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# Controlling the virulence of Pseudomonas aeruginosa through inhibiting the synthesis of pyoverdine

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Chapter 5

# *Galleria mellonella* larva as an infection model of PvdP inhibitor

Joko P. Wibowo, Rita Setroikromo, Frank J. Dekker, Wim J. Quax

*Manuscript in preparation.*

**Abstract**

*Galleria mellonella* larvae have been used as a representative infection model for many years. The advantages of using this animal are inexpensive, easy to handle, no special equipment needed, and no ethical constraint. Moreover, the immune response of this infection model is easy to be observed during the infection. In this report, we infected the larvae with *Pseudomonas aeruginosa*; then, we used the infected Galleria larvae to evaluate the efficacy of a newly synthesized PvdP inhibitor. We discovered that the PvdP inhibitor at a dose of 2 μg/g body weight can improve the survival rate of the infected group up to 50 % compared to the untreated group.

**Keywords:** *G. mellonella*, *P. aeruginosa,* PvdP inhibitor

## Introduction

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that causes infections in many organs of immunocompromised patients suffered from several diseases, such as cystic fibrosis, burn wounds, and HIV. The bacterium is widely known to be resistant to several antibiotics. The most recent report revealed that this bacterium has become resistant to carbapenem ($4^{th}$ generation beta-lactam)[1]. Moreover, the ability of the bacteria to form biofilm also contributes to the failure of the treatments[2]. Therefore, finding a new treatment against the infection is urgently needed.

However, the efforts to find the new treatment before it can be used in the clinical setting is a long journey. One crucial step in the early preclinical study of a novel compound is finding suitable animal models that are predictive and reliable. Up to now, vertebrates (e.g.: mice, rats, rabbits, etc.) are still the most reliable models since their physiology is close to the human one. The use of vertebrates as the only animal model, however, more and more deals with logistical, budgetary, and most importantly the ethical hurdles. Therefore, an alternative predictive animal model that accommodates those hurdles is needed.

*G. mellonella* larvae have been used as a predictive animal infection model to mammals for more than 15 years[3] and more specifically, it has been reported in studies on antimicrobial activity and antivirulence of candidate drugs[4]. On top of that, there are many advantages to using *G. mellonella* larvae as the animal model. More data can be obtained than using vertebrates since the price is low and many controls can be included in every experiment. The larvae can normally live in a 37 $^{o}$C incubator (no need for special equipment). Galleria larvae are large (length = 250-300 mm, weigh = 200-400 mg); therefore, it is easy to inject the tested compound intraperitoneally. More importantly, the pharmacokinetic data obtained with Galleria larvae (clearance time, elimination half-time, the maximum concentration of the drug) can be correlated directly to human data[5].

In a recent study, Galleria larvae are also used to evaluate the efficacy of some commercially available and current clinically used antibiotics (ampicillin, ciprofloxacin, rifampicin, and tetracycline) and the toxicity of widely used compounds such as amsacrine, ATP, chloramphenicol, chloroquine, ciprofloxacin, DMSO, doxorubicin, etoposide, glucose, streptomycin, tetracycline[6]. Due to these many advantages and successful results, the Galleria model has gradually been established as a model host to study infection[7]. It has also been used to evaluate a novel treatment of persisting pathogens[8] and to investigate the eradication of emerging pathogens[5].

Following our previous report, where we successfully designed and synthesized a phenylthiourea derivative (compound **3c**) (**Figure 1A**) as PvdP inhibitor. Also in a cell-based assay the inhibitor showed inhibition activity of pyoverdine production at a low micromolar dose and the binding mode to PvdP was simulated using a docking experiment (**Figure 1B**)[9]. In this study, we demonstrate the usage of Galleria larvae as an infection model to determine the efficacy of the inhibitor.

## Materials and Methods

### Bacterial strains, PvdP inhibitor, *G. mellonella* larvae

In this study, we used *P. aeruginosa* PAO1 and *P. aeruginosa* PAO1_PvdP knockout[10], compound **3c**[9], *G. mellonella* larvae ~250 mg in weight (purchased from 'Fritz Kuiper' Fishing Shop, Groningen). The bacterial strains were freshly inoculated prior to injection into the larvae, and the inhibitor has been analyzed to be a minimum of 95 % pure after the synthesis. The healthy (actively move, no indication of infection) larvae were selected to perform the experiments.

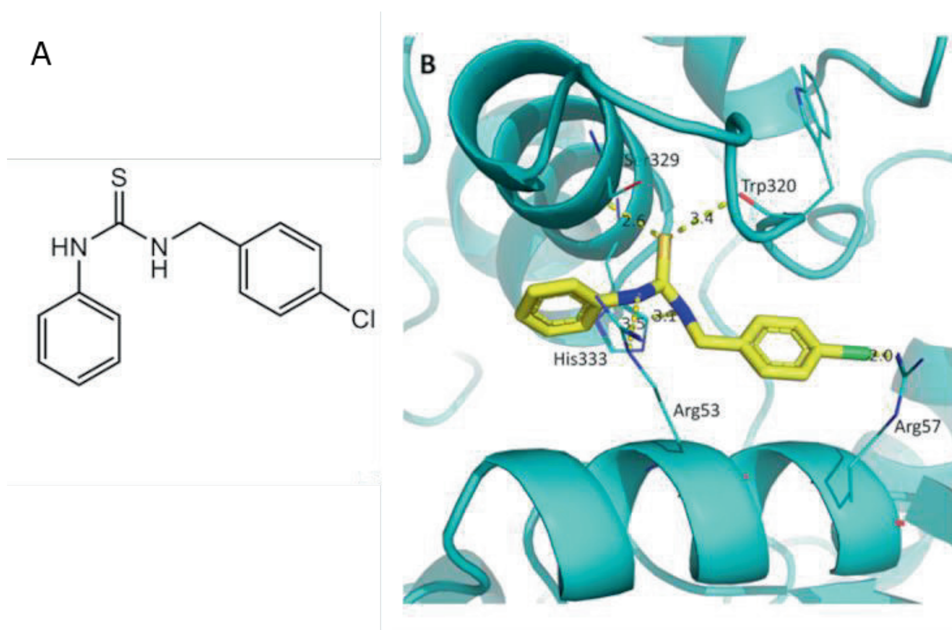


**Figure 1**. Compound 3c, a phenylthiourea derivative as an inhibitor of PvdP. (A) The 2D representation of compound **3c,** (B) the 3D representation of the docking result of compound **3c** on PvdP.

### Epithelial cell viability assay

The cell viability assay of the inhibitor was tested on the epithelial lung cell lines (A549 and H640). A serial dilution of the inhibitor (0- 50 μM) was prepared in 96-well plates (VWR Technology, The US). The MTS assay was performed in the presence of $10^5$ freshly prepared lung cells following the standard protocol in RPMI media. DMSO 1 % was used as a control. The plates were then incubated at 37 $^o$C for 48 hours. The experiments were conducted three times independently and the average of cell viability of each concentration of the inhibitor were calcuculated and presented from the obtained data.

**Preparation of the bacterial inoculum**

The preparation of the bacterial inoculum and the injection of the Galleria larvae as described in the following procedures are based on the protocol by Koch *et al*. with minor modifications[11]. An overnight culture of *P. aeruginosa* PAO1 in LB media was centrifuged (4000 rpm, 10 minutes, 4 °C) and the supernatant was replaced with fresh LB media then reinoculated at 37 °C in a 200-rpm shaking incubator. After the culture reached $OD_{600}$ = 0.1 (~$10^8$ CFU/ml), the culture was spinned down again and the medium was replaced with sterile PBS, and the concentration of the cells was adjusted to ~$10^3$ CFU/ml. Then the cell suspension was mixed with one of other solutions (PBS, DMSO 1% or 2 µg/g body weight of compound **3c**) corresponding to the treatments for the injection. The same procedure was conducted to prepare the inoculum of the *P. aeruginosa* PAO1_PvdP knockout except for the mixing step with other solutions.

**Galleria larvae injection procedure**

Twelve healthy larvae per group were selected randomly. Each solution, mixture, or suspension (as specified above) was injected (1 ml insulin syringe, needle 25G, volume 20 µL) into every larva of the groups via the last left proleg. PBS was also injected into a group of larvae as the second control (traumatic control) beside the untreated group. Subsequently, the larvae were stored in separate petri dishes and incubated at 37 °C for four days.

**Toxicity test of DMSO**

To know the concentration of DMSO can be tolerated by the Galleria larvae, four different concentrations of DMSO (1, 3, 5, 10 %) in PBS were injected into four groups of 12 larvae by the addition of two control groups (PBS and untreated). Each larva was injected carefully following the procedure mentioned above and then stored in the incubator at 37 °C for four days.

**Determination of moderate infection dose**

To know which concentration of *P. aeruginosa* PAO1 causes moderate infection to the Galleria larvae, we injected six groups of 12 larvae with successive ten times dilution of the bacterial cell suspension (10 – $10^6$ CFU/mL). The severity of infection is reflected by the response of the immune system that is visualized by the darkening of the cuticle [8]. After the injection, the larvae were incubated at 37 °C for four days to mimic the infection condition in humans. The moderate infective dose is the dose that caused 60-80 % lethality for 48 hours.

**PvdP inhibitor efficacy testing on *G. mellonella***

To test the efficacy of the PvdP inhibitor, we divided the larvae into six groups of 12 larvae (untreated, PBS, DMSO 1%, *P. aeruginosa* PAO1, *P. aeruginosa* PAO1_PvdP knockout, and *P. aeruginosa* PAO1 + 2 µg/g body weight of compound **3c**). Each larva of the group (except the untreated group) was injected following the procedure mentioned

above. The larvae were incubated at 37 $^{o}$C for four days, and the death rate was recorded daily.

## Results and Discussion

### Epithelial cell viability assay

Before we can administer a chemical compound, in our case, PvdP inhibitor, to the human body, we have to guarantee that the compound is not harmful to the human cell. Therefore, we tested our inhibitor against two epithelial lung cell lines to examine its toxicity. We tested seven different concentrations of the inhibitor on two cell lines. The result revealed that compound **3c** had no negative effect on the both cell lines up to 50 μM (**Figure 2**). It means up to 50 μM, the compound is not toxic to the lung cells. Unfortunately, we could not investigate the effect of the compound at a higher concentration since, at 100 μM, the inhibitor formed crystals that became visible under microscope observation. This crystal formation gave unreliable results at this highest concentration; therefore we omitted those data from Figure 2. The results show that the compound is not harmful to the human cell.

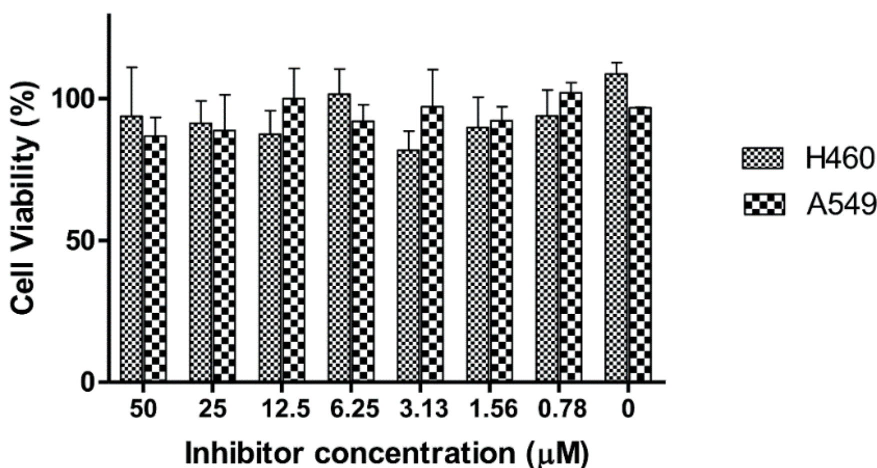


**Figure 2.** The viability of lung epithelial cells (H460 and A549) at seven different concentrations of PvdP inhibitor and 1 % DMSO was used as control.

### Effect of DMSO on Galleria

As our inhibitor has poor solubility in water, we had to use an organic solvent, DMSO in our case, which might be toxic to our animal model. As a stepwise flow of work, we firstly investigated the effect of different concentrations of DMSO on the Galleria larvae and determined the highest concentration of DMSO that can be tolerated by the animals in the *in vivo* assay. The results further confirmed that higher concentrations of DMSO showed more effects on the larvae, which is in line with previous reports[12]. In our hands, the highest concentration of the DMSO that can be tolerated by the larvae is 3 % (**Figure S1**). To be on the safe side, we eventually injected the inhibitor in 1%

DMSO. Fortunately, at this concentration, we were still able to dissolve the inhibitor up to 100 μM—the maximal concentration at which we want to inject the inhibitor into the larvae.

**Optimal infection dose of *P. aeruginosa* PAO1 on Galleria**

A critical step that needs to be done before determining the efficacy of the inhibitor is establishing a moderate infection condition on the G. *mellonella* larvae. A moderate infection condition causes an immune response showed by the darkening of the larvae and lethality of 60-80 % within 48 hours. Our results clearly showed a dose-dependent immune response and survival of the larvae upon injecting different concentrations of *P. aeruginosa*. The highest concentration ($10^6$ CFU/mL) resulted in 100 % lethality in 24 h; on the other hand, the lowest concentration (10 CFU/mL) showed light infection and the larvae survived until four days. Where the moderate infection condition can be reached at $10^4$ CFU/mL of *P. aeruginosa* (**Figure S2**).

Since we are going to inject a maximum of 1 % DMSO as a solvent for the inhibitor, next, we investigated the synergistic effect of DMSO and *P. aeruginosa* on the larvae. The same experiments as mentioned above by adding 1 % DMSO for each treatment were conducted. As we predicted, the addition of 1 % DMSO increases the lethality rate of each group. Here, we could reach the moderate infection at $10^3$ CFU/mL, which is ten times lower compared to the treatments in the absence of DMSO (**Figure 3**). Meaning the addition of 1 % DMSO increases lethality up to 10 times. Therefore, keeping the concentration of DMSO as low as possible is essential to get rid of the biased effect of the solvent on the final result. We discovered that 1% is the optimal balance between low toxicity and achieving high enough concentrations of the inhibitor.



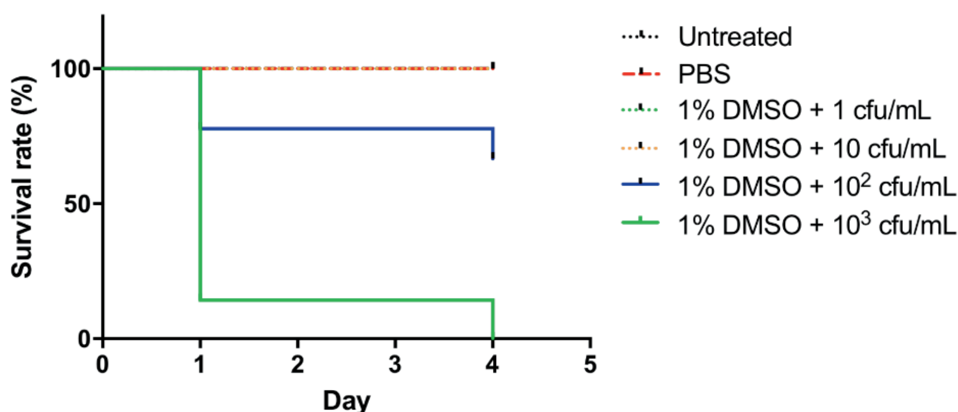


**Figure 3.** The survival rate of the Galleria larvae after the injection of different concentrations of *P. aeruginosa* PAO1 together with 1 % DMSO. A combination of 1 % DMSO and $10^3$ CFU/mL injection showed a moderate infection.

**Protection of Galleria larvae by PvdP inhibitor**

Our previous report has shown the efficacy of the PvdP inhibitor to interfere with the production of pyoverdine in the bacterial cell-based assay. The inhibitor could reduce the production of pyoverdine significantly at a micromolar dose ($IC_{50}$ = 44.9 µM)[9]. To further investigate the efficacy of the inhibitor in an infection scenario, we performed a study on *G. mellonella* larvae as the animal infection model.

In this report, the result shows that the inhibitor is active against P. *aeruginosa* infection in the Galleria model (**Figure 4**). At the dose of 2 µg/g body weight of Galleria larva, the inhibitor could increase the survival rate of the treatment group by 50 % until the 4$^{th}$ day compared to the untreated group. The result is in concert with our previous study in the bacterial cell-based assay, although in this study, we need a higher concentration of the inhibitor to reach a 50 % survival rate of the larvae. This can be due to reduced biological availability of the inhibitor in the larvae as compared to the bacterial cell-based assay. In general, we can conclude that the inhibitor is effective against *P. aeruginosa* infection in the Galleria model. The higher concentration of the inhibitor needed *in vivo* is apparently due to different conditions compared to the bacterial cell-based assay.

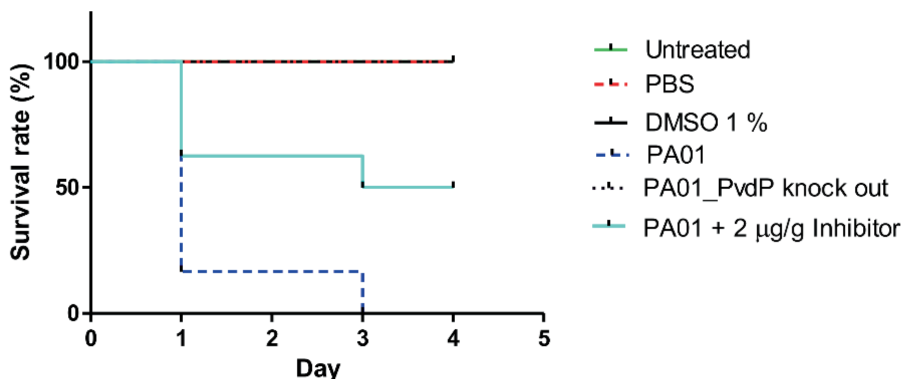


**Figure 4.** Percent survival of *G. mellonella* larvae after the injection of different solutions and/or suspension. Up to 4 days, no dead animals are found in the untreated, PBS, DMSO 1%, and *P. aeruginosa* PAO1_PvdP knockout groups. The addition of a 2 µg/g inhibitor improves the survival rate of the larvae up to 50 % compared to the *P. aeruginosa* PAO1 group.

One of the limitations of using Galleria is the need for experimental skills for injecting the solutions. We have found the benefit in using an insulin syringe (needle 25G, volume 20 µL) to inject the solution in the last left proleg. Once this is being performed with care, consistent and reliable results can be obtained.

The usage of Galleria larvae as the infection model has become more common in the past few years. Based on a recent review, at least 292 studies using Galleria as a

model host have been published during the period 2016 to 2018. This is almost half the number of the published reports (691) in the last decade[13]. The significantly increasing number of studies using Galleria as an infection model due to its advantages compared to the mammal model. Galleria model is cheap, easy to handle, no need for special equipment, the readout is easy to be observed[14]. Furthermore, it has evolved to be a standardized infection model host. Consequently, there will be more reports using Galleria larvae as the infection model in the coming years.

**Conclusions**

*G. mellonella* has been used for many years as an infection model and became more common recently. Overall experiments show *G. mellonella* can be used as an infection model to test the efficacy of PvdP inhibitor. Up to 50 μM the inhibitor is not toxic to the epithelial lung cells. The inhibitor can improve the survival rate of the infected larvae by up to 50 %.

**Acknowledgment**

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**References**

[1] E. Tacconelli *et al.*, "Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis," *Lancet Infect. Dis.*, vol. 18, pp. 318–327, 2018, doi: 10.1016/S1473-3099(17)30753-3.

[2] R. M. Donlan and J. William Costerton, "Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms," *Clin. Microbiol. Rev.*, vol. 15, no. 2, pp. 167–193, 2002, doi: 10.1128/CMR.15.2.167-193.2002.

[3] C. J.-Y. Tsai, J. Mei San Loh, and T. Proft, "Galleria mellonella infection models for the study of bacterial diseases and for antimicrobial drug testing," *Virulence*, vol. 7, no. 3, pp. 214–229, 2016, doi: 10.1080/21505594.2015.1135289.

[4] S. M. Cook and J. D. McArthur, "Developing Galleria mellonella as a model host for human pathogens," *Virulence*, vol. 4, no. 5, pp. 350–353, 2013, doi: 10.4161/viru.25240.

[5] R. J. Thomas *et al.*, "Galleria mellonella as a model system to test the pharmacokinetics and efficacy of antibiotics against Burkholderia pseudomallei," *Int. J. Antimicrob. Agents*, vol. 41, no. 4, pp. 330–336, Apr. 2013, doi: 10.1016/j.ijantimicag.2012.12.009.

[6] K. Ignasiak and A. Maxwell, "Galleria mellonella (greater wax moth) larvae as a model for antibiotic susceptibility testing and acute toxicity trials," *BMC Res. Notes*, vol. 10, p. 428, 2017, doi: 10.1186/s13104-017-2757-8.

[7] O. L. Champion, S. Wagley, and R. W. Titball, "Galleria mellonella as a model host for microbiological and toxin research," *Virulence*, vol. 7, no. 7, pp. 840–845, Oct. 2016, doi: 10.1080/21505594.2016.1203486.

[8] C. R. Harding, G. N. Schroeder, J. W. Collins, and G. Frankel, "Use of Galleria mellonella as a Model Organism to Study Legionella pneumophila Infection," *J. Vis. Exp*, vol. 81, p. 50964, 2013, doi: 10.3791/50964.

[9] J. P. Wibowo, Z. Xiao, J. M. Voet, F. J. Dekker, and W. J. Quax, "Development of phenylthiourea derivatives as allosteric inhibitors of pyoverdine maturation enzyme

PvdP tyrosinase," *Bioorg. Med. Chem. Lett.*, vol. 30, no. 17, p. 127409, Sep. 2020, doi: 10.1016/j.bmcl.2020.127409.

[10] I. L. Lamont and L. W. Martin, "Identification and characterization of novel pyoverdine synthesis genes in Pseudomonas aeruginosa," *Microbiology*, vol. 149, pp. 833–842, 2003, doi: 10.1099/mic.0.26085-0.

[11] G. Koch, P. N. Jimenez, R. H. Cool, and W. J. Quax, "Assessing Pseudomonas Virulence with Nonmammalian Host: Galleria mellonella," in *Pseudomonas Methods and Protocols, Methods in Molecular Biology*, vol. 1149, A. Folloux and J.-L. Ramos, Eds. New York: Humana Press, 2014, pp. 681–688.

[12] B. Suay-García, P. A. Alemán-López, J. Ignacio Bueso-Bordils, A. Falcó, G. Antón-Fos, and M. Teresa Pérez-Gracia, "New solvent options for in vivo assays in the Galleria mellonella larvae model," *Virulence*, vol. 10, no. 1, pp. 776–782, 2019, doi: 10.1080/21505594.2019.1659663.

[13] M. A. Cutuli *et al.*, "Galleria mellonella as a consolidated in vivo model hosts: New developments in antibacterial strategies and novel drug testing," *Virulence*, vol. 10, no. 1, pp. 527–541, 2019, doi: 10.1080/21505594.2019.1621649.

[14] N. Ramarao, C. Nielsen-Leroux, and D. Lereclus, "The Insect Galleria mellonella as a Powerful Infection Model to Investigate Bacterial Pathogenesis," *J. Vis. Exp.*, no. 70, pp. 1–7, 2012, doi: 10.3791/4392.

## Supporting Information

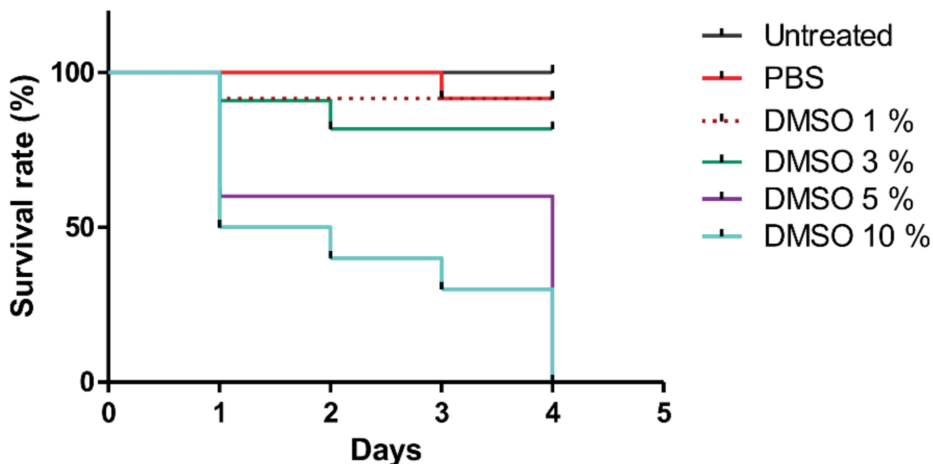


**Figure S1.** The effect of different concentrations of DMSO on the death rate of *G. mellonella*

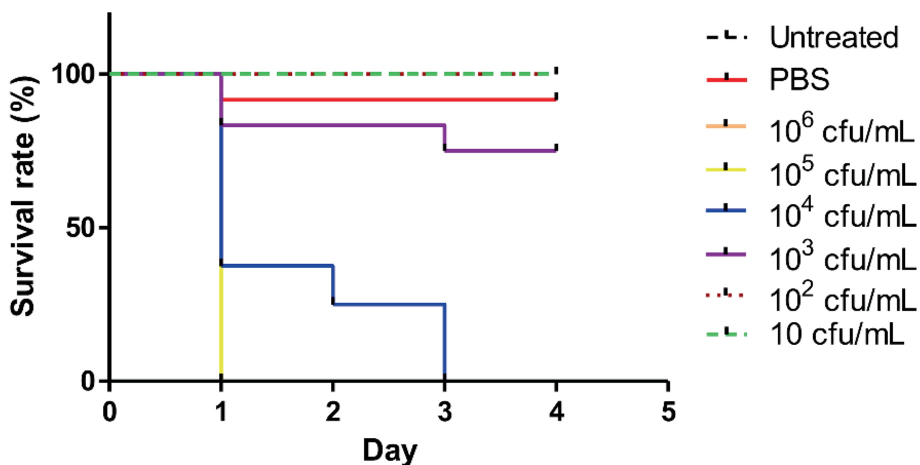


**Figure S2.** The determination of moderate infective dose of *P. aeruginosa* on *G. mellonella*

# Part B

# Inhibition of PvdQ Acylase