg leads to circulating concentrations $<1 \mu M$ in experimental animals¹⁸. This is likely in part a result of degradation as the compound passes the gut²². Thus, any effect induced by oral delivery of PGG must be mediated by an interaction in the submicromolar range.

Among published *in vitro* PGG targets, however, only a few mammalian proteins have IC_{50} 's in the submicromolar range¹⁷. These include inhibition of certain DNA polymerases¹⁷, and the H⁺, K⁺, ATPase²³. Molecular weight and solubility indicate that it is unlikely that PGG can penetrate the cell membrane and this is confirmed by the saturable

nature of the transport of PGG in Caco-2 monolayers²². As a result it seems unlikely that the *in vivo* effects of PGG are mediated by inhibition of DNA polymerase. This notion is confirmed by studies of PGG on a wide variety of cell lines, including our data on HMVEC, none of which show inhibition of proliferation with IC₅₀'s in the nanomolar range. It is possible that polymerase-inhibitory intracellular PGG concentrations are achieved by much higher extracellular concentrations. However, in endothelial cells we observe the cytostatic effects of PGG (which might be mediated by polymerase inhibition) at concentrations at least an order of magnitude above the concentrations at which inhibition of migration is observed. Thus, in this cell type antimigratory effects which can be mediated by CMG2 are observed at much lower concentrations than antiproliferative effects that could be mediated by DNA polymerase. Nevertheless it remains possible that endothelial cells are substantially more permeable to PGG *in vivo* than *ex vivo*. In that case, DNA polymerase activity may also play a significant role in the antiangiogenic activity of PGG. It is also possible that the antiangiogenic effects that we observed *in vivo* are mediated by the H⁺, K⁺, ATPase; however, there are no examples in the literature of angiogenesis modulation by the H⁺, K⁺, ATPase. In addition, the H⁺, K⁺, ATPase inhibitor omeprazole fails to inhibit angiogenesis at therapeutic concentrations²⁴.

In contrast, our results demonstrate that PGG has a submicromolar IC₅₀ in the *in vitro* FRET binding assay as well as in the *ex vivo* endothelial cell migration assay. In addition, both oral and IP administration results in inhibition of angiogenesis in the corneal micropocket assay. Together, these results demonstrate that PGG interacts directly with CMG2 and is a potent inhibitor of angiogenesis. Combined with our observation that an anthrax protective antigen and related CMG2 antagonist can inhibit endothelial cell migration *ex vivo* and angiogenesis *in vivo*, it seems likely that at least a portion of the antiangiogenic effects that we and others have observed *in vivo* are a result of the CMG2 inhibitory activity.

EXPERIMENTAL SECTION

General Methods

All NMR experiments were carried out on a Varian INOVA 600 MHz spectrometer. Digallic acid and PGG (1,2,3,4,6-penta-O-galloyl- β -D-glucopyranose) were purified from a "tannic acid" sample (Sigma Aldrich, catalog number 403040-100g, lot #: 06817CJ) on an Agilent 1100 series HPLC (Agilent Technologies) using a semi-preparative Phenomenex Luna Phenyl-hexyl column (25 cm $\times$ 10 mm, 5 μ m particle size) with a flow-rate of 10 mL/min (solvent A: H₂O with 0.1% formic acid; solvent B: acetonitrile with 0.1% formic acid). A second tannic acid sample from the same supplier (Sigma Aldrich, catalog number 403040-50G, lot# MKBC5527) produced a much simpler chromatogram suggesting higher purity. Commercially purchased PGG (Toronto Research Chemicals, Supplier's ID D270450 and AvaChem Scientific, San Antonio, TX, lot #: 100926) was characterized by NMR and LC-MS together with UV, and found to be authentic and >95% pure (Figure S1).

Purification of digallic acid and PGG

The "tannic acid" sample was dissolved in methanol as a 10 mg/mL solution, and filtered for HPLC study. A 1 mL solution was injected into a Phenyl-hexyl column, and the column was eluted with aqueous acetonitrile (10–100% in 30 min, and 100% for 10 min). Forty fractions were collected from 10 to 30 min, 1 fraction per 30 sec. All the forty fractions were tested in the FRET assay and only fraction SC1-129-18 (t_R 18.5 min) and SC1-129-26 (t_R 22.5 min) were active. The HPLC purification was repeated four times to obtain enough SC1-129-18 (1 mg) and SC1-129-26 (1.3 mg) for structure elucidation. SC1-129-18 and SC1-129-26 were identified as digallic acid and PGG, respectively, by comparison of the spectroscopic data with a commercially purchased digallic acid (Supplier's ID D445920) and authentic

PGG (Supplier's ID D270450) from Toronto Research Chemicals (See Figures S1 and S2). PGG identification was further achieved by the comparison of the spectroscopic data obtained with those in the literature^{25, 26}.

FRET assay

The CMG2-Protective antigen (PA) FRET assay was performed as described previously¹⁹. Briefly, site directed mutagenesis was used to generate plasmids coding for full length PA, with an E733C mutation (PA^{E733C}) and CMG2 residues 40–217 with C175A and R40C mutations (CMG2^{R40C C178A}). These proteins were produced in *E. coli* and purified as described previously, using combinations of ion exchange (HP Q-Sepharose; GE Healthcare), affinity (GST Bind Agarose; Novagen), and size exclusion chromatography (Sephacryl 200HR; GE Healthcare)²⁷. PA was labeled with Alexa fluor 488 C5 maleimide and CMG2 was labeled with Alexa fluor 546 C5 maleimide (Invitrogen). For compound characterization, serial dilutions of compound were prepared in a final volume of 10 μ l HBST (50 mM HEPES pH 7.4, 150 mM NaCl, 0.1 mM CaCl₂, 0.1% Tween-20) in wells of a 384-well black polypropylene plate (Corning). Following compound plate preparation, 20 μ l of a solution of 26 nM CMG2^{R40C C178A}*AF546 in HBST was added using a WellMate liquid handling robot (Matrix Technologies) with integrated stacker. Next, 10 μ l of a 30 nM PA^{E733C}*AF488 solution in HBS (50 mM HEPES pH 7.4, 150 mM NaCl, 0.1 mM CaCl₂) was then added to all wells using the Wellmate and plates were incubated for 3–4 hours. Final CMG2 concentration (13 nM) and PA concentration (7.5 nM) were sufficient to promote quantitative binding of CMG2 in the absence of effective inhibitors, based on the previously reported K_d (170 pM)²⁷. Following incubation, plates were read on an Envision (PerkinElmer) plate reader using a 485/14 excitation filter, with 535/25 and 595/60 emission filters. For each plate, positive controls were generated by including 16 wells containing 10mM EDTA in HBST and negative controls by including 16 wells containing 10 mM NaCl in HBST.

Mouse corneal micropocket assay

The corneal micropocket assay was performed as described²⁸ using pellets containing 80 ng of bFGF (Peprotech, Rocky Hill, NJ) in C57BL/6J mice. Groups of five mice each received 20 mg/kg PGG or carrier by daily oral gavage in 5% ethanol. Alternatively, PGG (AvaChem) was delivered intraperitoneally at 10 mg/kg/day in a vehicle consisting of 2.5% (v/v) DMSO, 5% (v/v) Cremaphor EL, 5% (v/v) Tween-80, 7.5% (v/v) N-methyl-2-pyrrolidone, and 0.9% (w/v) NaCl, with controls receiving vehicle alone. Treatment was started on the day of pellet implantation. The area of vascular response was assessed on the fifth postoperative day using a slit lamp. Typically 10 eyes per group were measured.

Proliferation assay

Human microvascular endothelial cells (Lonza, Walkersville, MD) were maintained in EGM-2 (Lonza, Walkersville, MD) according to the vendor's instructions and used before passage 7. On day 0, HMVEC-d cells were seeded into 96-well plates, at a concentration of 2000 cells per well. After attachment, media was exchanged for media containing the indicated treatment. Cells were allowed to grow for 1 or 3 days and then quantified using Cyquant (Invitrogen, Carlsbad, CA) according to manufacturer's directions. The degree of proliferation in culture was measured by comparing wells in each plate fixed in absolute ethanol on day 0 to experimental wells, with fold-proliferation calculated by dividing Cyquant fluorescence readout in experimental wells by that in day 0 wells. Groups were compared using Student's t-test, with Bonferroni correction where appropriate.

Migration assay

Human microvascular endothelial cells were maintained as above. Polycarbonate transwell inserts, 6.5 mm diameter with 8.0 μ m pores, were coated with fibronectin (BD BioScience, San Jose, CA). Cells were harvested and resuspended in EBM (Lonza, Rockland, ME) containing 0.1% BSA (Sigma Aldrich, St Louis, MO). 20,000 cells/well were plated onto the inserts and these were placed into wells of a 24-well plate containing full serum media alone, or media containing the molecule to be tested. Cells were allowed to migrate for 4 hours. Cells on the top of the membrane were removed using cotton-tipped applicators and membranes were fixed and processed using Diff-Quick (Dade diagnostics, Aguada, PR). Membranes were removed from the insert using a scalpel and mounted on slides, and the number of cells in each of 4 10X microscopic fields were counted using a Leica Galen III stereomicroscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NON-STANDARD ABBREVIATIONS USED

CMG2	Capillary Morphogenesis Gene 2
ANTXR2	anthrax toxin receptor 2
EGM2	Endothelial growth medium 2
bFGF	basic fibroblast growth factor, FGF2
HMVEC-d	human dermal microvascular endothelial cells
PTP1B	protein-tyrosine phosphatase 1B
PA	protective antigen
PA^{SSSR}	a protective antigen mutant in which 3 amino acids at the furin cleavage site are mutated to serine
PGG	1,2,3,4,6-penta-O-galloyl- β -D-glucopyranose, pentagalloyl glucose, 5GG
vWF	von Willebrand factor

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Figure 1. Identification of the active compounds in the "tannic acid" sample. The scheme used to purify CMG2-active compounds (A) is shown along with a representative HPLC-trace (B). CMG2 FRET assay results on serial dilutions of the starting material and crucial active fractions are also shown (C).

Figure 2. Effect of digallic acid and PGG in the CMG2 FRET assay. Serial dilutions of pure samples of digallic acid (A) and PGG (B) were included in the CMG2 FRET assay. Curves represent best fit binding isotherms. Error bars indicate standard deviation of mean from at least four independent replicates. Structures of the compounds are indicated on the right.

Figure 3.

Effects of PGG on endothelial cells *ex vivo*. A) Endothelial cell migration. Cells were allowed to migrate to media containing 5% serum for 4 hours. The average of two independent experiments ($n=3$ for each experiment), performed on separate days is presented. Data was normalized to the no-treatment control to account for day-to-day variation in number of cells migrated. Curve represents a standard binding isotherm with IC_{50} of 820 nM. B) Endothelial cell proliferation. Cells were plated in EGM2 or EGM2 with PGG with 2000 cells per well and assessed at 24 and 72 hours. The average of four independent experiments ($n=4$ wells for each experiment), performed on separate days is presented. Data was normalized to the average of four untreated wells to account for day-to-day variation in number of cells plated. T0 represents the readout from cells at the start of the assay without treatment, where wells were fixed in absolute ethanol on day 0. The point at 0 μ M represents a vehicle control. Error bars indicate standard deviation.

20mg/kg daily, oral gavage

Figure 4.

Effect of PGG treatment on corneal angiogenesis *in vivo*. (Top) Vessel area in response to an 80 ng pellet of bFGF following daily oral treatment with 20 mg/kg PGG or vehicle (5% ethanol) control. The observed difference is statistically significant ($P < 0.001$ by t-test, $n = 10$ eyes for control and 9 eyes for PGG). (Bottom) Picture of a representative eye for each group showing difference in vessel area elicited in control and PGG treated animals.

Table 1Polyphenols assessed for inhibition in the CMG2 FRET assay.^a

Compound	IC ₅₀ (μM)
Tannic acid	>300
PGG	0.3
Digallic Acid	50
Methyl Gallate	>300
Propyl Gallate	>300
Epigallocatechin	>300
Gallic Acid	>300
Dopamine	>300
Myricetin	>300
Vanillic Acid	>300
Quercetin	>300
3-(3,4-dihydroxy phenyl) propionic acid	>300
Methyl-3,4-dihydroxybenzoate	>300
(-)-Epigallocatechin	>300
Protocatechillic acid	>300
Curcumin	>300
(-)-Epicatechin	>300
Caffeic acid	>300

^aCompounds were assessed for inhibition of PA-CMG2 interaction by FRET at a single high concentrations, and then, where appropriate at 2-fold dilutions across a six order of magnitude concentration range, with IC₅₀ determined by nonlinear fitting to the Hill equation.