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L-methionine Anti-Biofilm Activity against *Pseudomonas Aeruginosa* Is Enhanced by the CFTR Potentiator, Ivacaftor

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Abstract

Objectives—Biofilms may contribute to refractory chronic rhinosinusitis (CRS), as they lead to antibiotic resistance and failure of effective clinical treatment. L-methionine is an amino acid with reported biofilm inhibiting properties. Ivacaftor is a CFTR potentiator with mild antimicrobial activity via inhibition of bacterial DNA gyrase and topoisomerase IV. The objective of this study is to evaluate whether co-treatment with ivacaftor and L-methionine can reduce the formation of *Pseudomonas aeruginosa* biofilms.

Methods—*P. aeruginosa* (PAO-1 strain) biofilms were studied in the presence of L-methionine and/or ivacaftor. For static biofilm assays, PAO-1 was cultured in a 48-well plate for 72 hours with stepwise combinations of these agents. Relative biofilm inhibitions were measured according to optical density of crystal violet stain at 590 nm. Live/dead assays (BacTiter-Glo™ assay, Promega) were imaged with laser scanning confocal microscopy. An agar diffusion test was used to confirm antibacterial effects of the drugs.

Results—L- methionine (0.5 μM) significantly reduced PAO1 biofilm mass (32.4 ± 18.0 %, n=4, $p<0.001$) compared to controls. Low doses of ivacaftor alone (4, 8, 12 μg/ml) had no effect on biofilm formation. When combined with ivacaftor (4 μg/ml), synergistic anti-biofilm effect was noticed at 0.05 μM and 0.5 μM of L-methionine (two-way ANOVA, $p = 0.0415$) compared to corresponding concentrations of L-methionine alone.

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Conclusion—Ivacaftor enhanced the anti-biofilm activity of L-methionine against the PAO-1 strain of *P. aeruginosa*. Further studies evaluating the efficacy of ivacaftor/L-methionine combinations for *P. aeruginosa* sinusitis are planned.

Keywords

Pseudomonas Aeruginosa; L-methionine; Ivacaftor; Biofilm; Chronic Rhinosinusitis; Sinusitis; CFTR; Cystic Fibrosis

INTRODUCTION

Chronic rhinosinusitis (CRS) is a chronic airway disease defined as persistent inflammation and infection of the nasal and sinus mucosa.[1] Mounting evidence suggests that biofilms may contribute to the pathophysiology of recalcitrant CRS.[2-5] Bacterial biofilm-positive CRS patients exhibit worse sinus symptoms and require multiple courses of antibiotic treatment.[4] In particular, *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms are known to confer poor clinical improvement following surgical intervention.[2] Biofilms create an inhospitable environment for antibiotic potency by downregulating the metabolic activity of "core" bacteria and creating a milieu conducive for antibiotic resistance.[6] Bacteria residing in biofilms are difficult to eradicate with antibiotics at standard doses, even when topical irrigation is considered. A rabbit model with *P. aeruginosa* sinusitis required tobramycin at approximately 400x the mean inhibitory concentration to eradicate the infection.[7] Such high doses can be ineffective in the long-term due to the heightened risk of the emergence of new antibiotic resistant strains as well as the risk of systemic side effects.

To treat bacterial biofilms without using such intense drug therapies, alternative approaches with unconventional agents that disrupt or inhibit biofilms have garnered significant attention.[8,9] Amino acids, such as D-amino acids and L-tryptophan are commonly secreted by biofilms during later stages of development and have been amongst several agents recently studied for their potential anti-biofilm activity.[10-13] D-amino acids are effective at disassembling existing biofilms, whereas L- tryptophan inhibits the formation of biofilms. L-methionine was recently shown to inhibit the formation of *P. aeruginosa* biofilms at low concentrations (0.5 μM) by increasing DNase activity and degrading extracellular DNA, which is required for biofilm formation. In addition, ivacaftor, a cystic fibrosis transmembrane conductance regulator (CFTR) potentiator that enhances Cl^- secretion in airway epithelia[14-18], was recently identified to have potentially beneficial off-target effects as a weak inhibitor of bacterial DNA gyrase and topoisomerase IV.[19,20] Alternative antibacterial agents against *P. aeruginosa* may provide a viable treatment option without risks of antibacterial resistance or antibiotic-related complications. The objective of the current study is to evaluate whether ivacaftor enhances the anti-biofilm activity of L-methionine against *P. aeruginosa*.

METHODS

Drugs

L-methionine (98+%, Acros Organics, Belgium, NJ) was added to Luria-Bertani (LB)-Miller broth (Fisher Scientific, Bridgewater, NJ) and immediately diluted to desired concentrations. Ivacaftor (VX-770; Selleckchem, Houston, TX) was dissolved in DMSO to create a stock solution (10 mg/ml).

Preparation of PAO-1 wild type P. aeruginosa Biofilm

P. aeruginosa (PAO-1 strain) was expanded from glycerol frozen stock by inoculating 50 ml of LB broth followed by growth overnight at 37°C on a shaker at 200 rpm. Cultures were streaked on LB agar plates according to the quadrant method and grown in a static incubator at 37°C overnight at least twice to confirm conformity of cultures. From the plate, an isolated colony was grown in 10 ml of LB-Miller broth at 37°C on a shaker at 200 rpm overnight. Cultures were diluted with fresh LB-Miller broth to an inoculation concentration of 1×10^4. Biofilm experiments were performed in a 48-well plate where 800 µl total LB-Miller broth containing different concentrations of L-methionine and ivacaftor were incubated at 30 °C for 72 hours. To evaluate impact on pre-existing biofilm mass, drugs were administered to the 48-well plate after 72 hours of bacterial incubation. Adequate biofilm growth for the positive control well was defined as a mean optical density $(OD)_{600}$ difference (OD_{600} at 6 h minus OD_{600} at 0 h) higher than 0.05.[21]

Crystal violet (CV) staining of biofilms

Crystal violet staining was used to quantify PAO-1 biofilms.[22] Briefly, after the incubation period, the media in each well was gently aspirated with a pipette and then rinsed in DI water 3 times to remove excess liquid and bacterial debris. After letting plates air dry, 900 µL of 0.1% (w/v) crystal violet diluted in DI water was added to the wells for 20 minutes at room temperature. The crystal violet was then aspirated by pipette and washed again three times in DI water to remove excess stain. Stain was dissolved using 900 µL of 30% acetic acid and the absorbance read at 590 nm using a microplate reader (Synergy HK, BIO-TEK Instruments, Winooski, VT).

Agar Diffusion Test

PAO-1 cultures were grown overnight to a concentration of 2×10^6. 100 µL of the culture was spread onto 100 mm petri dishes (Fisher Scientific, no. FB0875712) using 30 mm cell spreaders (Fisher Scientific, no. 08-100-10) on Mueller-Hinton agar (BD chemical, ref: 225250). Blank diffusion disks (ThermoFisher, ref: R55054) were soaked in pure LB broth, or LB broth with the desired drug concentrations of L-methionine and ivacaftor for 15 min before being transferred onto the agar with forceps. Plates were incubated at 30 °C overnight and measured the next day.

Bacterial Biofilm Viability

At the desired concentrations of L-methionine and ivacaftor, PAO-1 was cultured for 24 hours on 14mm glass coverslips within a 35mm dish (MatTek, Ashland, MA). PAO-1

biofilms were stained with SYTO9 and propidium iodide (PI) staining (BacLight™ Live/Dead Bacterial Viability Kit; Molecular Probes, Eugene, OR). The viability was measured with confocal laser scanning microscopy (A1R, Nikon, Tokyo, Japan) and image analyses were performed with ImageJ national institute of health (NIH) image processing software.[23] The quantitative structural parameters of the biofilms such as volume and thickness, were extracted from confocal stack images and analyzed. The average z-stacks of 1 μm were acquired from each biofilm horizontal plane with a maximum of five stacks at different fields of view. The quantification of biomass, representing overall volume of cells in the biofilm was carried out using free *bio*Image_L (www.bioimagel.com. Malmö, Sweden).[24]

Statistical Analysis

All experiments were performed at least 4 independent times. Normalized values for relative biofilm quantification were expressed as ± standard error of the mean. Statistical analyses were conducted with an included statistical tool of GraphPad Prism 6.0 software (La Jolla, Ca). For evaluating the combined effects of L-methionine and ivacaftor, a one-way ANOVA was performed with a post-hoc Dunnett's multiple comparison test with significance set at $p < 0.05$. For comparing the effects between different L-methionine concentrations, a two-way ANOVA was performed with a Tukey's multiple comparison test with significance set at $p < 0.05$.

RESULTS

Anti-biofilm activity of L-methionine and ivacaftor against PAO-1 biofilms

To determine the anti-biofilm activity of L-methionine against PAO-1 biofilms, escalating concentrations of 0.05, 0.5, 2.5, and 5 μM of L-methionine were used (Figure 1). Compared to control, statistically significant anti-biofilm activity was noted at concentrations of 0.05, 0.5 and 2.5 μM of L-methionine ($p = 0.036$, 0.003, 0.009, respectively). The highest dose-specific inhibition of biofilm growth was observed with 0.5 μM L-methionine (OD_{590} 0.29 ± 0.03), significantly lower than controls (OD_{590} 0.41 ± 0.06) ($p = 0.003$) (Figure 1), which was consistent with previous studies.[13] Anti-biofilm activity diminished as the concentration of L-methionine increased from 2.5 μM (OD_{590} 0.307 ± 0.023, $p=0.009$) to 5 μM (OD_{590} 0.39 ± 0.033, $p = 0.9$).

Anti-biofilm activity of ivacaftor was evaluated with escalating concentrations 4, 8, and 12 μg/ml. These doses were selected based on our previous experiments (unpublished) to avoid dimethyl sulfoxide (DMSO)'s antibacterial effect. DMSO vehicle controls reduced PAO-1 concentrations with escalating doses above 16 μg/ml. As expected, there was no dose-dependent inhibition of biofilm mass at the 3 selected concentrations of ivacaftor in the current study (Figure 2).

Synergistic effect on PAO-1 biofilm formation by L-methionine and ivacaftor

PAO-1 biofilms were grown in the presence of the 2 chosen L-methionine concentrations (0.05 μM, 0.5 μM) combined with the concentrations of ivacaftor used in the previous experiments (Figure 3). L-methionine at 0.05 μM showed significant reduction in biofilm mass when combined with ivacaftor at all concentrations vs. L-methionine alone.

Interestingly, the greatest decrease was observed with the lowest ivacaftor concentration [4 μg/ml ivacaftor = 31.5 ± 3.8 % reduction (n = 4, $p < 0.001$), 8 μg/ml ivacaftor = 17.2 ± 2.2 % reduction (n = 4, $p < 0.001$), 12 μg/ml ivacaftor = 10.7 ± 9.7 % reduction (n = 4, $p < 0.05$)].

When combining ivacaftor with 0.5 μM L-methionine, a similar pattern was observed. All concentrations of ivacaftor significantly inhibited relative biofilm biomass with the lowest concentration providing the greatest decline [4 μg/ml ivacaftor = 54.5 ± 14.1 % reduction (n=4, $p<0.001$), 8 μg/mL ivacaftor = 37.9 ± 13.2 % reduction (n=4, $p<0.001$), 12 μg/ml ivacaftor = 32.3 ± 6.3 % reduction (n=4, $p<0.001$)]. Synergistic anti-biofilm effect of L-methionine and ivacaftor was noticed at 0.05 μM and 0.5 μM of L-methionine with 4 μg/ml of ivacaftor (two-way ANOVA, $p = 0.0415$).

Eradication of PAO-1 biofilm by L-methionine and ivacaftor

The capability of 0.5 μM of L-methionine plus 4 μg/ml of ivacaftor to eradicate pre-existing PAO-1 biofilms was evaluated. Drugs were administered 72 hours after PAO-1 incubation. Combining L-methionine with ivacaftor significantly decreased biofilm mass (co-treatment (0.5 μM of L-methionine + 4 μg/ml ivacaftor) = 57.5 ± 0.07 % eradication (n = 6), $p = 0.043$) compared to either agent alone (0.5 μM of L-methionine = 18.7% ± 0.17 % eradication, 4 μg/ml ivacaftor = 27.4% ± 0.09 % eradication) (Figure 4). There was no statistically significant difference in eradication of biofilm mass by single agent compared to control ($p > 0.05$).

Agar Diffusion Tests

To evaluate the synergy of L-methionine and ivacaftor against planktonic growth of PAO-1, blank diffusion disks were impregnated with pure LB broth, 0.5 μM L-methionine, 4 μM ivacaftor, or ivacaftor (4 μg/mL) + L-methionine (0.5 μM). Disks (n = 4, per condition) were inserted onto evenly streaked plates of MH agar and incubated for 24 hours. The radii of the clear zones around the disks were measured with ImageJ (Figure 5). An increase in the susceptibility of PAO-1 was observed from single to combined drug concentrations (clear zone radius (cm), control = 0 ± 0.00, ivacaftor = 0.28 ± 0.16, L-methionine = 0.59 ± 0.12, L-methionine + ivacaftor = 0.92 ± 0.05, $p < 0.05$). The mean radius of the clear zone of ciprofloxacin (2μg) was 1.16 +/− 0.01 cm and there was no statistical difference between ciprofloxacin and L-methionine + ivacaftor.

Confocal Laser Scanning Microscopy (CLSM)

Total biofilm area and bacterial viability were calculated in the presence of L-methionine (0.5 μM) or L-methionine with ivacaftor (4 μg/mL) for 3 days under static biofilm conditions (Figure 6). Image analysis using *bio*Image_L revealed the biofilm area was significantly smaller with L-methionine (3.3 ± 0.45%) compared to controls (19.1 ± 0.85%, $p < 0.001$), but adding ivacaftor reduced the biofilm area to 1.2 ± 0.19%. This was significantly reduced compared to L-methionine alone (n = 12 each, $p < 0.001$). When we calculated the thickness of the biomass, there were significant differences among groups (controls = 21.6 ± 1.3 μm: L-methionine = 11.1 ± 0.6 μm: L-methionine + ivacaftor = 6.8 ± 1.4 μm, $p < 0.0001$).

DISCUSSION

Bacteria adhered to a surface form the complicated structural architecture of a biofilm, endowing resistance against antibacterial therapy. Bacterial biofilms are a conducive environment where bacterial cells can perceive antibiotics, communicate with each other, and change gene and metabolic activity to create less susceptibility to antibiotics.[25,26] Biofilms are linked to the onset of recalcitrant CRS, and have substantial impact to the clinical course of the disease due to induction of strong antibiotic resistance.[27-29] New medical therapies directed towards biofilm formation are clearly required to treat recalcitrant CRS infections. In the current study, a synergistic effect against anti-biofilm formation was noted with low concentrations of L-methionine (0.5 μM) and ivacaftor (4 μg/ml) without the addition of traditional antibiotics. A strategy using topical delivery of L-methionine and ivacaftor to the sinuses as therapy for recalcitrant biofilm-forming *P. aeruginosa* infections would help avoid expansion and promotion of resistant strains of bacteria and assist with clearance of biofilm forming niduses that inevitably result in acute exacerbations.[6]

L-methionine is an amino acid not directly associated with antibacterial therapy, but was recently shown to inhibit and disassemble *Pseudomonas* biofilms at low concentrations (0.5 μM). The mechanism is thought to be related to induction of bacterial DNase expression which then degrades extracellular DNA within the biofilm.[13] However, the mechanism behind DNase induction is not fully understood. Since L-methionine is an amino acid and can be utilized by bacteria as a nutrient source, higher concentrations may not provide the specific cue to induce DNase. At significantly low concentrations, bacteria may not be capable of detecting L-methionine as a signal for the cue.[13] The biofilm inhibitory effect observed at lower concentrations of L-methionine may also be linked to quorum sensing – a mechanism highly sensitive to signaling molecules.[13,30] Notably, DNase treatments have been exploited to improve infections in the lower and upper airways of cystic fibrosis patients. The mucolytic, dornase alfa, once daily (2.5 mg) administered intranasally was found to be improve sinusitis symptoms in cystic fibrosis patient in several clinical trials.[15,31-36]

Ivacaftor enhances Cl^- secretion in airway epithelia, including the sinonasal mucosa through CFTR ion channels.[14-17] Past studies have reported off target anti-bacterial effects of ivacaftor as having an MIC against *P. aeruginosa* above 32 μg/ml.[19] Indeed, the lower concentrations (4, 8 and 12 μg/ml) used in the present study did not have an effect on biofilm formation unless combined with L-methionine indicating a synergistic effect. A low concentration of ivacaftor (e.g. 4 μg/ml) significantly enhanced the activity of L-methionine at 0.5 μM, suggesting that this drug combination holds promise as a treatment for *P. aeruginosa* biofilm formation associated with CRS.

While the reasons for this phenomenon are currently unknown, biofilm disassembly by L-methionine through DNase induction could provide access to resident bacteria for the antimicrobial effects of ivacaftor. Previous studies indicate that the outer membrane of *P. aeruginosa* limits the diffusion of hydrophobic substances, including antibiotics.[19] Therefore, we hypothesized that the outer membrane of *P. aeruginosa* might serve as a barrier for ivacaftor penetration, which may require dissolution by L-methionine (DNase)

first. To test this hypothesis, L-methionine (0.5 μM) was administered, followed by L-methionine 48 hours later. However, we did not observe a dose-dependent reduction in biomass with ivacaftor at 0.5 μM L-methionine (Supplement Figure 1). The highest inhibition was still observed at the lower dose of ivacaftor (4 and 8 μg/ml). Although the MIC of ivacaftor has been reported above 32 μg/ml,[19] we were unable to dissolve ivacaftor into regular LB broth without the addition of higher concentrations of DMSO, which would affect the behavior of the PAO1 strain. It is important to note that the agar diffusion test clearly shows that both L-methionine and ivacaftor have activity against planktonic PAO1 bacteria indicating other undescribed mechanisms may be involved.

Methionine is an indispensable amino acid and must be supplied from the diet. Long term human studies did not demonstrate serious toxicity, except at very high levels of intake.[37] Despite the function of methionine as a precursor of homocysteine, and the role of homocysteine in vascular damage and cardiovascular disease, there is no evidence that dietary intake of methionine within reasonable limits will cause cardiovascular damage. A single dose of 100 mg/kg body weight has been shown to be safe, but this is about 7 times the daily requirement for sulfur amino acids. In infants, methionine intakes of 2–5 times normal resulted in impaired growth and extremely high plasma methionine levels, but no adverse long-term consequences were observed.[37,38]

There are several limitations to this study. *P. aeruginosa* is known for its intrinsic resistance to a variety of antimicrobial agents and toxic compounds, but only the PAO1 strain was tested.[39] In addition, DNase activity was not measured in the supernatants of the cell cultures, which may have provided clues to the underlying pathophysiology at each setting. Finally, these *in vitro* experiments only demonstrated the inhibitory effect of biofilm formation rather than disruption of previously existing biofilms. Further studies are planned to 1) test activity against multi-drug resistant strains of *P. aeruginosa* from human isolates, 2) evaluate the underlying mechanism of action, including generation of DNase, 3) administer to a co-cultured model of PAO1 on nasal epithelial cell culture,[29] and 4) validate the efficacy of L-methionine/ivacaftor topical therapy in a rabbit model of *P. aeruginosa* sinusitis.

CONCLUSION

Ivacaftor enhanced L-methionine's anti-biofilm activity against the PAO-1 strain of *P. aeruginosa*. This combination therapy represents an exciting treatment strategy for recalcitrant biofilm-associated sinus infections. Translations of these findings to preclinical and clinical trials are planned.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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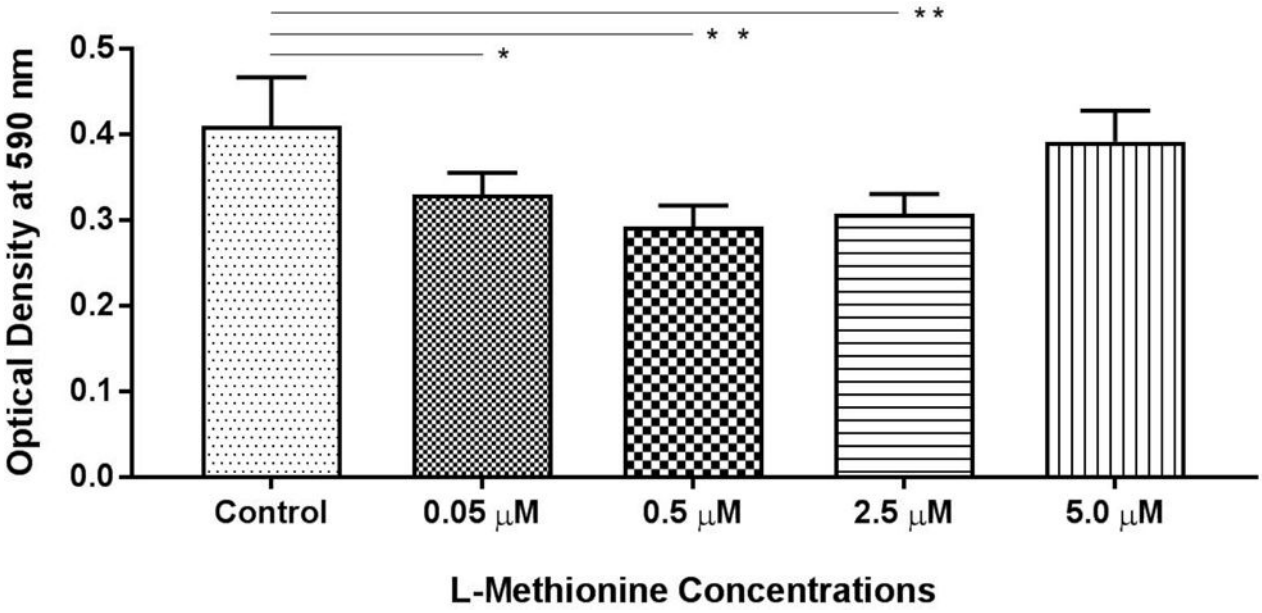

Figure 1. Effect of L-methionine on PAO1 Biofilm formation

Dose-dependent inhibition of PAO-1 biofilm growth was observed at 0.05, 0.5, and 2.5 μM L-methionine, with the largest difference occurring at 0.5 μM.

* represents statistical significance of $p<0.05$. ** represents statistical significance of $p<0.01$ when compared to the control condition. Analysis was performed by one-way ANOVA tests with Dunnett's multiple comparisons. All conditions consisted of at least 4 experiments.

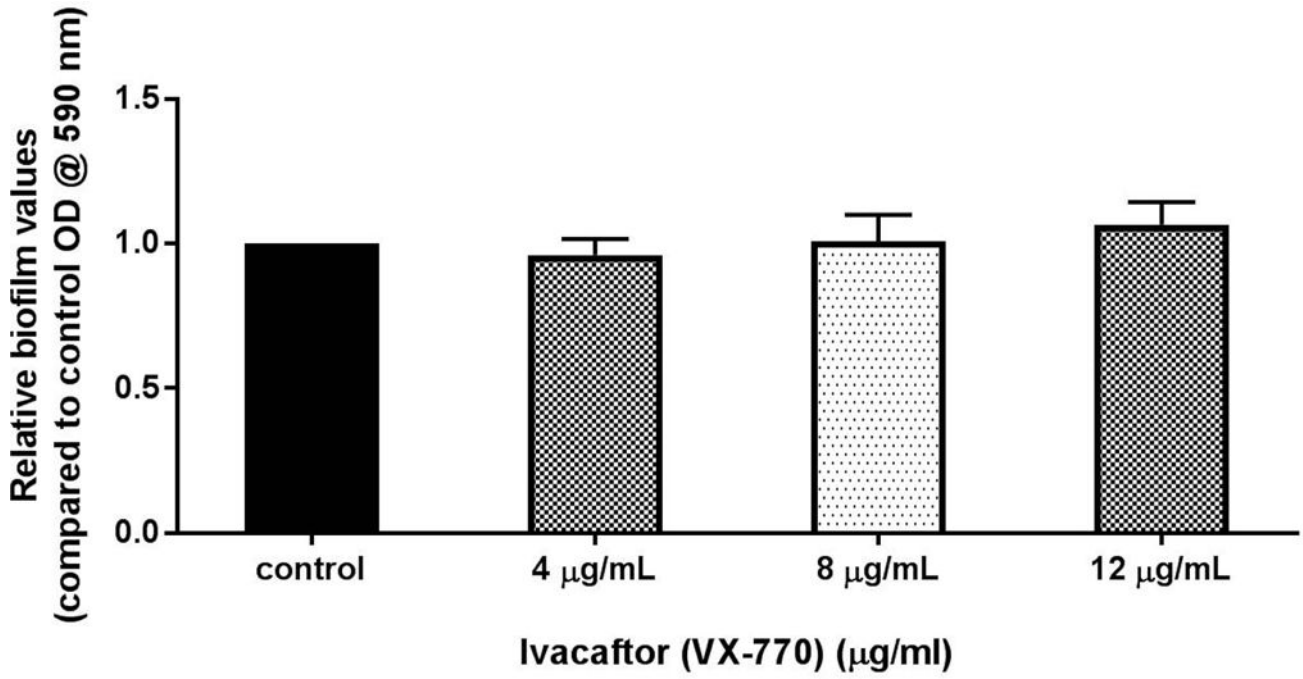

Figure 2. Effect of ivacaftor on PAO-1 biofilm formation

There was no observable effect on biofilm biomass with any ivacaftor concentration tested.

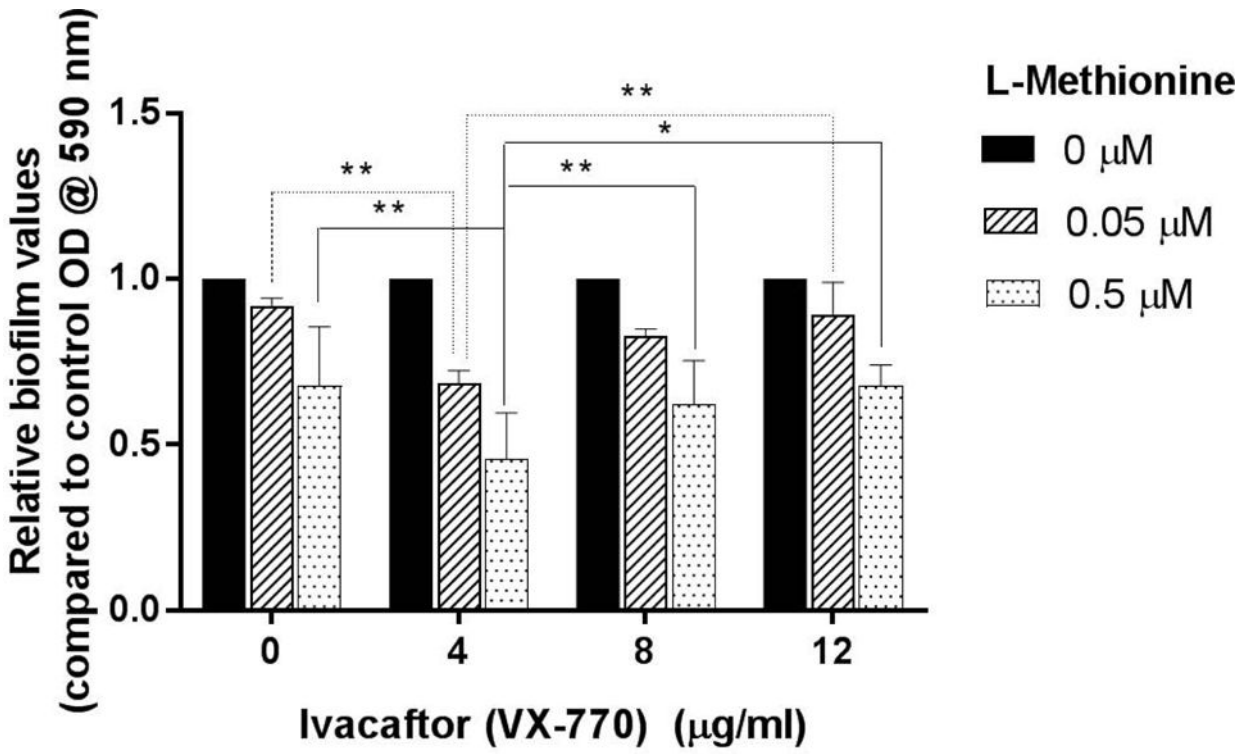

Figure 3. Enhanced anti-biofilm formation activity of L-methionine against *P. aeruginosa* PAO-1 with ivacaftor

L-methionine anti-biofilm activity was detected at all concentrations ivacaftor.

* represents a statistical significance of $p<0.05$. ** represents a statistical significance of $p<0.01$. Significance was measured using a two-way ANOVA test with a Tukey's post-hoc multiple comparisons test. All conditions consisted of at least 4 experiments.

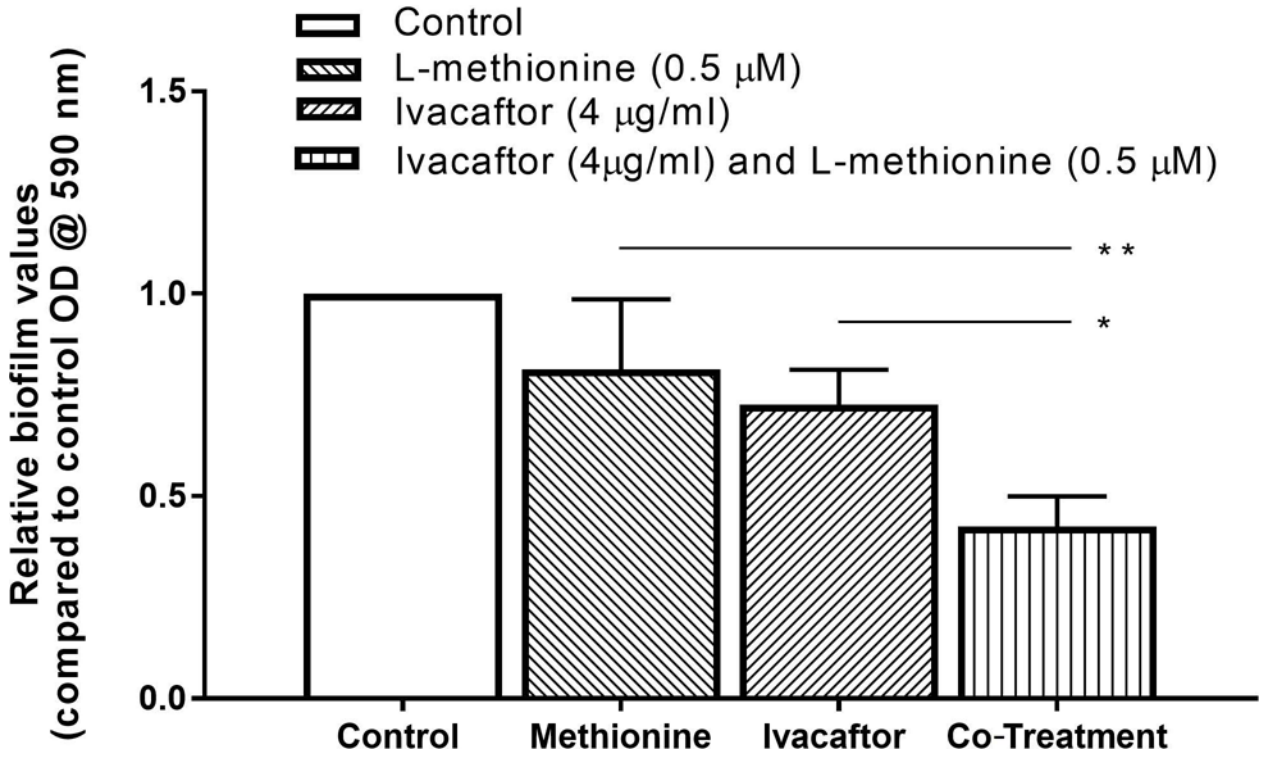

Figure 4. Enhanced anti-biofilm eradication activity of L-methionine against *P. aeruginosa* PAO-1 with ivacaftor

* represents a statistical significance of $p<0.05$. ** represents a statistical significance of $p<0.01$. Significance was measured using one-way ANOVA test with a Tukey's post-hoc multiple comparisons test. All conditions consisted of at least 4 experiments.

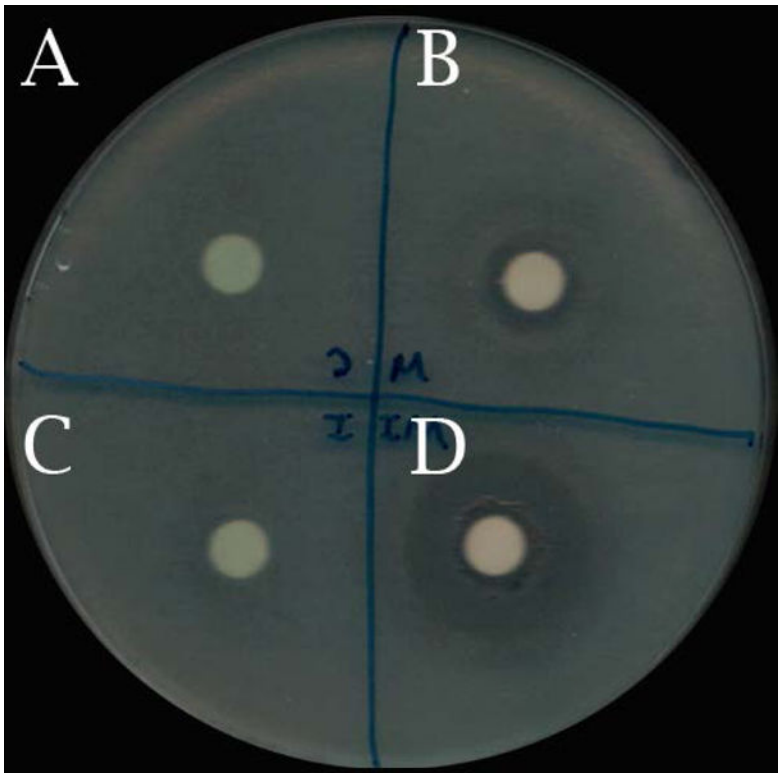

Figure 5. Representative image of agar susceptibility test for 18 hours

A: Control

B: L-methionine 0.5 μM

C: Ivacaftor 4 μg/mL

D: L-methionine 0.5 μM and ivacaftor 4 μg/mL

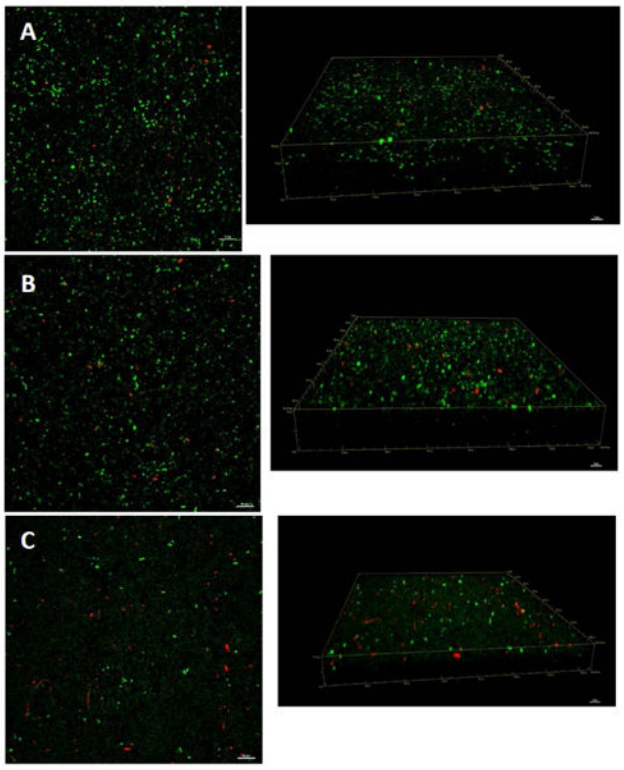

Figure 6. Representative confocal laser scanning microscopy (CLSM) images of *Pseudomonas aeruginosa* PAO-1 biofilms with 3D structures by CLSM z-stacks

A: Control

B: L-methionine 0.5 μM

C: L-methionine 0.5 μM plus ivacaftor 4 μg/ml

Green - live cells, Red - dead cells. Scale bars indicate 20 μM.