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Membrane-Permeabilizing Activity of Reverse-Amide 2-Aminoimidazole Antibiofilm Agents Against *Acinetobacter baumannii*:

Permeabilizing Activity of Reverse Amide 2-AI Agents

Sean D. Stowe¹, Richele J. Thompson¹, Lingling Peng^{2,*}, Zhaoming Su^{2,**}, Meghan Blackledge², G. Logan Draughn¹, William H. Coe¹, Eva Johannes³, Valerie K. Lapham⁴, John Mackenzie⁴, Christian Melander², and John Cavanagh^{1,#}

¹ Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, North Carolina 27695, United States of America

² Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States of America

³ Cellular and Molecular Imaging Facility, Department of Plant Biology, North Carolina State University, Raleigh, North Carolina 27695, United States of America

⁴ Center for Electron Microscopy, Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695, United States of America

Abstract

Acinetobacter baumannii has quickly become one of the most insidious and prevalent nosocomial infections. Recently, the reverse-amide class of 2-aminoimidazole compounds (RA-2AI) was found both to prevent *A. baumannii* biofilm formation and also to disperse preexisting formations, putatively through interactions with cytosolic response regulators. Here we focus on how this class of antibiofilm agent traverses cellular membranes. Following the discovery of dosage-dependent growth rate changes, the cellular effects of RA-2AI were investigated using a combination of molecular assays and microscopic techniques. It was found that RA-2AI exposure has measureable effects on the bacterial membranes, resulting in a period of increased permeability and visible structural aberrations. Based on these results, we propose a model that describes how the structure of RA-2AI allows it to insert itself into and disrupt the fluidity of the membrane, creating an opportunity for increased molecular permeability.

Graphical Abstract

Corresponding Author: John Cavanagh, john_cavanagh@ncsu.edu, phone: 919-513-4349, fax: 919-515-2047.

* Current address for Dr. Lingling Peng: Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, USA

** Current address for Dr. Zhaoming Su: Department of Chemistry, The Scripps Research Institute, Jupiter, Florida 33458, USA

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Reverse-amide 2-aminoimidazole compounds are known to prevent and disperse *Acinetobacter baumannii* biofilms. During its initial activity, this antibiofilm agent traverses membranes and increases permeability, acting as a delivery adjuvant.

Keywords

2-aminoimidazole; *Acinetobacter baumannii*; biofilm; membrane permeabilization; reverse amide; SEM

Introduction

The Gram-negative pathogen, *Acinetobacter baumannii*, has rapidly become one of the leading sources for multidrug-resistant (MDR) nosocomial infections, especially for immuno-compromised patients[1-3]. Found in numerous environments in the clinical setting, *A. baumannii* can persist for weeks without desiccating[4] and is able to readily infect patients through contact with contaminated surfaces and medical devices. Its persistence has been [5] linked to its ability to form resilient biofilms on both biotic and abiotic surfaces[6-9], a process regulated in part by BfmR, the cytosolic response regulator of the two-component signal transduction (TCS) module controlling pili-dependent biofilm formation[9-10]. TCS mechanisms are ubiquitous methods by which bacteria interact with their surroundings and are highly sought after therapeutic targets in antimicrobial drug design[11-12].

Over the last few years, several methods have emerged to combat *A. baumannii* biofilm-based infections, involving a wide-range of potential therapeutic scaffolds[12-17]. As a part of this crusade, we have focused on enhancing the antibiofilm properties of promising 2-aminoimidazole (2AI) derivatives[5, 16, 18-20]. One particular class of these 2AI molecules, referred to as the reverse-amides or RA-2AIs (named for the ‘flipped’ orientation of the amide linkage used to synthesize this family of derivatives from the original parent molecule, oroidin[21]) not only efficiently inhibit *A. baumannii* biofilm formation but also disperse previously formed biofilms[21-22]. They have also been found to be non-toxic at working concentrations using both *C. elegans* and human keratinocytes[23]. In our previous work[24], we showed how one RA-2AI representative (referred to here as RA-2AI-1, Fig.1) was able to penetrate the *A. baumannii* membrane barriers and interact with BfmR.

It is well known that a significant chemical barrier for bacterial cells is the cellular envelope, providing rigid structural support and hydrophobic shielding against the surrounding aqueous environment. For Gram-negative bacteria species, the envelope is defined by dual

membranes and the periplasmic space between them[25]. While the inner plasma membrane can act as a hydrophobic permeability barrier, this layer has a higher fluidity than its external counterpart, the outer membrane (OM), resulting in higher toxin permeability[25-26]. The outer membrane (OM) offers a higher level of defense against antimicrobial agents due to a lipopolysaccharide (LPS) coated surface significantly limiting the diffusion of many compounds[27-28]. The LPS surface layer is responsible for blocking majority of the external toxins through the crystalline nature of well-packed saturated fatty acid chains, a lack of fluidity due to the absence of phospholipids, a variety of branching glycans acting as charged netting along the cellular surface, and stabilizing divalent cation linkages holding LPS molecules together[28-31]. Since many antibiotics are either hydrophobic or large hydrophilic molecules, there has been significant focus on increasing their permeation rates across the outer membrane.

Here, we present evidence that the biomimetic structure of RA-2AI molecules enables them to interact with the outer membrane of *A. baumannii*, disrupting its fluidity and easing the barrier's restrictive nature.

Materials and Methods

Bacterial strains

The *Acinetobacter baumannii* (ATCC 19606) and *E. coli* strains (ML-35, ATCC 43827) used in this study were purchased from ATCC (Manassas, VA). Stock cultures were stored in glycerol stock media (65% v/v glycerol, 100 mM MgSO₄, 25 mM Tris, pH 8.0) when the bacteria reached mid-log phase (OD₆₀₀ ~ 0.5) and maintained at -80°C. Prior to use, each strain was subcultured twice on LB agar, once with 100 µg/mL ampicillin (Acros Organics) and then without any selective agent.

Compound preparation

The compounds under investigation, referred to as RA-2AI-1 (**1**) and RA-2AI-F (**2**) (Fig. 1) were synthesized as previously described[21, 24]. RA-2AI-1 represents the leading antibiofilm agent from the reverse-amide library of 2AI compounds (*A. baumannii* biofilm IC₅₀ = 26 µM). , and RA-2AI-F represents the FITC-labeled analog of RA-2AI-1 (*A. baumannii* biofilm IC₅₀ = 21.7 µM). RA-2AI-1 was stored as a 10 mM stock in DMSO at 4°C, while RA-2AI-F was kept as a 100 mM stock in DMSO at -20°C. Prior to use, all solutions were diluted using ddH₂O to the desired concentration.

Membrane permeability

The permeabilization ability of RA-2AI-1 was evaluated using a simple colorimetric assay modified for nitrocefin (EMD Millipore) in place of the original PADAC reagent[32-33]. *E. coli* ML-35 was grown in LB media without antibiotic selection at 37°C and harvested during mid-log phase (OD₆₀₀ = 0.5) at 2,000×g. The bacteria were washed once in PBS pH 7.5 (Amresco) and resuspended in PBS containing 50 µg/mL nitrocefin. The mixture was added to serially diluted RA-2AI-1 (2-fold dilutions starting at 500 µM and ending at 488 nM) in a 96-well microtiter plate to a total sample volume of 100 µL/well. Polymyxin B (Sigma-Aldrich) was used as a positive control. During incubation at 25°C, the plate was

scanned every 2 minutes at 486 nm, with a reference absorbance of 660 nm, for 1 hour using a TECAN® Sunrise plate reader (Tecan) and A_{486} endpoints for each well were recorded, subtracting the blank values. Each sample was run in duplicate on the same plate, and three biological replicates were performed across three plates. All of the A_{486} values were normalized with the untreated values (0 μ M RA-2AI-1) be set to 0.0. The averages and standard deviations for the normalized endpoints were determined, and error bars were calculated using $\alpha = 0.05$.

Confocal Laser Scanning Microscopy (CLSM)

Four *A. baumannii* 19606 cultures were treated with 100 μ M RA-2AI-1 at an OD_{600} of 0.5 and incubated at 37°C and 160 rpm for 3 hours. The samples consisted of 1 mL from each culture that was treated with the BacLight™ LIVE/DEAD (LD) bacteria viability stain (Life Technologies) according to the manufacturers instructions. Prior to observation, 5 μ L aliquots were mounted on a glass slide using #1.5 borosilicate coverslips (Fisher Scientific). For the RA-2AI-F permeation images, both *A. baumannii* 19606 and 1605 were dosed with 10 μ M RA-2AI-F and incubated in the dark at room temperature for 1 h. *A. baumannii* 19606 biofilms were grown under static conditions on Nunc® Lab-Tek® II 8-well chambered coverglass (Fisher) using a 1:100 dilution from an overnight culture and incubated at 37°C for 24 to 30 hours. Media was exchanged every 8 hours to maintain viability. Prior to visualization, the wells were aspirated and extensively washed with PBS, pH 7.5 to remove any planktonic cells from the biofilm. The biofilms were stained with 0.01% (w/v) crystal violet solution to confirm presence of biofilm, LIVE/DEAD stain to confirm biofilm viability, or 12.5 μ M RA-2AI-F. All RA-2AI-F stained samples were allowed to incubate at 37°C for 30 minutes and compared to a fluorescein control. All images were acquired using a Zeiss LSM 710 system attached to a Zeiss Axio Observer microscope (Carl Zeiss Microscopy, LLC). The cells were imaged using either a 40 $\times$ 1.1 numerical aperture (N.A.) water immersion or a 63 $\times$ 1.4 N.A. oil immersion objective; while, biofilm samples were imaged using either a 40 $\times$ 1.1 N.A. or 63 $\times$ 1.2 N.A. water immersion objective. The samples were excited with 488 nm argon laser light. Fluorescence emission was collected between 493 – 576 nm for FITC, 493 – 545 nm for SYTO9 and 605 – 690 nm for propidium iodide. Images were analyzed using both Zeiss Zen 2009 (Carl Zeiss Microscopy, LLC) and ImageJ (NIH). Cell counts for the LD time-course study were made using the Cell Counter plugin for ImageJ (NIH). Cell counts were averaged and error bars were calculated using their standard deviations. Adobe Photoshop (v. 13.0.4) was used to perform level adjustment and enhance the contrast on select images.

Scanning Electron Microscopy (SEM)

After removing the CLSM sample from each RA-treated culture, the remaining culture was harvested at 2,000 $\times$ g for 5 min and washed with PBS (pH 7.5). The pellets were then resuspended in PBS with 3.0% (v/v) glutaraldehyde (Ladd Research) and fixed for 24 h at 4°C. Following fixation, the samples were syringe-filtered onto a Nuclepore® membrane (Whatman) and washed three times with PBS to remove excess fixative. The filters were then dehydrated using graded ethanol steps and subjected to critical point-drying (CPD). Immediately after CPD, the filter was mounted on an aluminum stub (Ladd Research) and

sputter-coated with a layer of palladium-gold for analysis with SEM. All images were taken using a JEOL JSM-5900LV scanning electron microscope at 15 kV.

Growth rate analysis

A. baumannii 19606 was cultivated overnight in LB media with 100 µg/mL ampicillin at 30°C. The overnight culture was used to aseptically inoculate fresh LB media without a selective agent and incubated at 37°C and 160 rpm. For growth rate analysis, cultures were treated with varying concentrations of RA-2AI-1 (0, 25, 62.5, and 100 µM) when the cultures reached an OD₆₀₀ of 0.5. After every hour of incubation time, a 500 µL sample was removed, and the OD₆₀₀ was measured. In addition to OD readings, the CFUs for the untreated and 100 µM treated cultures were counted after the plates were incubated overnight at 37°C. The analysis was performed using three biological replicates. The linear growth rates were defined as the change in OD₆₀₀ over time (i.e. the slope of the linear regression) during log phase growth. The linear regression was performed using Excel™ (Microsoft). The generation times for the untreated and 100 µM treated cultures were determined using a standard geometric progression over the length of the exponential growth phase as observed in the OD₆₀₀ growth curves and the CFU counts.

Results and Discussion

Permeabilization effects of RA-2AI

The cellular envelope enclosing Gram-negative bacterial cells serves as a significant chemical barrier against antimicrobial agents due to the combination of the limited permeability of the outer LPS leaflet and hydrophobic nature of the remaining phospholipid layers of both the inner and outer membrane structures [27-28]. The goal of this study was to provide the first insight into how reverse amide 2AI molecules, responsible for *A. baumannii* biofilm eradication [21, 34], affect bacterial membranes and gain access to cytosolic targets as seen in our previous work [24]. Due to its amphipathic design, it was no surprise that RA-2AI-1 readily interacts with the bacterial membranes, but it was not clear how the compound overcame its membrane affinity. The first step was to determine whether the compound had any direct effect on the membrane permeability. To this end, an assay was selected with the capability to demonstrate changes in both inner and outer membrane permeability. This assay was based on the levels of nitrocefin, a membrane impermeable molecule, that were hydrolyzed by the β-lactamases sequestered in the cytosol. *E. coli* ML-35, a permease-free strain, was used, because *A. baumannii* has active permease expression that allows β-lactamases to translocate from the cytosol to the periplasmic space, disrupting the assay mechanism [32-33]. The hydrolysis reaction only occurred when the permeability of both the inner and outer membranes increased to allow nitrocefin access to the β-lactamases and was typically triggered by changes in the membrane structure or fluidity[33]. Based on this assay, RA-2AI-1 was found to be a moderate permeabilizing agent, and the permeabilization activity increased in direct relation to the increase in RA-2AI-1 concentration and approached a saturation point at 62.5 µM (Fig. 2), indicating that the compound had reached maximum activity. These results indicated that the accumulation of RA-2AI-1 was changing the permeability of the protective membrane layers. As a biofilm inhibitor, RA-2AI-1 had an IC₅₀ of 26 µM (± 3 µM) against *A.*

baumannii and reduced biofilm formation by >95% at 100 μ M[21]. When combined, the nitrocefin and anti-biofilm data indicate that the permeabilization of the bacterial membranes may play a role in the activity of the compound against *A. baumannii*.

Structural effects of RA-2AI exposure

Because the nitrocefin assay revealed that RA-2AI-1 increased the permeability of the *E. coli* ML-35 membranes, a combination of confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) was used to elucidate the cellular effects of RA-2AI-1 and to correlate any changes in *A. baumannii* permeability with structural changes in response to treatment. First, the RA-treated *A. baumannii* 19606 cultures were assessed using Live/Dead® (LD) staining and experienced a rapid increase in their propidium iodide uptake post treatment; in fact > 98% of the samples were stained within an hour of incubation (Fig. 3), indicative of membrane disruption. LD stain is composed of two intercalating fluorescent dyes, SYTO9 and propidium iodide. Because only SYTO9 was membrane permeable, this method quickly separated the cells with damaged membranes from the surrounding healthy cells and provided a simple way for monitoring the permeabilization of the bacteria in solution[35]. Due to the small size of the bacterial cells (ranging from 0.5 μ m to 1.2 μ m, depending on orientation), the available CLSM technology was unable to offer the resolution necessary to observe any detailed cellular alterations caused by RA-2AI-1 treatment. Due to this lack of single cell resolution, the time course was repeated and combined with the resolving power of SEM to reveal the drastic emergence of membrane blebbing in response to treatment (Fig. 4). As quickly as 15 minutes post-treatment, the cells presented a variety of bleb morphologies that seemed to localize near the septa of couplets and the ends of long chains (Fig. 4E). As the incubation progressed, these blebs increased in number and changed morphology to larger, rougher structures that localized towards the center of the cells (Fig. 4F). Membrane blebbing is a natural phenomenon in Gram-negative bacterial species, and is typically generated in response to environmental stresses[36-37]. Because surface blebs were never detected under normal growth conditions in *A. baumannii* 19606, the appearance of the surface blebs can be ascribed to the effects of RA-2AI-1 on the bacteria, which coincides with the increased permeability of the cellular membranes. It remains unclear whether RA-2AI-1 directly causes the blebbing morphology through its interaction with the membranes or if it is the result of an unknown stress response.

Membrane Localization

In order to demonstrate that the molecule was interacting with the bacterial membranes prior to accessing the cytosol in live cells, CLSM was revisited using a low dose of a fluorescein isothiocyanate (FITC) analog of the compound (RA-2AI-F (2), Fig. 1). Previously, the FITC-analog had been shown to retain its biological function and was found to remain in the cellular envelope after a series of stringent washes and formaldehyde fixation, resulting in a well-defined “halo” around the cells and the observation of isolated “hotspots” of compound[24]. The cultures were treated with 10 μ M RA-2AI-F and imaged using CLSM. To confirm that the RA-2AI-1 component was responsible for any interactions, the samples were compared to a FITC-only control. While a portion of the observed cells had the uniform distribution typical of compound permeation[24], there was large number of

observed cells that displayed a “halo” of fluorophore that was the result of sequestered molecules in the membranes rather than inside the cell (Fig. 5A); while, only background fluorescence was observed in the FITC-only samples (Fig. 5D). These images also showed the appearance of the RA-2AI hotspots around septa and specific regions of the cells as discussed in previous work[24]. Due to the resolution limitations caused by the small size of the bacteria, the observations were subject to minor distortion, but the distortions did not affect the appearance of the fluorophore halos. To confirm that this localization was not an artifact of the bacterial strain, MDR *A. baumannii* strain ATCC 1605 and the 19606 biofilm state were both visualized with RA-2AI-F and found to have the same “halo” appearance (Fig. 5B & 5C). The appearance and location of the bleb formation on the cells in the SEM images also agreed with the “hotspots” seen during the CLSM studies. Based on this agreement, the potential for a specific cellular target is currently being pursued.

Growth rate response to RA-2AI

In previous studies[21], RA-2AI-1 treatment was observed to result in a bacteriostatic period, where no changes in the OD₆₀₀ values were observed for several hours in the *Acinetobacter baumannii* growth curve after treatment, but this effect was not explored any further at that time as the population growth was able to recover and resume normal growth. After discovering that RA-2AI-1 was modifying the permeability of the bacterial membranes, it was important to determine if these growth effects could be correlated to the observed permeabilization using a series of *A. baumannii* growth studies. After dosing the culture with RA-2AI-1, a temporary interruption of exponential growth (measured using OD₆₀₀) was observed, corroborating the growth effects in the initial biofilm inhibition studies[21]. The length of the disruption was directly related to the RA-2AI-1 concentration, ranging from <1 hour at 25 μ M up to approximately 3 hours at 100 μ M (Fig. 6A). Using standard linear regression to model the log-phase growth of the samples, the average growth rates were 0.58 AU/h for the control culture and 0.51, 0.18, and 0.21 AU/h for the 25, 62.5, and 100 μ M treatments, respectively (Fig. 6B). Further, RA-2AI-1 caused the growth rate to decrease by 65% ($\pm$ 3%) at concentrations above 62.5 μ M, coinciding with the plateaued permeation activity seen in the nitrocefin assays (Fig. 2). To validate bacterial viability, colony forming unit counts (CFU/mL) were measured for the 0 and 100 μ M RA-2AI-1 treated cultures (Fig. 7A). After treatment, a 40-fold decrease was observed in the CFU/mL of culture, which consistently reached their lowest point around 3.5×10^6 ($\pm 1.05 \times 10^6$) CFU/mL after 30 minutes of exposure of RA-2AI-1 (Fig. 7A). The cells then entered a recovery period with a generation time of approximately 0.768 hour. When compared to the untreated generation time of 0.655 hour (Fig. 7B), the treatment caused a 17.3% increase in the generation time of the recovering population, supporting the decrease in growth rate observed in the OD₆₀₀ growth curves. To confirm that the bacteria were not developing a RA-resistant phenotype, the recovered *A. baumannii* populations were used to seed new cultures that were re-treated under the initial conditions. These cultures exhibited the same growth effects after dosage as seen in the initial treatments (data not shown).

Through this study, the bacteriostatic interval was found to be the result of the widespread disruption of normal cellular growth and viability shortly after treatment as indicated by the drop in CFU/mL. Despite the initial shock caused by RA-2AI-1, the population was able to

gradually recover, but it experienced a significantly reduced growth rate and increased membrane permeability in the presence of the compound. In addition, the reproducibility of the results using recovered cells from RA-2AI-1 treated cultures lends credence to the belief that the drop in viability was the result of localized saturation immediately after treatment. Our current hypothesis for the entry mechanism is based on a combination of the lipomimetic nature of the RA-2AI molecules and the general mechanism of action for antimicrobial peptides[14, 35, 38]. The amphipathic nature of the molecule should draw it to the LPS layer to escape the aqueous environment. At the LPS leaflet, the positive nature of the 2AI head group could allow it to interact with polyanionic moieties, and the pendant chain would be able to insert itself into the outer leaflet, mimicking a fatty acid. Once in contact with the Lipid A portion of the LPS molecule, RA-2AI would be capable of disrupting the crystalline packing either by displacing divalent cations bridging the LPS molecules or by interacting with the Lipid A fatty acids. The length of the RA-2AI chain could play a role in the molecules' interactions with the fatty acids in the outer membrane leaflet, which is supported by the decreased activity of RA-2AI species with chains lengths outside the range of 11 and 14 carbons[34]. As the concentration of RA-2AI increases on the surface, the molecules would readily increase leaflet fluidity and allow the permeation of previously impermeable molecules.

Conclusion

The discovery that the RA-2AI class of molecules can act as permeabilizing agents in addition to possessing antibiofilm properties is an important step forward in the development of small molecules that can be used to control biofilm formation and also as adjuvants to overcome antibiotic resistance[39]. In addition to targeting regulatory proteins within the bacteria, future variations of the RA class that possess the ability to disrupt the membranes of Gram-negative bacteria may significantly increase the diffusion rate of therapeutic agents into the cell, thus increasing their efficacy. By allowing impermeable molecules as large as propidium iodide (MW = 668.5 g/mol) and nitrocefin (MW = 516.5 g/mol) to penetrate *Acinetobacter baumannii*'s cellular envelope, RA-2AI-1 permeabilization has the potential to readily increase the permeation of many conventional antibiotics that have been “shelved” due to the rapid rise of bacterial resistance, since many of these molecules are smaller molecules (500 g/mol or less)[40-42]. With this in mind, we are currently exploring the use of RA-2AI molecules as a new “drug delivery” mechanism in a variety of systems.

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Figure 1.
The structure of the 2-aminoimidazole reverse-amide, RA-2AI-1 (1) and its FITC-analog, RA-2AI-F (2).

Figure 2. Results of nitrocefin-based membrane permeabilization assay using RA-2AI-1
Average A_{486} values for the assay endpoints at each concentration of Polymyxin B (PMB), RA-2AI-1 and DMSO. Error bars were calculated using $\alpha = 0.05$.

Figure 3. Results of RA-2AI treatment of *A. baumannii* 19606 using Live/Dead staining
Average cell counts of viable (green or SYTO9-stained) and permeabilized (red or propidium iodide-stained) bacteria visualized using CLSM post-treatment with 100 μ M RA-2AI-1. Cell counts were made using ImageJ Cell Counter plugin, and error bars were calculated using standard deviations.

Figure 4. Effects of RA-2AI treatment of *A. baumannii* 19606 membrane
 (A) Untreated *A. baumannii* 19606 prior to treatment with 100 μ M RA; (B) 15 minutes post-treatment showing a mixture of cells with increased PI uptake; (C) 1 hour post-treatment where the culture has reached maximum PI uptake. SEM images were all taken at 7500x magnification; (D) Untreated *A. baumannii* 19606 prior to treatment with 100 μ M RA-2AI showing normal cell morphology; (E) 15 minutes post-treatment showing the development of small blebs around the septa between cells and along the terminal ends of long chains; (F) 1 hour post-treatment where the bleb morphology has transitioned into larger rough blebs in more central cellular locations. Red arrows were used to indicate a few examples of the blebbing morphology seen during the 15 minute and 1 hour timepoints.

Figure 5. Membrane localization of RA-2AI-1

(**A**) *A. baumannii* strain 19606 treated with low dose of RA-2AI-F; (**B**) *A. baumannii* MDR strain 1605 in planktonic state showing a similar outlining as seen with 19606; (**C**) *A. baumannii* strain 19606 in biofilm state (orthogonal slices of 4 μm thick slab; center: x,y, bottom: x,z, right: y,z); (**D**) *A. baumannii* strain 19606 FITC-only control.

Figure 6. OD₆₀₀ Growth Curve Analysis for RA-2AI-treated *A. baumannii*

(A) Comparison the effect of varying RA-2AI dosages on the growth of *A. baumannii* 19606 (error bars calculated using standard deviation); (B) Comparison of the log phase growth rates for the dosed cultures (◆ = untreated control, $R^2 = 0.9929$; ■ = treated with 25 μM, $R^2 = 0.9879$; ▲ = treated with 62.5 μM, $R^2 = 0.9893$; ● = treated with 100 μM, $R^2 = 0.9886$).

Figure 7. CFU Analysis for RA-2AI-treated *A. baumannii*

(A) CFU/mL comparison of untreated *A. baumannii* 19606 and *A. baumannii* 19606 treated with 100 μ M RA (error bars calculated using standard deviation); (B) Comparison of exponential growth rates for the untreated and treated bacterial culture recovery using the CFU count results ($\blacklozenge$ = untreated culture, $R^2 = 0.9612$; $\bullet$ = treated culture, $R^2 = 0.9893$).