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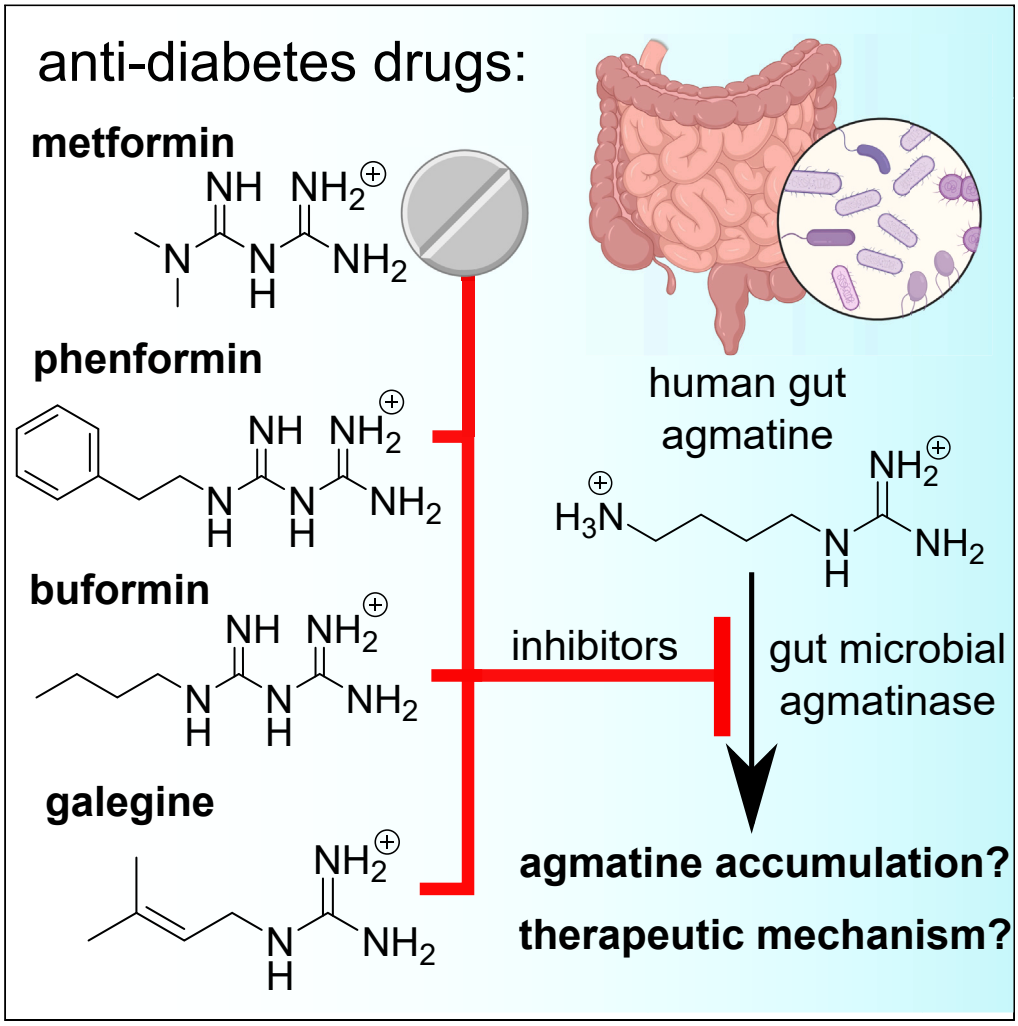

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Highlights

Metformin inhibits gut microbial agmatinases at a level relevant in the human gut

Buformin, phenformin, and the natural product galegine are more potent inhibitors

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Insights into the action of the pharmaceutical metformin: Targeted inhibition of the gut microbial enzyme agmatinase

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SUMMARY

Metformin is the first-line treatment for type 2 diabetes, yet its mechanism of action is not fully understood. Recent studies suggest metformin's interactions with gut microbiota are responsible for exerting therapeutic effects. In this study, we report that metformin targets the gut microbial enzyme agmatinase, as a competitive inhibitor, which may impair gut agmatine catabolism. The metformin inhibition constant (K_i) of *E. coli* agmatinase is 1 mM and relevant in the gut where the drug concentration is 1–10 mM. Metformin analogs phenformin, buformin, and galegine are even more potent inhibitors of *E. coli* agmatinase (K_i = 0.6, 0.1, and 0.007 mM, respectively) suggesting a shared mechanism. Agmatine is a known effector of human host metabolism and has been reported to augment metformin's therapeutic effects for type 2 diabetes. This gut-derived inhibition mechanism gives new insights on metformin's action in the gut and may lead to significant discoveries in improving metformin therapy.

INTRODUCTION

Metformin is the first-line treatment for type 2 diabetes with an unknown mechanism and prescribed to hundreds of millions of patients annually. Beyond the prescribed usage, metformin has been identified to also have anti-tumor and anti-obesity properties.[1,2] The versatility of the drug to promote healthy outcomes in treatment has prompted intense scrutiny of its mechanism of action in the past two decades. Recent studies identify metformin's interaction with human gut microbes to be responsible for the drug's therapeutic effects.[3–6] In the human gut, the concentration of metformin is estimated to vary between 1 and 10 mM while the concentration of the drug once absorbed into the portal vein, entering the liver, is reported to be between 10 and 40 μM.[5] This level of concentration in human tissue is orders of magnitude less than the inhibitory concentrations found for metformin acting on certain human targets. In addition, a study that conducted intravenous administration of metformin to treat type 2 diabetes patients, which mainly circumvents the human gut, showed little or no effectiveness.[7]

Human gut microbes are known to be a key indicator in human health and modulation of the gut microbiome is seen as a new frontier for therapeutic intervention.[8] Targeting the human gut can be problematic due to the variation of the gut microbiome from patient to patient which may cause variable side effects or potency.[9] Approximately 30% of patients treated with metformin suffer from gastrointestinal symptoms and another 20% of type 2 diabetes patients are non-responders to the drug and require alternative medication.[10,11] Elucidating the direct mode of action of metformin in the gut could help reveal key improvements that can improve the therapy and ameliorate side effects. The recommended dose for metformin is 1000–2000 mg daily for patients which is not metabolized by human enzymes and >80% is excreted into wastewater, making the drug one of the most pervasive pollutants in the world.[12]

Recently, we discovered the genes and enzyme in microbes responsible for transforming metformin to guanylurea in wastewater treatment plants.[13] This was an effort to determine metformin metabolic gene markers that could be used to identify similar genes in human gut metagenomes and thus possibly reveal gut drug metabolism. The discovered enzyme, metformin hydrolase, is homologous to members of the ureohydrolase protein superfamily like agmatinase and arginase which are metal dependent using binuclear, divalent metals. Several metformin-degrading bacteria, encoding metformin hydrolase (seq. id. > 97%), have been isolated from wastewater treatment plants across three continents (North America, Europe, and Asia) suggesting the enzyme is widespread.[13,14,15] Surprisingly, we were able to identify metformin-degrading genes in freshwater, but not wastewater, metagenome sequences.

The metformin hydrolase genes *mfmA* and *mfmB* encode a nickel-dependent binuclear metalloenzyme that is made from the complex of MfmA of and MfmB proteins with the MfmA subunit containing the active site (Figure 1A).[13] Using the MGnify database, which allows for a protein sequence to be searched against hundreds of thousands translated metagenome datasets, found no close matches (>30% seq. id.) to MfmA or MfmB sequences in recorded human gut metagenomes, as of August 19th, 2023 (Figure 1A).[16] This inconclusive result led us to

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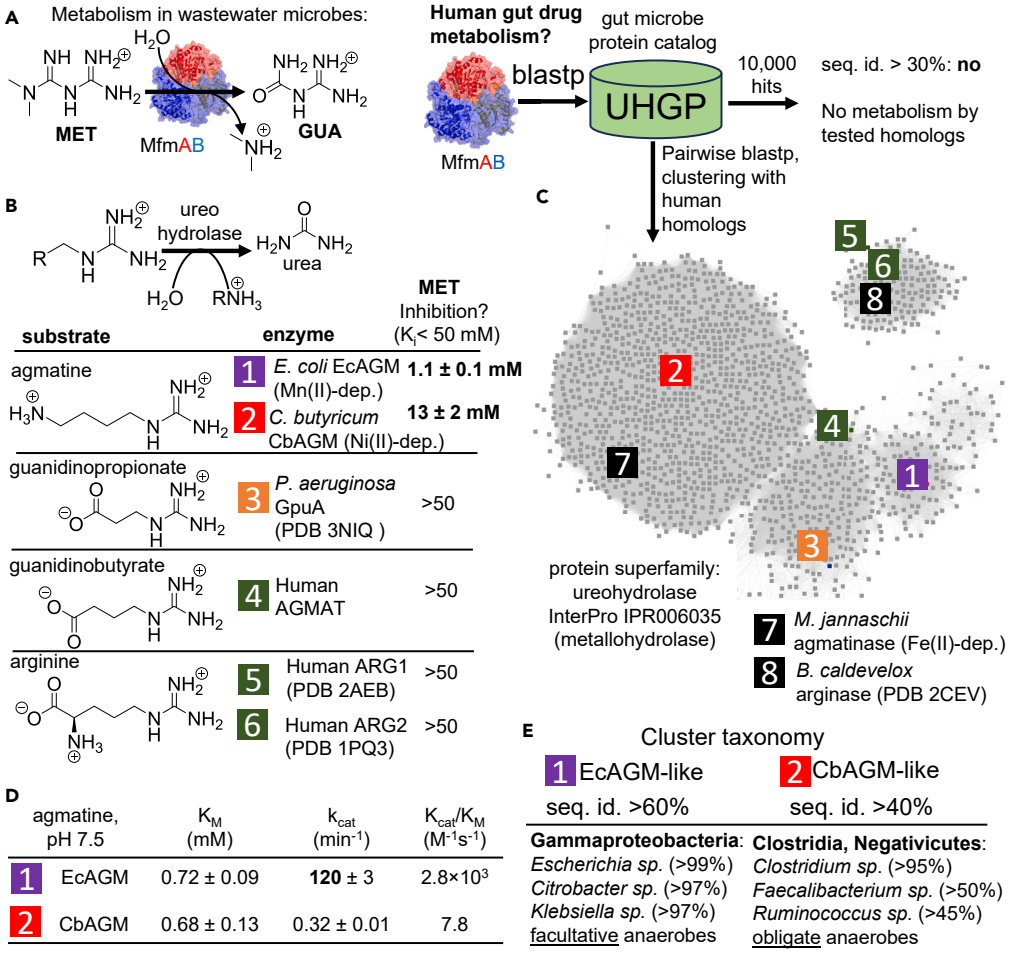


Figure 1. Targeted inhibition of gut bacterial agmatinases by metformin

(A) Searching for metformin-degrading enzymes in the human gut microbiome. Metformin is transformed by wastewater microbes to guanylurea and dimethylamine using the metformin hydrolase enzyme, MfmAB. BLAST search for gut homologs of MfmAB from the Unified Human Gastrointestinal Protein (UHGP) catalog retrieved 10,000 hits but none shared more than 30% sequence identity with MfmA or MfmB sequences.

(B) Gut and human homologs of MfmAB tested for inhibition by metformin. Protein homologs of MfmAB are from the ureohydrolase superfamily that hydrolyzes guanidine substrates to form urea and alkylamines. Select human and gut microbe homologs were expressed heterologously in *E. coli*, purified, and screened for inhibition by metformin in competition with their native substrates. Only the gut homologs, EcAGM and CbAGM, that are active on agmatine were potently inhibited by metformin (K_i < 50 mM). Metformin was not degraded by these homologs as determined by an HPLC method. Error bars denote one standard deviation of the mean from two technical replicates.

(C) Sequence similarity network (SSN) of gut and human homologs of MfmAB. 10,000 sequences from the UHGP along with human homologs and experimentally characterized proteins were clustered and indicate that human gut microbes have agmatinase, guanidinopropionase, guanidinobutyrase, and arginase activities. The gut agmatinases EcAGM and CbAGM come from two different clusters of sequences. EcAGM is manganese dependent while CbAGM is nickel dependent and metal preferences may explain their divergence. The E-value cutoff for this SSN was set to 61.

(D) Kinetic parameters of EcAGM and CbAGM agmatinases. EcAGM has a superior k_{cat} over CbAGM with an approximately 350-fold difference in catalytic efficiency (k_{cat}/K_M). Error bars denote one standard deviation of the mean from two technical replicates.

(E) Taxonomy of EcAGM-like and CbAGM-like sequences. Sequences pertaining to the clusters with CbAGM or EcAGM were extracted from the SSN and taxonomic metadata was evaluated. EcAGM-like sequences shared >60% sequence identity with EcAGM and came from Gammaproteobacteria that are facultative anaerobes which includes notable *Escherichia*, *Citrobacter*, and *Klebsiella* microbes that have an agmatinase with >97% sequence identity to EcAGM. CbAGM-like sequences are more divergent and share >40% sequence identity with CbAGM and come from Clostridia and Negativicutes that are obligate anaerobes which includes notable *Clostridium*, *Faecalibacterium*, and *Ruminococcus* microbes.

develop an alternative hypothesis that, since metformin hydrolase evolved from the ureohydrolase protein superfamily, there could be very poor activity or possibly inhibition of gut ureohydrolase homologs with metformin.

Agmatinase is an example of a gut microbial ureohydrolase that, in a recent host-microbe study by Pryor et al., was linked to metformin action to increase the lifespan of *C. elegans* with *Escherichia coli* (*E. coli*) as an endosymbiont.[17] In the study, deletion of the agmatinase gene in *E. coli* simulated metformin's positive effect and this finding suggested that metformin inhibits agmatinase from *E. coli*, a known gut microbe in humans.[17]

We now report that metformin targets gut bacterial agmatinases, which catabolize agmatine, as a potent, competitive inhibitor that is relevant in the human gut context. We also find that related metformin analogs buformin, phenformin, and galegine competitively, and more potently, inhibit agmatinase, indicating a shared mechanism. Revealing that metformin targets agmatine metabolism in the gut may have profound implications in type 2 diabetes therapy which is discussed in this report.

RESULTS

Metformin inhibits human gut agmatinases

Metformin is a potent, competitive inhibitor of *E. coli* agmatinase (EcAGM) with an inhibition constant (K_i) of 1 mM at near physiological pH, 7.5 (Figure 1B). This level of inhibition is relevant in the context of the human gut as the concentration of metformin in the gut is estimated to be between 1 and 10 mM.[18] This result prompted us to explore if metformin can inhibit other gut ureohydrolases or possibly even homologs found in human cells.

To discern this, we clustered 10,000 human gut protein sequences from the Unified Human Gastrointestinal Protein catalog that are homologous to EcAGM, along with experimentally characterized ureohydrolases, to create a sequence similarity network (SSN) that can visualize clusters of sequences with unique functions (Figure 1C). Annotating the SSN, using the characterized ureohydrolases, the SSN revealed clusters of ureohydrolases, present in the human gut, that likely utilize 3-guanidinopropioniate, 4-guanidinobutyrate, and arginine along with agmatine. There are three ureohydrolase homologs in humans which include arginases, ARG1 and ARG2 as well as the enzyme AGMAT that is active on guanidino acids like 4-guanidinobutyrate.[19–21] The largest cluster, cluster 2, of gut sequences in the SSN contains a separate subfamily of agmatinase enzymes which have been found to be Ni^{2+}, Co^{2+}, or Fe^{2+} dependent and are divergent from the *E. coli* agmatinase which is Mn^{2+} dependent (Figure 1C). This was determined when characterizing a sequence from this divergent cluster, from the gut microbe, *Clostridium butyricum* (CbAGM), with 32% sequence identity to EcAGM, which was most activated with the addition of Ni^{2+} but not Mn^{2+} cations (Figure S1). In addition, previously characterized agmatinases from hyperthermophilic archaea, *Pyrococcus horikoshii* and *Methanocaldococcus jannaschii*, were found to be Co^{2+} and Fe^{2+} dependent, respectively, and are also part of the agmatinase subfamily that includes CbAGM (Figure 1C; Table S1).[22,23]

After assessing the possible functional landscape of ureohydrolases in the human gut, a select set of sequences were screened for inhibition by metformin, which could represent most of the sequence space. No metabolism of metformin was found for any gut or human ureohydrolase homolog tested. The screening found no significant inhibition (K_i < 50 mM) by metformin with ureohydrolase function other than agmatinase (Figure 1B). The competitive inhibition constant of metformin for the Ni^{2+}-dependent CbAGM was 13 mM and was less potent than metformin's effect on EcAGM with a K_i of 1.1 mM. Comparing the kinetic parameters of the two enzymes, EcAGM has more than a 350-fold higher k_{cat} but similar K_M compared to CbAGM (Figure 1D). The cluster of sequences in the SSN that contains CbAGM, or CbAGM-like sequences, are encoded primarily in Clostridia and Negativicutes microbes (e.g. *Clostridium*, *Faecalibacterium*, and *Ruminococcus*) that are obligate anaerobes whereas the cluster of sequences that contains EcAGM-like sequences are encoded in Gammaproteobacteria (e.g. *Escherichia*, *Citrobacter*, and *Klebsiella*) that are facultative anaerobes (Figures 1C and 1E).

To estimate the level of inhibition that metformin may have on these agmatinases in the gut, Michaelis-Menten kinetic equations may be used, assuming a steady state. Taking into account the concentration of agmatine in the gut, which has been reported to be in the micromolar range (e.g. 20 μM), and an average metformin concentration of 5 mM, the reduction in the maximum velocity of EcAGM-like and CbAGM-like enzymes is predicted to be 83% and 27%, respectively (Figure S2).[24] This indicates that inhibition of EcAGM-like agmatinases by metformin is significant while for CbAGM-like it appears to be less so.

Inhibition of *E. coli* agmatinase by metformin analogs and its parent compound, galegine

To determine whether agmatinase inhibition is a mechanism of action reserved only for metformin, more potent anti-diabetic analogs, phenformin, buformin, and metformin's parent compound, galegine, were tested as competitive inhibitors of EcAGM. This revealed that phenformin, buformin, and galegine were more potent inhibitors of EcAGM, compared to metformin, with K_i of 0.6, 0.1, and 0.007 mM, respectively (Figure 2A). The potency of the analogs to inhibit *E. coli* agmatinase appears to be proportional to their recommended doses to treat type 2 diabetes (Figure 2B). The recommended dose for metformin is 1000–2000 mg daily while for phenformin and buformin the dose is more than 5-fold less than metformin.[25] Galegine has not been tested clinically but in an experiment by Muller et al., galegine was administered to several diabetic patients with doses between 25 and 100 mg as treatment.[26]

The competitive inhibition of EcAGM by metformin and its analogs indicates that the inhibitors bind in the enzyme's active site which contains a binuclear manganese cluster. Attempts were made to get an X-ray crystal structure to show the inhibition complex of metformin or galegine in the EcAGM active site but were not successful. Instead, the complex was modeled *in silico* by docking the inhibitors into the EcAGM active site. The docking suggests that the biguanide inhibitors can bind so that some of its guanidinium nitrogen atoms may chelate the binuclear manganese metals whereas other nitrogen atoms can make polar contacts with EcAGM residues Thr242, Thr244, and Glu274 (Figure 2C). The exquisite potency of galegine suggests that it too may chelate the binuclear metals, and it is possible that the sp^2 character of the alkenyl functionality of galegine may act as a chelator in an η^2 fashion while the guanidinium nitrogen atoms could interact with the same EcAGM residues that may bind the biguanide inhibitors (Figure 2D). The mechanism of EcAGM, and other members of the ureohydrolase superfamily, involves metal-activated hydroxide to act as the nucleophile in substrate hydrolysis which is demonstrated by the enzyme being the most active in alkaline conditions, where the concentration of hydroxide is higher than neutral pH.[27] To see if the inhibitors are affected by pH and compete with, or are assisted, by the metal-bound hydroxide, we determined the inhibition kinetics of metformin and galegine in

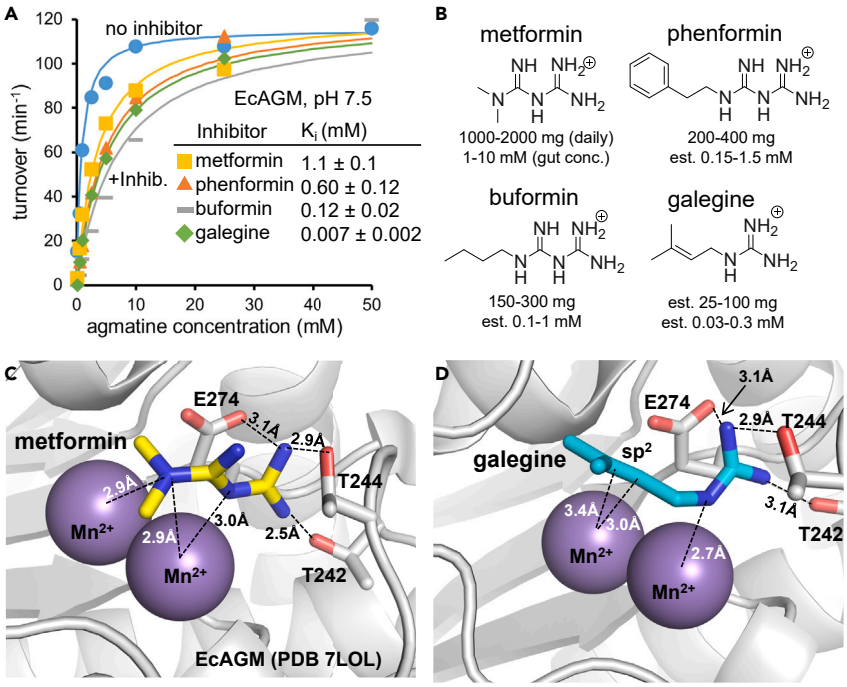


Figure 2. Inhibition of *E coli* agmatinase by metformin analogs

(A) Anti-diabetic analogs phenformin, buformin, and galegine are competitive inhibitors of EcAGM. Michaelis-Menten kinetics was obtained for EcAGM in the presence or absence of inhibitor and the inhibition constants (K_i) were determined. Activity at several agmatine concentrations was measured by observing urea release using a colorimetry assay in 100 mM HEPES pH 7.5, 2 mM $MnCl_2$. Error bars denote one standard deviation of the mean from two technical replicates. Plot lines show the fit to the Michaelis-Menten equation. The K_i for each inhibitor is expressed as a mean and one standard deviation.

(B) Recommended dosage and estimated gut drug concentration of metformin and analogs. Drug potency appears to be correlated with the K_i of the different analogs and estimated gut concentrations are determined relative to metformin's dosage and assuming 75% drug absorption for buformin, phenformin, and galegine compared to the known 50% drug absorption for metformin.

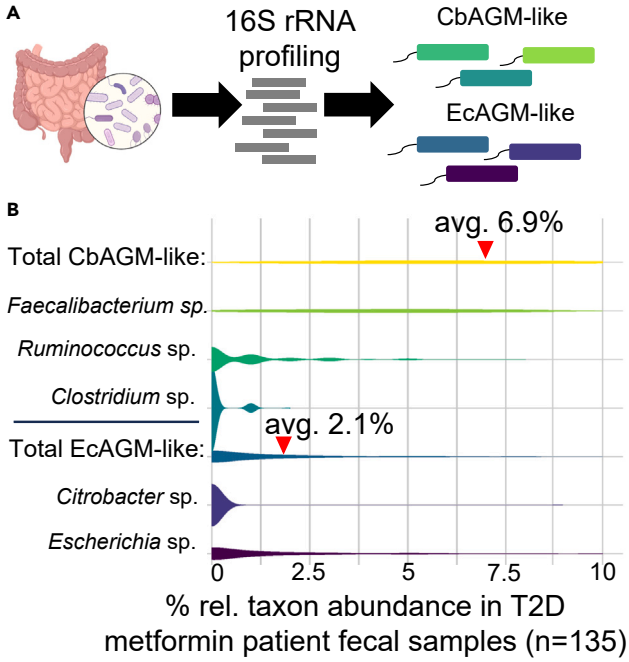
(C and D) Docking of metformin and galegine into the EcAGM active site. Docking of metformin indicates some of its guanidinium atoms may have polar contacts with residues T242, T244, and E274 of EcAGM and other guanidinium atoms chelate the binuclear manganese in the active site. Galegine docked into the active site may have its guanidinium atoms make similar polar contacts to EcAGM residues like metformin and may be possible that the sp^2 character of the alkene bond of galegine chelates the metal to be a potent inhibitor. See Figure S3 for docking models of buformin and phenformin.

more acidic, pH 6.5 and more alkaline pH of 8.5. The lower or higher pH resulted in no significant change in the inhibition constants for metformin or galegine that were measured at pH 7.5 (Table S2).

Metformin inhibition of agmatinase in the context of the human gut

The inhibition by metformin appears most significant for EcAGM-like enzymes and, while there are two subfamilies of agmatinases in the human gut, their relative abundance to the whole microbiome and to each other is not clear. To get a good estimate of relative abundance, the taxonomic information of the EcAGM-like and CbAGM-like sequence clusters was cross referenced to the 16S rRNA gene-based profiling of 136 fecal samples obtained from several cohorts of type 2 diabetes patients that were prescribed metformin.[17] The profiling data indicated that the most abundant microbes containing EcAGM-like sequences are *Escherichia*, *Citrobacter*, and *Klebsiella* from Gammaproteobacteria (Figure 3A). While for the CbAGM-like cluster, the profiling data indicated that it is comprised of Clostridiales microbes *Faecalibacterium prausnitzii*, *Ruminococcus* sp., and *Clostridium* sp. The distribution of the relative abundances for CbAGM-like encoding microbes in fecal samples was broad between 0% and 10% while for EcAGM-like microbes the distribution was more narrow between 0% and 2.5% (Figure 3B). A comparison of the means of the two distributions suggests that the CbAGM-like encoding microbes may be more abundant than EcAGM-like encoding microbes in fecal samples. Although EcAGM-like microbes may still contribute the majority of gut agmatinase activity as the turnover number (k_{cat}) of EcAGM at physiological conditions is several orders of magnitude greater than that of characterized CbAGM-like enzymes (Figure 3C; Table S1). More investigation is necessary to understand levels of gut agmatinase activity *in situ* and what the likely contributions come from CbAGM-like and EcAGM-like agmatinases.

Agmatine produced in the gut is derived from decarboxylation of L-arginine in microbes that encode arginine decarboxylase. L-arginine comes from dietary or endogenous sources and decarboxylase activity in the gut is highest in the colon, where most gut microbes reside and it is an anaerobic environment.[28] Agmatine can be catabolized into putrescine by agmatinase but also by an alternate pathway that is initiated with the agmatine deiminase (AgDI) enzyme (Figure 4). Agmatine deiminase is a hydrolytic



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AGM cluster	est. k_{cat} (min^{-1})	est. gut AGM fraction	est. gut AGM activity
EcAGM-like	120	23%	>90%
CbAGM-like	3-0.3	77%	<10%

Figure 3. Relative fecal abundances of microbes encoding a CbAGM-like or EcAGM-like agmatinase

(A) Profiling human microbiome using 16S rRNA amplicon sequencing reads. Sequencing reads from microbes with genus-species that matched the taxonomy of EcAGM-like or CbAGM-like protein sequences were counted to estimate relative abundances.

(B) Violin plot of relative abundance of CbAGM-like or EcAGM-like encoding microbes in fecal samples of type 2 diabetes patients taking metformin. The three most abundant CbAGM-like microbes were *Faecalibacterium*, *Ruminococcus*, and *Clostridium* species while for EcAGM-like microbes the two most abundant were *Citrobacter and Escherichia* sp. The distribution of relative abundances of CbAGM-like encoding microbes was broad between 0 and 10% while for EcAGM-like microbes, the distribution was more narrow between 0 and 2.5%. The average relative fecal abundance of CbAGM-like microbes was approximately 3-fold more than EcAGM-like microbes. See Table S7 for the data points shown in this plot.

(C) Estimation of the human gut agmatinase activity contribution by microbes encoding EcAGM-like or CbAGM-like agmatinases. Compounding the relative abundance of CbAGM-like and EcAGM-like microbes with the estimated turnover numbers (k_{cat}) of the enzymes suggests that the majority of gut agmatinase activity (>90%) is contributed by EcAGM-like enzymes in the gut.

enzyme like agmatinase but is different in that it is not metal dependent and yields ammonium and N-carbamoylputrescine as products of the reaction.[29] N-carbamoylputrescine is then transformed into putrescine either by putrescine carbamoyltransferase or N-carbamoylputrescine amidase with the latter being hydrolytic and the former produces carbamoylphosphate that can be used by carbamate kinase to yield ATP (Figure 4).[30,31] To see if agmatine catabolism via AgDI is inhibited by metformin or galegine, the previously characterized AgDI homolog from *Enterococcus faecalis* was expressed in *E. coli* and tested *in vitro*.[31] The purified enzyme was active on agmatine but not inhibited by either 50 mM metformin or 5 mM galegine (Figure S4). Putrescine can be utilized by microbes in catabolism to produce metabolites such as γ-aminobutyric acid or in biosynthesis of polyamines like spermidine, spermine. The targeted inhibition of gut agmatinase by metformin gives insight that either agmatine, putrescine catabolites, or polyamines may be effectors of the drug's therapy for type 2 diabetes (Figure 4).

DISCUSSION

Metformin targets gut agmatinase activity as a potent, competitive inhibitor

Metformin potently inhibited gut agmatinase activity and not any other homolog of the ureohydrolase protein superfamily nor agmatine deiminase that also uses agmatine as a substrate. The agmatinase inhibition constant (K_i) for metformin, at pH 7.5 was around 1 mM which is

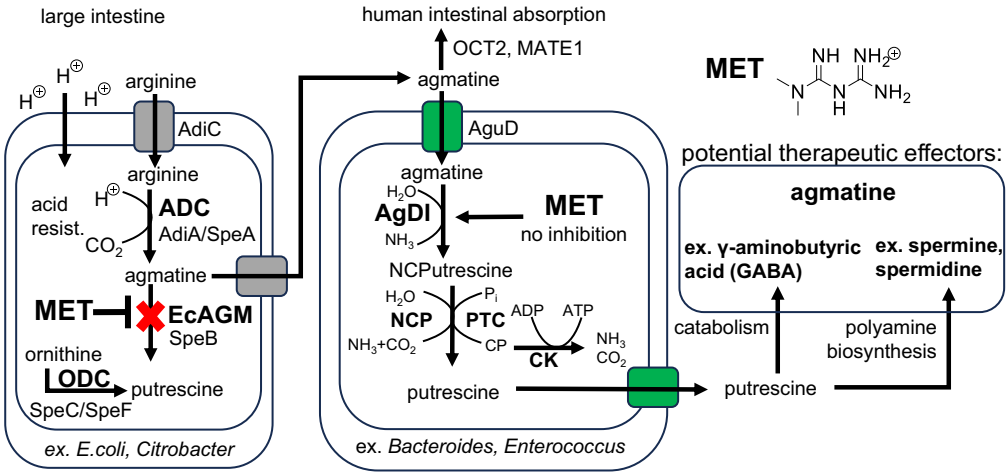

Figure 4. Metformin inhibition of agmatinase in the context of the human gut

Agmatine is a product of L-arginine decarboxylation which in *E. coli* or *Citrobacter* serves as an acid resistance mechanism. Arginine and agmatine are known to be transported in and out of the cell by the AdiC antiporter. Metformin inhibition of EcAGM prevents agmatine degradation to putrescine although *E. coli* are able to synthesize putrescine from ornithine via ornithine decarboxylase (ODC). Agmatine is known to be preferentially absorbed into human tissue via the OCT2 and MATE1 transporters. Agmatine can also be catabolized by gut microbes into putrescine using an alternate pathway that is initiated with agmatine deiminase (AgDI) to produce N-carbamoylputrescine (NCPutrescine). Agmatine deiminase from *Enterococcus faecalis* was not inhibited by metformin or galegine when tested *in vitro* (Figure S4). An enzyme, N-carbamoylputrescine amidase (NCP), can hydrolyze this to putrescine in a *Bacteroides* species for example. Alternatively, in *E. faecalis*, N-carbamoylputrescine can be converted to putrescine via putrescine transcarbamylase (PTC) which forms carbamoyl phosphate that can be used by carbamate kinase to form an ATP energy equivalent. In these microbes, agmatine and putrescine can be transported in an out of the cell by the AguD antiporter. Putrescine is utilized by microbes in catabolism to produce metabolites such as γ-aminobutyric acid (GABA) or in biosynthesis of polyamines like spermidine, spermine. The targeted inhibition of gut agmatinase by metformin gives insight that either agmatine, putrescine catabolites, or polyamines are effectors of the drug's therapy for type 2 diabetes.

relevant in the gut where the concentration of metformin is several millimolar and the lumen pH of the small and large intestines varies between pH 6.5 and 7.5.[32]

Inhibition of enzymes *in vitro* by metformin has been described in several prior studies but shows less potent inhibition. Metformin competitively inhibits dihydrofolate reductase from *E. coli* but with a reported K_i of 24 mM.[33] For a human enzyme, serine hydroxymethyltransferase 2 showed mixed inhibition by metformin with a K_i of 13 mM although the concentration of metformin entering the liver from the portal vein has been measured to be between 10 and 40 μM.[5,34] A study by Ma et al. found that metformin can bind the human PEN2 protein with a binding constant in the micromolar range and this can cause AMP kinase activation, a hallmark of metformin therapy.[35] However, in the same study, biguanide analogs phenformin and buformin were shown to not act in the same manner as metformin. A few studies report metformin inhibiting mitochondrial respiration proteins like complex IV in the hundreds of micromolar but rely on the assumption that metformin can accumulate in the mitochondria of hepatocytes at concentrations several fold higher than in the plasma.[36,37]

Agmatine, putrescine, and its metabolites as effectors of metformin therapy

The inhibition of gut agmatinase activity suggests either agmatine, putrescine, or related metabolites may be effectors in the therapy that metformin delivers (Figure 4). Agmatine is known to act like metformin and lower plasma glucose levels in diabetic mice and also have anti-tumor, anti-aging, and neuroprotective effects.[17,38] In a study by Kotagale et al., diabetic mice were treated with agmatine and metformin which saw synergistic effects to lower blood glucose and treat impaired cognition of the mice.[39] Agmatine can bind and is an agonist to human imidazoline receptors (I_1, I_2) in the micromolar range and it is purported that this may stimulate the therapeutic effects of the molecule.[40,41] The I_2 receptors specifically are located on the outer membrane of mitochondria but it is still unclear what effects are caused when agmatine binds to these receptors. Another mechanism independent of imidazoline receptors is the effect of agmatine to indirectly inhibit ornithine decarboxylase activity in certain human cell lines and thus polyamine biosynthesis and cell proliferation.[42–44] The effective concentrations for agmatine used in *in vitro* studies to see decreases in cell proliferation were in the range of tens of micromolar. This effect of agmatine is seen as a mechanism to inhibit cancer growth.[43]

Metformin does not inhibit agmatine deiminase in the gut which suggests that agmatine can still be catabolized even with metformin treatment. A study by Kitada et al. observed that putrescine production in a complex community of gut microbes can be simplified to a collaboration of *E. coli* and *Enterococcus faecalis*, of which the latter encodes agmatine deiminase.[45] The study determined that *E. coli*'s role is to produce agmatine as part of an acid resistance mechanism and then expel agmatine into the media to be consumed by *E. faecalis* to produce putrescine. AgDI from *E. faecalis* has superior kinetics parameters (K_M, k_{cat}) compared to EcAGM with a K_M of 35 μM and k_{cat} of 840 s^{-1} which may explain why this synergy is more productive.[31] The study also tested *E. coli* with an agmatinase gene deletion and saw an approximate

50% increase in putrescine production in combination with *E. faecalis*.[45] This experiment was done in culture and it is important to note that in the context of the gut, the increase in the amount of agmatine exported, due to *E. coli* deficient in agmatinase activity, may be absorbed into the colonic epithelium and only be partially taken up by other gut microbes such as *E. faecalis*. Agmatine is known to be preferentially absorbed into human cells by the human OCT2 and MATE1 transporters.[46]

While the *E. coli* agmatinase appears to be a target in the gut for metformin, it is important to mention that a key observation in metformin's effect in altering the gut microbiome of treated patients is the increased abundance of bacterial genera *Escherichia*, *Enterobacter*, and *Citrobacter*, all which encode EcAGM-like agmatinases.[3,17,47] This suggests that inhibition of gut agmatinase activity is not toxic to these microbes with one explanation being that they are still able to produce putrescine and polyamines from another pathway via ornithine decarboxylase (Figure 4). In growth studies of *E. coli* deficient in both pathways to biosynthesize polyamines, it was observed that the polyamine spermidine is not needed for aerobic growth but is required for growth in strictly anaerobic conditions, like the large intestine of the human gut.[48] If agmatinase activity is the only target of metformin therapy in the gut, then it is possible that positive feedback is coming from the host or other microbes which increases the abundance of EcAGM-like containing microbes. The other key modulations of the gut microbiome by metformin, across many studies, are the increase in short-chain fatty acid (SCFA)-producing and mucin-degrading microbiota.[3,6,49] In the aforementioned study by Kitada et al., the addition of SCFA-producing *Bifidobacterium* microbes in co-culture with *E. coli* and *Enterococcus faecalis* led to an acidification of the medium and increased putrescine production, from *E. coli* and *Enterococcus*, more than 2-fold. The acid resistance mechanism of *E. coli* suggests that SCFA producers in the gut would also promote agmatine production.[45] How metformin treatment reinforces these modulations of the gut microbiome and whether they are linked remains to be seen but may possibly be probed with this new insight that metformin targets gut agmatinase activity.

According to several reports, putrescine, polyamines, or putrescine catabolites derived from the gut may have a myriad of effects that have been relatively understudied compared to agmatine.[50,51] Targeted metabolomics of these compounds in the gut and human tissue is necessary to understand their change in response to metformin treatment. Testing using gnotobiotic and diabetic mice inoculated with *E. coli* deficient in agmatinase activity may be important in revealing metformin's true mechanism in the gut. If agmatine is the true effector of metformin therapy, there are possible opportunities to improve the therapy for the many millions of patients taking metformin. A potential therapy for type 2 diabetes may be to administer agmatine in combination with metformin that may not require as high of dosage that is needed for treatment when using metformin or agmatine alone. A lower dosage would also limit gastrointestinal symptoms of metformin which occurs in 30% of patients.[11]

Probiotics like *Bifidobacteria*, which ferment acid metabolites like lactate and SCFAs, lower intestinal pH and may increase agmatine production of *E. coli*-like bacteria in the gut and could also be co-administered with metformin.[45] One other approach is to develop effective inhibitors of agmatine deiminase to test for type 2 diabetes treatment in combination with metformin.[52]

Metformin gut drug metabolism

Gut ureohydrolase homologs tested in this study were not active with metformin and were distant in shared sequence identity (<30%) with metformin hydrolase, MfmAB. This still does not indicate that metformin is not metabolized by gut microbes. The lack of a detected signal may be due to metagenomic sequence coverage in human fecal samples that is not adequate to capture the genes from low-abundance microbes (<1%).[53,54] In the human gut microbiome, microbes present in low-abundance have been shown to be important in causing extensive changes to the overall community, affecting pathogenicity and secondary metabolism.[55] Quantitative PCR may be a great tool to detect the MfmAB genes in fecal samples and determine if gut drug metabolism may be present.

Limitations of the study

Despite sampling a functionally diverse set of ureohydrolase homologs for metformin inhibition, the study may not have sampled enough agmatinase homologs from the CbAGM-like or EcAGM-like clusters to be able to generalize accurately the level of metformin inhibition and enzyme kinetics for each.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- METHOD DETAILS
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  - Computational modeling and Bioinformatics
  - Bioinformatics

- Enzyme kinetics of agmatine deiminase
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.108900.

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AUTHOR CONTRIBUTIONS

L.J.T. and L.P.W. designed research; L.J.T. performed research; L.J.T. and L.P.W. analyzed the data; and L.J.T. and L.P.W. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

1. Tzoulaki, I., Molokhia, M., Curcin, V., Little, M.P., Millett, C.J., Ng, A., Hughes, R.I., Khunti, K., Wilkins, M.R., Majeed, A., and Elliott, P. (2009). Risk of cardiovascular disease and all cause mortality among patients with type 2 diabetes prescribed oral antidiabetes drugs: Retrospective cohort study using UK general practice research database. BMJ *339*, b4731.
2. Bo, S., Ciccone, G., Rosato, R., Villois, P., Appendino, G., Ghigo, E., and Grassi, G. (2012). Cancer mortality reduction and metformin: A retrospective cohort study in type 2 diabetic patients. Diabetes, Obes. Metab. *14*, 23–29.
3. Forslund, K., Hildebrand, F., Nielsen, T., Falony, G., Le Chatelier, E., Sunagawa, S., Prifti, E., Vieira-Silva, S., Gudmundsdottir, V., Pedersen, H.K., et al. (2015). Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature *528*, 262–266.
4. Tobar, N., Rocha, G.Z., Santos, A., Guadagnini, D., Assalin, H.B., Camargo, J.A., Gonçalves, A.E.S.S., Pallis, F.R., Oliveira, A.G., Rocco, S.A., et al. (2023). Metformin acts in the gut and induces gut-liver crosstalk. Proc. Natl. Acad. Sci. USA *120*, e2211933120.
5. He, L., and Wondisford, F.E. (2015). Metformin action: Concentrations matter. Cell Metab. *21*, 159–162.
6. Foretz, M., Guigas, B., and Viollet, B. (2023). Metformin: update on mechanisms of action and repurposing potential. Nat. Rev. Endocrinol. *19*, 460–476.
7. Sum, C.F., Webster, J.M., Johnson, A.B., Catalano, C., Cooper, B.G., and Taylor, R. (1992). The effect of intravenous metformin on glucose metabolism during hyperglycaemia in type 2 diabetes. Diabet. Med. *9*, 61–65.
8. Schmidt, T.S.B., Raes, J., and Bork, P. (2018). The human gut microbiome: From association to modulation. Cell *172*, 1198–1215.
9. Zimmermann, M., Patil, K.R., Typas, A., and Maier, L. (2021). Towards a mechanistic understanding of reciprocal drug–microbiome interactions. Mol. Syst. Biol. *17*, e10116.
10. Kahn, S.E., Haffner, S.M., Heise, M.A., Herman, W.H., Holman, R.R., Jones, N.P., Kravitz, B.G., Lachin, J.M., O'Neill, M.C., Zinman, B., et al. (2006). Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. N. Engl. J. Med. *355*, 2427–2443.
11. Diabetes Prevention Program Research Group, Edelstein, S.L., Crandall, J.P., Aroda, V.R., Franks, P.W., Fujimoto, W., Horton, E., Jeffries, S., Montez, M., Mudaliar, S., et al. (2012). Long-term safety, tolerability, and weight loss associated with metformin in the diabetes prevention program outcomes study. Diabetes Care *35*, 731–737.
12. Wilkinson, J.L., Boxall, A.B.A., Kolpin, D.W., Leung, K.M.Y., Lai, R.W.S., Galbán-Malagón, C., Adell, A.D., Mondon, J., Metian, M., Marchant, R.A., et al. (2022). Pharmaceutical pollution of the world's rivers. Proc. Natl. Acad. Sci. USA *119*, e2113947119.
13. Tassoulas, L.J., Rankin, J.A., Elias, M.H., and Wackett, L.P. (2023). Di-nickel enzyme evolved to metabolize the pharmaceutical metformin: Implications for wastewater and human microbiomes. Proc. Natl. Acad. Sci. USA.
14. Chaignaud, P., Gruffaz, C., Borreca, A., Fouteau, S., Kuhn, L., Masbou, J., Rouy, Z., Hammann, P., Imfeld, G., Roche, D., and Vuilleumier, S. (2022). A methylotrophic bacterium growing with the antidiabetic drug metformin as its sole carbon, nitrogen and energy source. Microorganisms *10*, 2302.
15. Li, T., Xu, Z.J., and Zhou, N.Y. (2023). Aerobic degradation of the antidiabetic drug metformin by Aminobacter sp. strain NyZ550. Environ. Sci. Technol. *57*, 1510–1519.
16. Richardson, L., Allen, B., Baldi, G., Beracochea, M., Bileschi, M.L., Burdett, T., Burgin, J., Caballero-Pérez, J., Cochrane, G., Colwell, L.J., et al. (2023). MGnify: the microbiome sequence data analysis resource in 2023. Nucleic Acids Res. *51*, D753–D759.
17. Pryor, R., Norvaisas, P., Marinos, G., Best, L., Thingholm, L.B., Quintaneiro, L.M., De Haes, W., Esser, D., Waschina, S., Lujan, C., et al. (2019). Host-Microbe-Drug-Nutrient screen identifies bacterial effectors of metformin therapy. Cell *178*, 1299–1312.e29.
18. Tucker, G.T., Casey, C., Phillips, P.J., Connor, H., Ward, J.D., and Woods, H.F. (1981). Metformin kinetics in healthy subjects and in patients with diabetes mellitus. Br. J. Clin. Pharmacol. *12*, 235–246.
19. Di Costanzo, L., Sabio, G., Mora, A., Rodriguez, P.C., Ochoa, A.C., Centeno, F., and Christianson, D.W. (2005). Crystal structure of human arginase I at 1.29-Å resolution and exploration of inhibition in the immune response. Proc. Natl. Acad. Sci. USA *102*, 13058–13063.
20. Cama, E., Colleluori, D.M., Emig, F.A., Shin, H., Kim, S.W., Kim, N.N., Traish, A.M., Ash, D.E., and Christianson, D.W. (2003). Human arginase II: Crystal structure and physiological role in male and female sexual arousal. Biochemistry *42*, 8445–8451.
21. Sinn, M., Stanoppi, M., Hauth, F., Fleming, J.R., Funck, D., Mayans, O., and Hartig, J.S. (2022). Guanidino acid hydrolysis by the human enzyme annotated as agmatinase. Sci. Rep. *12*, 22088.
22. Miller, D., Xu, H., and White, R.H. (2012). A new subfamily of agmatinases present in methanogenic archaea is Fe(II) dependent. Biochemistry *51*, 3067–3078.

23. Goda, S., Sakuraba, H., Kawarabayasi, Y., and Ohshima, T. (2005). The first archaeal agmatinase from anaerobic hyperthermophilic archaeon Pyrococcus horikoshii: Cloning, expression, and characterization. Biochim. Biophys. Acta *1748*, 110–115.
24. Molderings, G.J., Burian, M., Homann, J., Nilius, M., and Göthert, M. (1999). Potential relevance of agmatine as a virulence factor of Helicobacter pylori. Dig. Dis. Sci. *44*, 2397–2404.
25. Luft, D., Schmülling, R.M., and Eggstein, M. (1978). Lactic acidosis in biguanide-treated diabetics - A review of 330 cases. Diabetologia *14*, 75–87.
26. Müller, H., and Reinwein, H. (1927). Zur pharmakologie des galegins. Archiv f. experiment. Pathol. u. Pharmakol. *125*, 212–228.
27. Salas, M., Rodríguez, R., López, N., Uribe, E., López, V., and Carvajal, N. (2002). Insights into the reaction mechanism of Escherichia coli agmatinase by site-directed mutagenesis and molecular modelling: A critical role for aspartate 153. Eur. J. Biochem. *269*, 5522–5526.
28. Pereira, F.C., and Berry, D. (2017). Microbial nutrient niches in the gut. Environ. Microbiol. *19*, 1366–1378.
29. Nakamura, A., Ooga, T., and Matsumoto, M. (2019). Intestinal luminal putrescine is produced by collective biosynthetic pathways of the commensal microbiome. Gut Microb. *10*, 159–171.
30. Shimokawa, H., Sakanaka, M., Fujisawa, Y., Ohta, H., Sugiyama, Y., and Kurihara, S. (2023). N-Carbamoylputrescine Amidohydrolase of Bacteroides thetaiotaomicron, a Dominant Species of the Human Gut Microbiota. Biomedicines *11*, 1123.
31. Llácer, J.L., Polo, L.M., Tavárez, S., Alarcón, B., Hilario, R., and Rubio, V. (2007). The gene cluster for agmatine catabolism of Enterococcus faecalis: Study of recombinant putrescine transcarbamylase and agmatine deiminase and a snapshot of agmatine deiminase catalyzing its reaction. J. Bacteriol. *10*, 1128.
32. Evans, D.F., Pye, G., Bramley, R., Clark, A.G., Dyson, T.J., and Hardcastle, J.D. (1988). Measurement of gastrointestinal pH profiles in normal ambulant human subjects. Gut *29*, 1035–1041.
33. Gabel, S.A., Duff, M.R., Pedersen, L.C., DeRose, E.F., Krahn, J.M., Howell, E.E., and London, R.E. (2017). A Structural Basis for Biguanide Activity. Biochemistry *56*, 4786–4798.
34. Tramonti, A., Cuyàs, E., Encinar, J.A., Pietzke, M., Paone, A., Verdura, S., Arbusà, A., Martin-castillo, B., Giardina, G., Joven, J., et al. (2021). Metformin is a pyridoxal-5′-phosphate (PLP)-competitive inhibitor of SHMT2. Cancers *13*, 4009.
35. Ma, T., Tian, X., Zhang, B., Li, M., Wang, Y., Yang, C., Wu, J., Wei, X., Qu, Q., Yu, Y., et al. (2022). Low-dose metformin targets the lysosomal AMPK pathway through PEN2. Nature *603*, 159–165.
36. LaMoia, T.E., Butrico, G.M., Kalpage, H.A., Goedeke, L., Hubbard, B.T., Vatner, D.F., Gaspar, R.C., Zhang, X.M., Cline, G.W., Nakahara, K., et al. (2022). Metformin, phenformin, and galegine inhibit complex IV activity and reduce glycerol-derived gluconeogenesis. Proc. Natl. Acad. Sci. USA *119*, e2122287119.
37. Madiraju, A.K., Erion, D.M., Rahimi, Y., Zhang, X.M., Braddock, D.T., Albright, R.A., Prigaro, B.J., Wood, J.L., Bhanot, S., MacDonald, M.J., et al. (2014). Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature *510*, 542–546.
38. Piletz, J.E., Aricioglu, F., Cheng, J.T., Fairbanks, C.A., Gilad, V.H., Haenisch, B., Halaris, A., Hong, S., Lee, J.E., Li, J., et al. (2013). Agmatine: Clinical applications after 100 years in translation. Drug Discov. Today *18*, 880–893.
39. Kotagale, N., Rahangdale, S., Borkar, A., Singh, K., Ikhar, A., Takale, N., Umekar, M., and Taksande, B. (2021). Possible involvement of agmatine in neuropharmacological actions of metformin in diabetic mice. Eur. J. Pharmacol. *907*, 174255.
40. Hwang, S.L., Liu, I.M., Tzeng, T.F., and Cheng, J.T. (2005). Activation of imidazoline receptors in adrenal gland to lower plasma glucose in streptozotocin-induced diabetic rats. Diabetologia *48*, 767–775.
41. Li, G., Regunathan, S., Barrow, C.J., Eshraghi, J., Cooper, R., and Reis, D.J. (1994). Agmatine: An endogenous clonidine-displacing substance in the brain. Science, 966–969.
42. Satriano, J., Matsufuji, S., Murakami, Y., Lortie, M.J., Schwartz, D., Kelly, C.J., Hayashi, S., and Blantz, R.C. (1998). Agmatine suppresses proliferation by frameshift induction of antizyme and attenuation of cellular polyamine levels. J. Biol. Chem. *273*, 15313–15316.
43. Wolf, C., Brüss, M., Hänisch, B., Göthert, M., von Kügelgen, I., and Molderings, G.J. (2007). Molecular basis for the antiproliferative effect of agmatine in tumor cells of colonic, hepatic, and neuronal origin. Mol. Pharmacol. *71*, 276–283.
44. Isome, M., Lortie, M.J., Murakami, Y., Parisi, E., Matsufuji, S., and Satriano, J. (2007). The antiproliferative effects of agmatine correlate with the rate of cellular proliferation. Am. J. Physiol. Cell Physiol. *293*, C705–C711.
45. Kitada, Y., Muramatsu, K., Toju, H., Kibe, R., Benno, Y., Kurihara, S., and Matsumoto, M. (2018). Bioactive polyamine production by a novel hybrid system comprising multiple indigenous gut bacterial strategies. Sci. Adv. *4*, eaat0062-89.
46. Winter, T.N., Elmquist, W.F., and Fairbanks, C.A. (2011). OCT2 and MATE1 provide bidirectional agmatine transport. Mol. Pharm. *8*, 133–142.
47. Wu, H., Esteve, E., Tremaroli, V., Khan, M.T., Caesar, R., Mannerås-Holm, L., Ståhlman, M., Olsson, L.M., Serino, M., Planas-Fèlix, M., et al. (2017). Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. Nat. Med. *23*, 850–858.
48. Chattopadhyay, M.K., Tabor, C.W., and Tabor, H. (2009). Polyamines are not required for aerobic growth of Escherichia coli: Preparation of a strain with deletions in all of the genes for polyamine biosynthesis. J. Bacteriol. *191*, 5549–5552.
49. De La Cuesta-Zuluaga, J., Mueller, N.T., Corrales-Agudelo, V., Velásquez-Mejía, E.P., Carmona, J.A., Abad, J.M., and Escobar, J.S. (2017). Metformin is associated with higher relative abundance of mucin-degrading akkermansia muciniphila and several short-chain fatty acid-producing microbiota in the gut. Diabetes Care *40*, 54–62.
50. Bui, T.I., Britt, E.A., Muthukrishnan, G., and Gill, S.R. (2022). Probiotic induced synthesis of microbiota polyamine as a nutraceutical for metabolic syndrome and obesity-related type 2 diabetes. Front. Endocrinol. *13*, 1094258.
51. Kulkarni, A., Anderson, C.M., Mirmira, R.G., and Tersey, S.A. (2022). Role of Polyamines and Hypusine in β Cells and Diabetes Pathogenesis. Metabolites *12*, 344.
52. Jones, J.E., Causey, C.P., Lovelace, L., Knuckley, B., Flick, H., Lebioda, L., and Thompson, P.R. (2010). Characterization and inactivation of an agmatine deiminase from Helicobacter pylori. Bioorg. Chem. *38*, 62–73.
53. Rodriguez-R, L.M., and Konstantinidis, K.T. (2014). Estimating coverage in metagenomic data sets and why it matters. ISME J. *8*, 2349–2351.
54. Quince, C., Curtis, T.P., and Sloan, W.T. (2008). The rational exploration of microbial diversity. ISME J. *2*, 997–1006.
55. Cena, J.A.d., Zhang, J., Deng, D., Damé-Teixeira, N., and Do, T. (2021). Low-abundant microorganisms: The human microbiome's dark matter, a scoping review. Front. Cell. Infect. Microbiol. *11*, 1.
56. Almeida, A., Nayfach, S., Boland, M., Strozzi, F., Beracochea, M., Shi, Z.J., Pollard, K.S., Sakharova, E., Parks, D.H., Hugenholtz, P., et al. (2021). A unified catalog of 204,938 reference genomes from the human gut microbiome. Nat. Biotechnol. *39*, 105–114.
57. Irwin, J.J., Tang, K.G., Young, J., Dandarchuluun, C., Wong, B.R., Khurelbaatar, M., Moroz, Y.S., Mayfield, J., and Sayle, R.A. (2020). ZINC20 - A free ultralarge-scale chemical database for ligand discovery. J. Chem. Inf. Model. *60*, 6065–6073.
58. Maturana, P., Orellana, M.S., Herrera, S.M., Martínez, I., Figueroa, M., Martínez-Oyanedel, J., Castro-Fernandez, V., and Uribe, E. (2021). Crystal structure of escherichia coli agmatinase: Catalytic mechanism and residues relevant for substrate specificity. Int. J. Mol. Sci. *22*, 4769.
59. Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T.L. (2009). BLAST+: Architecture and applications. BMC Bioinf. *10*, 421.
60. Gerlt, J.A., Bouvier, J.T., Davidson, D.B., Imker, H.J., Sadkhin, B., Slater, D.R., and Whalen, K.L. (2015). Enzyme function initiative-enzyme similarity tool (EFI-EST): A web tool for generating protein sequence similarity networks. Biochim. Biophys. Acta *1854*, 1019–1037.
61. Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Res. *13*, 2498–2504.
62. Eberhardt, J., Santos-Martins, D., Tillack, A.F., and Forli, S. (2021). AutoDock Vina 1.2.0: New docking methods, expanded force field, and Python bindings. J. Chem. Inf. Model. *61*, 3891–3898.
63. Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S., and Olson, A.J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem. *30*, 2785–2791.

64. Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., and Mori, H. (2005). Complete set of ORF clones of Escherichia coli ASKA library (A complete set of E. coli K-12 ORF archive): unique resources for biological research. DNA Res. *12*, 291–299.
65. Nishihara, K., Kanemori, M., Kitagawa, M., Yanagi, H., and Yura, T. (1998). Chaperone coexpression plasmids: Differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese cedar pollen, Cryj2, in Escherichia coli. Appl. Environ. Microbiol. *64*, 1694–1699.
66. Williams, S., Shah, S., Hakki, Z., and Stewart, A. (2018). Compounds for the Treatment of Respiratory Diseases.
67. Geyer, J.W., and Dabich, D. (1971). Rapid method for determination of arginase activity in tissue homogenates. Anal. Biochem. *39*, 412–417.
68. Neves, R.P.P., Sousa, S.F., Fernandes, P.A., and Ramos, M.J. (2013). Parameters for molecular dynamics simulations of manganese-containing metalloproteins. J. Chem. Theory Comput. *9*, 2718–2732.
69. Karlsson, F.H., Tremaroli, V., Nookaew, I., Bergström, G., Behre, C.J., Fagerberg, B., Nielsen, J., and Bäckhed, F. (2013). Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature *498*, 99–103.
70. Magnúsdóttir, S., Heinken, A., Kutt, L., Ravcheev, D.A., Bauer, E., Noronha, A., Greenhalgh, K., Jäger, C., Baginska, J., Wilmes, P., et al. (2017). Generation of genome-scale metabolic reconstructions for 773 members of the human gut microbiota. Nat. Biotechnol. *35*, 81–89.
71. Lin, M.C., Lin, J.H., and Wen, K.C. (2020). Detection and determination of phenformin in chinese medicinal capsules by GC-MS and HPLC. J. Food Drug Anal. *9*, 139–144.
72. Martinez-Vaz, B.M., Dodge, A.G., Lucero, R.M., Stockbridge, R.B., Robinson, A.A., Tassoulas, L.J., and Wackett, L.P. (2022). Wastewater bacteria remediating the pharmaceutical metformin: Genomes, plasmids and products. Front. Bioeng. Biotechnol. *10*, 1086261.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli BL21 (DE3)	New England Biolabs	Cat# C2527H
Chemicals, peptides, and recombinant proteins		
agmatine sulfate	Fluka	Cat# 05083
metformin hydrochloride	Cayman Chemical	Cat# 13118
buformin hydrochloride	Enamine	Cat# EN300-123753
phenformin hydrochloride	Cayman Chemical	Cat# 14997
2-methyl-2-thiopseudourea hemisulfate	Sigma-Aldrich	Cat# M84445
3-methyl-2-buten-1-amine	A2B Chem	Cat# AA57254
L-glutamic acid dehydrogenase from bovine liver	Sigma-Aldrich	Cat# G7882
Bradford Protein Assay Dye Reagent	Bio-Rad	Cat# 5000006
thiosemicarbazide	Acros Organics	Cat# 138901000
2,3-butanedione monoxime	Sigma-Aldrich	Cat# 31550
urea	Fluka	Cat# 51456
4-guanidinobutyric acid	Fluka	Cat# 51018
L-arginine hydrochloride	Acros Organics	Cat# 105001000
3-guanidinopropionic acid	Sigma-Aldrich	Cat# G6878
E. coli agmatinase (EcAGM)	Prepared in study	NCBI: WP_000105566.1
C. butyricum agmatinase (CbAGM)	Prepared in study	NCBI: QGH27404.1
E. faecalis agmatine deiminase (AgDI)	Prepared in study	NCBI: WP_002363185.1
P. aeruginosa guanidinopropionase (GpuA)	Prepared in study	NCBI: WP_003112934.1
human arginase 1 (ARG1)	Prepared in study	NCBI: NP_000036.2
human arginase 2 (ARG2)	Prepared in study	NCBI: NP_001163.1
human guanidino acid hydrolase (AGMAT)	Prepared in study	NCBI: NP_079034.3
Critical commercial assays		
Gibson Assembly Cloning Kit	New England Biolabs	Cat# E5510S
Deposited data		
Unified Human Gastrointestinal Protein Catalog v(2.0.1)	Almeida et al.[56]	http://ftp.ebi.ac.uk/pub/databases/metagenomics/mgnify_genomes/human-gut/v2.0.1/
ZINC20 ligand restraints	Irwin et al.[57]	https://zinc.docking.org/
E. coli agmatinase (EcAGM)	Maturana et al.[58]	PDB: 7LOL
Mapped microbial abundances for metagenome-based cohort data	Pryor et al.[17]	https://data.mendeley.com/datasets/crmtpmd622/draft?a=ef347ccd-7532-44b0-8925-d2c04a71b419
Recombinant DNA		
pET28a(+)	EMD Millipore	Cat# 69864-3
pGro7	Takara Bio	Cat# 3340
Software and algorithms		
MGnify	Richardson et al.[15]	https://www.ebi.ac.uk/metagenomics
BLAST+	Camacho et al.[59]	https://blast.ncbi.nlm.nih.gov/doc/blast-help/downloadblastdata.html
EFI-EST	Gerlt et al.[60]	https://efi.igb.illinois.edu/efi-est/

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cytoscape	Shannon et al.[61]	https://cytoscape.org/
AutoDock Vina	Eberhardt et al.[62]	https://autodock-vina.readthedocs.io/
AutoDockTools	Morris et al.[63]	https://autodock.scripps.edu/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Lawrence P. Wackett (wacke003@umn.edu).

Materials availability

- This study did not generate new unique reagents.

Data and code availability

- Data showing enzyme kinetics reported in this paper will be shared by the lead contact upon request. This paper analyzes existing, publicly available datasets and their accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

METHOD DETAILS

Cloning, expression and purification of ureohydrolase homologs

The agmatinase from *Escherichia coli* gene (EcAGM, WP_000105566.1) was cloned from the ASKA[64] clone, JW2904, that contained the *speB* gene on an overexpression plasmid which was PCR amplified and cloned into *Escherichia coli* BL21 DE3 cells (New England Biolabs) using a pET28 vector derivative with kanamycin resistance. The gene was cloned with an N-terminal a 6x His-tag and inserted, by Gibson assembly, into the multiple cloning site using the NdeI and HindIII restriction sites. The *Clostridium butyricum* agmatinase (CbAGM, QGH27404.1), *Pseudomonas aeruginosa* guanidinopropionase (GpuA, WP_003112934.1), *Enterococcus faecalis* agmatine deiminase (AgDI, WP_002363185.1), and human arginase 1 (ARG1, NP_000036.2) were codon-optimized and cloned in the same procedure as EcAGM. The homologs, human arginase 2 (ARG2, NP_001163.1) and human guanidino acid hydrolase (AGMAT, NP_079034.3) were cloned as truncation variants for optimal expression in *E. coli* as the native sequences contain signal peptide sequences to direct their proteins to the mitochondria. Truncation variants ARG2 $\Delta_{1\text{-}24}/\Delta_{331\text{-}354}$ and AGMAT $\Delta_{1\text{-}35}$ were made as done in prior studies and codon-optimized, cloned in the same procedure as above.[20,21]

The proteins EcAGM, GpuA, CbAGM, and AgDI were expressed by growing cells in lysogeny broth (LB) medium supplemented, with either 0.5 mM $MnSO_4$ (EcAGM, GpuA), 0.5 mM $NiSO_4$ (CbAGM), or no metal (AgDI) and 50 μg/mL kanamycin at 37°C and 200 rpm to an OD_{600} of 0.6 in a shake flask. The culture was cooled to 16°C and induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and, with the same agitation, incubated for 20 h. Cell pellets were harvested by centrifugation at 1,500 x g for 20 min and stored at −80°C. The human arginases ARG1, ARG2 were expressed by growing cells in terrific broth (TB) medium supplemented with 0.5 mM $MnSO_4$, 50 μg/mL kanamycin and the cells were grown, induced and cell pellets harvested as done for the other proteins.

The soluble expression of AGMAT required the co-expression of GroES-GroEL chaperones using a co-transformed plasmid, pGro7 (Takara Bio) with chloramphenicol resistance and expression induced by arabinose.[65] AGMAT was expressed by growing cells in TB medium, supplemented with 0.5 mM $MnSO_4$, and 50 μg/mL kanamycin and 30 μg/mL chloramphenicol at 37°C and 200 rpm to an OD_{600} of 0.4 in a shake flask. The culture was cooled to 16°C and GroES-GroEL chaperones were induced with 0.005% (w/v) arabinose and 1 h later, AGMAT was induced with 1 mM IPTG and, with the same agitation, incubated for 20 h. Cell pellet of AGMAT was harvested as stored as done for the other proteins.

For purification, cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, 500 mM NaCl, 10 mM beta-mercapatoethanol pH 7.4). The cells were lysed using a French Press with three passes at 10,000 psi and the lysate was then clarified by centrifugation at 20,000 x g for 1 h. The proteins were purified from the lysate by using fast protein liquid chromatography (FPLC) and immobilized metal affinity chromatography (IMAC). Using a GE-AKTA FPLC and a GE HisTrap 5 mL column, proteins were purified after running an imidazole gradient from 25 mM to 500 mM and fractions collected. Pooled fractions from the FPLC were buffer exchanged into storage buffer (20 mM HEPES-NaOH, 200 mM NaCl pH 8) using a 15-mL Amicon 10 kDa centrifugal filter. Protein concentrations were determined by Bradford reagent. Aliquots of concentrated protein were flash frozen in liquid nitrogen and stored at −80°C to be then used for kinetic assays. For metal reconstitution experiments, CbAGM enzyme was stripped of metal by buffer exchanging the enzyme with 1 mM 1,10-phenanthroline, 2 mM EDTA in storage buffer using a 15-mL Amicon 10 kDa centrifugal filter. See Figure S5 for a stained, denaturing polyacrylamide gel of the proteins used in the study.

Substrates and NMR

Metformin hydrochloride (Cayman Chemical), buformin hydrochloride (Enamine), phenformin hydrochloride (Cayman Chemical), L-arginine hydrochloride (Acros), agmatine sulfate (Fluka), 4-guanidinobutyric acid (Fluka) and 3-guanidinopropionic acid (Sigma) were obtained with high purity (>97%).

Galegine hemisulfate was synthesized via the condensation reaction of a thiourea with the alkylamine using a procedure adapted from Williams et al.[66] 2-methyl-2-thiopseudourea hemisulfate, 7 mmol, (Sigma) was added to 8 mmol 3-methyl-2-buten-1-amine (A2B Chem) in 5 mL of water and stirred overnight at room temperature. The reaction produced precipitate which was dried on a porcelain plate and washed with methanol. The solids were then recrystallized in methanol to produce galegine hemisulfate in high purity (>95%) as determined by NMR. ^{1}H-NMR (DMSO-d6, 400 MHz) δ (ppm) 1.62 (s, 3H), 1.68 (s, 3H), 3.64 (br, 2H), 5.16 (t, 1H), 7.62 (br, 4H), 8.46 (br, 1H). ^{1}H-NMR experiments were conducted using the Varian Unity Inova 400 MHz NMR system and VnmrQ 2.2 software.

Enzyme kinetics and inhibition

Agmatinase, arginase, guanidinopropionase and guanidinobutyrase activity was measured by tracking urea production using a fixed time-point, colorimetric assay.[67] Enzyme assays were mainly done at room temperature in 100 mM HEPES-NaOH pH 7.5 with 2 mM $MnCl_2$ or 0.1 mM $NiCl_2$ for the Ni^{2+}-dependent CbAGM. Enzyme reactions with 25 μL volume, initiated by enzyme, were quenched with 75 μL of the colorimetric acid reagent (120 μM $FeCl_3$, 10 mM phosphoric acid in 20% w/v H_2SO_4) and then to this mixture, 50 μL of the colorimetric color reagent (62 mM 2,3-butanedione monoxime, 3.6 mM thiosemicarbazide in water) was added. The samples were then heated in a PCR thermocycler for 15 min at 96°C, in capped tubes, to produce color. Samples were cooled to room temperature and then transferred to 96-well flat-bottom microplates and diluted to 200 μL with deionized water. Using an Agilent BioTek Synergy HTX microplate reader, the absorbance value at 520 nm was measured for all of the samples. Urea standards from 0 μM to 200 μM and no enzyme controls were treated in the same way as the enzyme reactions to quantify urea concentrations and determine background levels. Enzyme assays done at pH 6.5 or pH 8.5 were done in 100 mM MOPS-NaOH or 100 mM HEPES-NaOH, respectively.

Initial screening for metformin inhibition of ureohydrolase proteins involved measuring activity with 1 mM of the preferred substrate with or without 50 mM metformin. Inhibition constants (K_i) for were determined for the inhibitors by first obtaining the apparent K_M (K_M^{app}) of agmatine in the presence of inhibitor. The activity of EcAGM and CbAGM was measured at multiple agmatine concentrations (0–50 mM) with or without fixed inhibitor concentrations (0–50 mM) of metformin, phenformin, buformin and galegine. The measured K_M, K_M^{app} for each inhibitor were then used to determine the K_i using Equation 1, assuming purely competitive inhibition.

$$K_M^{app} = K_M\left(1 + \frac{[I]}{K_i}\right) \quad \text{(Equation 1)}$$

Computational modeling and Bioinformatics

Docking metformin, buformin, phenformin and galegine into the active site of EcAGM was done using AutoDock Vina (Version 1.2.5) and ligand restraints were obtained from the ZINC20 database (https://zinc.docking.org/, ZINC000012859773, ZINC000004097425, ZINC000005851063, ZINC000000897460, respectively).[57,58,62] The protein receptor (PDB 7LOL) was prepared using AutoDockTools4 (Version 4.2.6) with polar hydrogens added, using default charges for standard residues and the partial charges for manganese ions were set to 0.580 according to Neves et al..[63,68] The docking was done using the AutoDock4 forcefield with default parameters except the exhaustiveness was set to 100 and the ligand guanidinium torsions were set to rotatable. The AutoDock scores for the best binding mode when docking EcAGM with metformin, buformin, phenformin and galegine were −6.7, −8.6, −4.9, and −6.0, respectively.

Bioinformatics

Ureohydrolase protein superfamily human gut homologs were mined from the Unified Human Gastrointestinal Protein (UHGP) v.2.0.1 catalog (http://ftp.ebi.ac.uk/pub/databases/metagenomics/mgnify_genomes/human-gut/v2.0.1/) by first performing a protein BLAST search to retrieve 10,000 sequences that are most similar to the protein sequence of *E. coli* agmatinase (EcAGM).[56,59] A sequence similarity network (SSN) was then generated from this set of sequence hits using the EFI-EST tool that performed pairwise BLAST comparisons on the 10,000 related sequences.[60] Cytoscape was used to visualize the clustering in the SSN and identify sequence clusters encoding unique functions.[61] The SSN also included protein sequences of experimentally characterized ureohydrolase enzymes with known function to help annotate the hypothetical function of these clusters. Using this SSN, the cluster of sequences associated with EcAGM and the cluster associated with CbAGM were extracted to identify the taxonomy of the gut microbes that contain an agmatinase related to EcAGM or CbAGM. See Tables S3 and S4 for lists of EcAGM-like and CbAGM-like protein sequences with taxonomic information.

To understand the likely relative abundances of EcAGM-like, and CbAGM-like functions in the human gut microbiome, the 16S rRNA profiling data from fecal samples of type-II diabetes patients, taking metformin, as analyzed by Pryor et al. was used (https://data.mendeley.com/datasets/crmtpmd622/1).[3,17,47,69] The analysis consisted of mapping 16S rRNA reads from three separate cohort studies (Danish,[3] Swedish[69] and Spanish[47]) to 773 human gut microbial genomes used in the metabolic reconstruction tool AGORA (Assembly of Gut Organisms through Reconstruction and Analysis).[70] The mapped reads to microbial genomes were normalized to the total amount of reads mapped in each sample to calculate % relative abundances. Microbes with genus and species that matched the organismal taxonomy

of the protein sequences pertaining to EcAGM-like, and CbAGM-like functions were put into separate bins. See Tables S5 and S6 for relative abundances of microbes that encode an EcAGM-like or CbAGM-like agmatinase.

Enzyme kinetics of agmatine deiminase

Agmatine deiminase activity by AgDI was measured by following ammonia production using a spectrophotometric, coupled-enzyme assay. Agmatine deiminase produces N-carbamoylputrescine and ammonia which the latter can be used with bovine liver L-glutamic acid dehydrogenase (GDH) to reductively aminate 2-oxoglutarate to form L-glutamate, which causes NADH oxidation that can be measured by absorbance at 340 nm. For reactions, a 10X coupled enzyme assay master mix was prepared in 50 mM HEPES-NaOH pH 7.5 which had the following components and final concentrations: 0.3 mM NADH disodium salt (Sigma), 5 mM 2-oxoglutarate sodium salt (Aldrich), 0.8 mM adenosine diphosphate sodium salt (Sigma), 2.5 U/mL GDH from lyophilized powder (Sigma). The master mix was then diluted with buffer (150 mM HEPES-NaOH pH 7.5) and purified AgDI enzyme into wells of 96-well flat-bottom microplates and the reaction was initiated by adding substrate to make a total sample volume of 200 μL. The reactions were monitored, continuously, by absorbance at 340 nm using a microplate reader and initial rates were recorded. Rates of NADH oxidation were calculated assuming a pathlength of 0.56 cm and a molar extinction coefficient for NADH, at 340 nm, of 6220 $M^{-1}cm^{-1}$. Negative controls for the assays included no-enzyme and enzyme with no substrate that resulted in an unchanging amount of NADH in the assay over time.

HPLC analysis for metformin transformation

A reversed-phase HPLC method to separate guanylurea from metformin was adapted from Lin et al. and detailed by Martinez-Vaz et al. which, in brief, used a C18 column and an isocratic mobile phase of 75:25 (v/v) acetonitrile:10 mM potassium phosphate buffer pH 6.6.[71,72] To determine if metformin could be transformed by enzymes EcAGM, CbAGM, GpuA, AGMAT, ARG1 and ARG2, 1 mM metformin was incubated with 10 μg of purified enzyme in 20 mM CHES pH 9 overnight and then assayed by HPLC. No transformation of metformin was seen for any of the enzymes tested.