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Short Communication

Dictyostelium discoideum as a surrogate host–microbe model for antivirulence screening in *Pseudomonas aeruginosa* PAO1

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ABSTRACT

The interest of the pharmaceutical industry in developing new antibiotics is decreasing, as established screening systems which identify compounds that kill or inhibit the growth of bacteria can no longer be used. Consequently, antimicrobial screening using classical minimum inhibitory concentration (MIC) measurements is becoming obsolete. The discovery of antimicrobial agents that specifically target a bacterial pathogen without affecting the host and its beneficial bacteria is a promising strategy. However, few host–microbe models are available for in vivo screening of novel antivirulence molecules. Here we designed high-throughput developmental assays in the social amoeba *Dictyostelium discoideum* to measure *Pseudomonas aeruginosa* virulence and to screen for novel antivirulence molecules without side effects to the host and its beneficial bacteria *Klebsiella aerogenes*. Thirty compounds were evaluated that had been previously selected by virtual screening for inhibitors of *P. aeruginosa* PAO1 polyphosphate kinase 1 (*Pa*PPK1) and diverse compounds with combined PPK1 inhibitory and antivirulence activities were identified. This approach demonstrates that *D. discoideum* is a suitable surrogate host for preliminary high-throughput screening of antivirulence agents and that PPK1 is a suitable target for developing novel antivirulence compounds that can be further validated in mammalian models.

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1. Introduction

The increased incidence and spread of multidrug-resistant pathogens that have evolved resistance mechanisms against specific antibiotics are a cause for alarm [1]. Today, most existing antimicrobials on the market or in clinical development focus on only a small number of essential bacterial processes such as protein synthesis, central metabolic pathways and cell membrane function [2]. With an increasing rate of resistance to different drugs that act upon these processes, there is an urgent need to search for novel targets. Thus, selection of the correct cellular or molecular non-vital target, which is related to the production of virulence factors, is very important [2]. Recently, novel antivirulence molecules targeting bacterial communication (quorum sensing) and toxin production systems have been developed [3], demonstrating that identification of new biological targets and approaches that could lead to antivirulence via novel mechanisms of action are critical and active areas of research.

In bacteria, inorganic polyphosphate (polyP) metabolism is not essential for growth in a nutrient broth, however polyP deficiency affects various cellular processes such as bacterial virulence and susceptibility toward antibiotics, including persistence [4–6]. Consequently, polyphosphate kinase 1 (PPK1), the enzyme responsible for polyP synthesis in many bacterial pathogens, has been proposed as a potential drug target for novel antivirulence molecules [7,8].

Pseudomonas aeruginosa remains the most important cause of nosocomial infections. Currently, first-line therapies based on the use of antibiotics show increased resistance and persistence, leading to clinical ineffectiveness. Consequently, the increasing frequency of multidrug-resistant *Pseudomonas* strains is concerning, as successful antimicrobial options are severely limited [9]. In addition, mutants of *P. aeruginosa* PAO1 lacking PPK1 are defective in motility, quorum sensing, biofilm formation, virulence and antibiotic resistance [10].

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Although antivirulence drugs represent a novel and effective antimicrobial approach, virulence is difficult to quantify in a high-throughput screening manner using animal models. Recently, a number of alternative non-mammalian host models have been established enabling a more efficient means of evaluating bacterial virulence [11]. Consequently, antivirulence molecules have rarely been systematically searched for and thus may represent an unexploited source of antibacterial compounds [12].

The social amoeba *Dictyostelium discoideum* feeds on bacteria by phagocytosis and its growth and development is affected when faced by pathogenic micro-organisms [13]. Previously, a host model system based on *D. discoideum* was successfully used to identify *P. aeruginosa* virulence genes [14,15]. In addition, *P. aeruginosa* has been shown to use similar virulence factors when infecting mammalian hosts or *Dictyostelium* amoebae [15]. Interestingly, *D. discoideum* is among the few eukaryotes that possess bacterial PPK1 orthologues (*Dd*PPK1), and mutants of *Dd*PPK1 are deficient in development, predation and sporulation [16]. Specifically, on a *P. aeruginosa* lawn, *D. discoideum* is killed leaving the lawn intact. However, on a lawn of the bacterial PPK1 mutant, the social amoeba is an effective predator, as observed by the phagocytic plaques formed [16].

In this study, *D. discoideum* was used as a host–pathogen model with the aim of discovering antivirulence molecules targeting inorganic polyP synthesis in *P. aeruginosa* PAO1. The screening approach focused on the identification of molecules that (i) do not kill the bacterial pathogen, (ii) avoid affecting the host and its beneficial bacteria and (iii) disrupt the production of virulence factors that damage the host.

2. Materials and methods

2.1. Pseudomonas aeruginosa PAO1 polyphosphate kinase 1 (PaPPK1) modelling and in silico screening of putative inhibitors

A model of *Pa*PPK1 was generated by comparative modelling using the reported crystal structure of *Escherichia coli* PPK1 (*Ec*PPK1) (PDB id 1XDP, resolution 2.5 Å) in complex with ATP as template using MODELLER as implemented in the Build Homology Models protocol in Discovery Studio v.2.1 (Accelrys Inc., San Diego, CA). The National Cancer Institute (OpenNCI) diversity set II program containing 260 174 compounds was selected for virtual screening, and docking was performed by using FRED v.3.01 (OpenEye Scientific Software, Santa Fe, NM). These compounds were docked into the ATP binding site of *Pa*PPK1, which was prepared using FRED receptor GUI. Structure-based interaction schemes were obtained using LigandScout v.3.1 (Inte:Ligand GmbH, Vienna, Austria).

The top 100 candidate conformations for each ligand within the receptor site were obtained and optimised using the Chemgauss4 scoring function. Finally, the best-ranked binding models for the top 300 compounds were minimised using the CHARMM22 forcefield in Discovery Studio v.2.1. The consensus score protocol within Discovery Studio was used to select the compound for biological assays, and the top 30 compounds were obtained from the National Cancer Institute's Developmental Therapeutics Program (DTP-NCI).

In addition, a set of Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties was computed using the ADMET and TOPKAT modules available in Discovery Studio v.2.1. The ADMET Descriptors protocol uses the QSAR models to estimate a range of ADMET-related properties for small molecules.

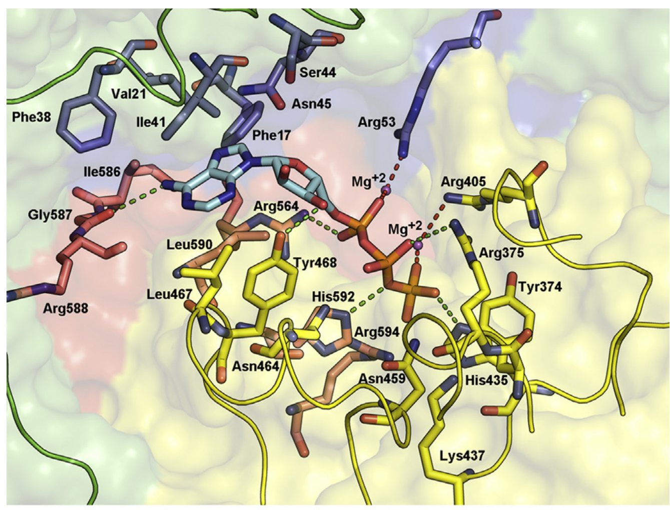
2.2. PaPPK1 purification and enzymatic inhibition assays

To overexpress the *ppk1* gene from *P. aeruginosa* PAO1 strain (*Pa*PPK1, UniProtKB ID: PPK_PSEAE) in *E. coli*, the sequence of *Pa*PPK1 was used to design a synthetic gene with codon optimisation for *E. coli* using OptimumGene® technology (GenScript, Piscataway, NJ). The synthetic *Pa*PPK1 gene was commercially subcloned into the pUC57 vector. In-frame insertion of an amino-terminal 6xHis-tag was included by cloning the *Nde*I/*Hin*dII fragment of the synthetic *Pa*PPK1 gene into the pET-TEV vector to facilitate the overexpression and his-tag-based purification of the enzyme. All constructs carrying synthetic *Pa*PPK1 gene were confirmed by bidirectional dideoxy sequencing (Macrogen, Seoul, South Korea). For purification, *E. coli* BL21-DE3 cells transformed with the derived plasmid (pET-PaPPK1) were aerobically grown in Luria–Bertani (LB) medium at 37 °C until an optical density at 600 nm (OD_{600}) of 0.5. After 2 h of induction with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), the cells were harvested by centrifugation (10 min, 3000 × g, 4 °C) and were re-suspended in B-PER™ Bacterial Protein Extraction Reagent (Thermo Scientific, Waltham, MA). The cellular extract was applied to a HisTrap column (GE Healthcare, Fairfield, CT), which had been previously charged with $NiSO_4$ according to the manufacturer's instructions. The bound proteins were eluted using a buffer containing 50 mM Tris–HCl (pH 8.2) and 10 mM $MgCl_2$ supplemented with increasing concentrations of imidazole (40–600 mM). The fractions were pooled and dialysed against a buffer containing 25 mM Tris–HCl (pH 8.0), 50 mM NaCl and 10% glycerol.

PPK1 activity was assessed by incubating the pure enzyme (5 μg protein/reaction) in assay buffer [50 mM HEPES-KOH (pH 7.5) containing 40 mM ammonium sulfate, 4 mM magnesium sulfate, 60 mM creatine phosphate and 50 μg creatine kinase] with substrate (5 mM ATP; total reaction volume = 200 μL) for 1 h at 37 °C. This was followed by addition of 4′,6-diamidino-2-phenylindole (DAPI) at a final concentration of 50 μM. Fluorescence intensity measurements using DAPI were obtained on a Multi-Modal Synergy™ 2 Microplate Reader (BioTek, Winooski, VT) at an excitation wavelength of 400 nm (bandwidth of 10 nm). Fluorescence emission was measured at 540 nm (bandwidth of 25 nm). All experiments were performed at room temperature using black 96-well fluorescence microplates (Thermo Scientific). The values obtained were corrected by subtracting the blank reading (all ingredients except enzyme). Standard curves were prepared with known concentrations (1–50 μM) of $polyP_{45}$ (Sigma–Aldrich, St Louis, MO) and the amount of poly-P synthesised was expressed as μmol of polyP produced (phosphates/min/mg protein). The half maximal inhibitory concentration (IC_{50}) was determined using dose–response curves evaluating the effect of various concentrations of the inhibitor on the activity of the *Pa*PPK1 enzyme.

2.3. Dictyostelium discoideum developmental virulence assays

Dictyostelium AX4 cells from dictyBase (http://DictyBase.org) were grown axenically in HL5 medium or in association with *Klebsiella aerogenes* on SM broth plates [17]. For the virulence assay, *P. aeruginosa* PAO1 and *K. aerogenes* were grown overnight in LB medium. After washing, bacteria were re-suspended with nematode growth medium (NGM) buffer and the OD_{600} was determined. After adjusting to 0.5 OD units, 300 μL of *Klebsiella* and *Pseudomonas* at the indicated proportions (25–50%) were plated on SM agar plates with ca. 300 *D. discoideum* cells. Amoeba predation and social development phases (vegetative growth and aggregation, elevation and culmination) were evaluated for 10 days. The effective (antivirulence) concentration was defined as the minimum concentration that allowed culmination of *D. discoideum* social development on a bacterial lawn with 50% *P. aeruginosa*. More detailed experimental procedures can be found in the Supplementary data.



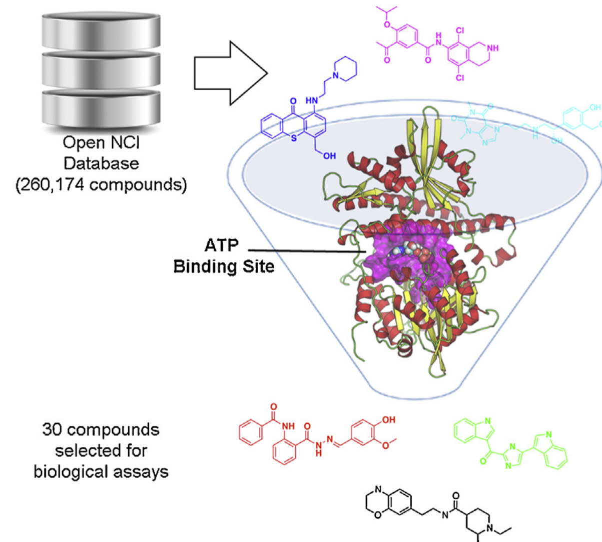


Fig. 1. Virtual screening for the identification of inhibitors against *Pseudomonas aeruginosa* PAO1 polyphosphate kinase 1 (*Pa*PPK1). ATP binding site of a *Pa*PPK1 monomer. Carbon atoms and secondary structure from residues at the binding site are coloured according to defined structural domains of *Escherichia coli* PPK1 (*Ec*PPK1) [18]. Schematic representation of the virtual screening procedure to identify novel *Pa*PPK1 inhibitors from the OpenNCI database.

3. Results and discussion

First, a virtual screening approach was conducted to identify compounds that inhibit *Pa*PPK1. To this end, a three-dimensional homology model of *Pa*PPK1 was constructed using the X-ray crystal structure of *Ec*PPK1 as template. The *Pa*PPK1 model was validated and the structure was used to screen the OpenNCI database in search for putative inhibitors that block the binding of ATP (Fig. 1; Supplementary Fig. S1) [19].

3.1. In silico and enzymatic screening of PaPPK1 inhibitors

The top 30 compounds based on the consensus scoring results (Supplementary Table S1) were obtained from the DTP-NCI. In addition, a set of ADMET properties was computed to assess the pharmaceutical profile of the compounds (Supplementary Table S2). The compounds obtained from the DTP-NCI were further experimentally evaluated to assess their ability to inhibit *Pseudomonas* virulence in *D. discoideum*.

All compounds were assayed against purified recombinant *Pa*PPK1. The inhibitory effect of 80% of the compounds was confirmed in vitro on the *Pa*PPK1 enzyme activity, with IC_{50} values of 2.0–50 μM (Table 1).

3.2. Dictyostelium discoideum developmental virulence and antivirulence assays

Prior to testing the antivirulence efficacy of the compounds, optimal conditions for the in vivo developmental assay in *D. discoideum* cells were established (Fig. 2). *Dictyostelium* is a surrogate host model with several advantages because of its simplicity and reproducibility compared with other host–microbe systems [14]. Fig. 2a shows a schematic representation of the assay in which axenic *D. discoideum* cells were grown for 10 days in association with bacteria on 24–48-well SM plates. *Klebsiella aerogenes* was routinely used as an appropriate food supply and *P. aeruginosa* were mixed at the indicated proportions (Fig. 2b). In addition, a *ppk1* mutant strain from *P. aeruginosa* PAO1 was used to confirm its attenuated virulence phenotype [16].

When axenic *Dictyostelium* cells were plated on a *K. aerogenes* lawn, each amoeba cell created a phagocytic plaque and culmination of social development (spore formation) was achieved in 5 days (Fig. 2a). In contrast, when 50% wild-type *P. aeruginosa* PAO1 were added to the *K. aerogenes* lawn, development of *D. discoideum* was completely inhibited. Initial social development was more favourable when 25% *Pseudomonas* was used, but culmination was not completely achieved (Fig. 2b and c). In contrast, the *ppk1* mutant allowed the elevation phase, but not the culmination of social development (Fig. 2b and c). Given the contrasting phenotype when 50% *P. aeruginosa* was added to the plates, this condition was used for subsequent antivirulence assays.

The results of the developmental assays are in agreement with previous predation studies which showed that *P. aeruginosa* PAO1 is a virulent growth substrate for *D. discoideum* and that polyP synthesis is essential for the virulence of *P. aeruginosa* toward the social amoeba [15,16].

Next, the antivirulence capability of the predicted *Pa*PPK1 inhibitors was further examined in the aforementioned *D. discoideum* developmental assay (Fig. 3). Of the 30 compounds tested, 14 allowed amoeba predation at a concentration of 10 μM (47% active), but only 5 of these compounds allowed the culmination phase of the social development of *D. discoideum* (Fig. 3; Table 1). A *D. discoideum* lawn together with its beneficial bacterium (*K. aerogenes*) was also used for the safety assays, whereby the cytotoxicity of each compound was tested without the bacterial pathogen. None of the compounds with antivirulence properties affected the normal development of *D. discoideum* (Fig. 3; Table 1).

Finally, the growth of *P. aeruginosa* was not affected at 10 μM by any of the 14 compounds with antivirulence activity as confirmed by conventional agar. We conclude that none of the identified antivirulence compounds possess either bactericidal activity against *P. aeruginosa* or cytotoxic activity toward *D. discoideum*. Table 1 summarises all biological properties (antivirulence, antibiotic and cytotoxicity) determined for the 14 antivirulence compounds.

Among the compounds with the highest antivirulence activity, s-triazine derivative compound 28 (NSC-696924; Table 1) appears as the most promising for several reasons. Other triazine derivatives have shown antimicrobial and particularly

Table 1
Chemical structures of antivirulence molecules identified using the *Dictyostelium discoideum* development assays and their corresponding antibiotic activities and secondary effects.

Compound	NSC ID	Structure	IC_{50} (μM)	Antivirulence[a]	Antibiotic[b]	Safety[b]
3	293892		32	+	–	–
7	65375		15	+	–	–
8	618160		2	+	–	–
10	166366		10	++	–	–
18	380323		20	++	–	–
19	205574		8	+++ (EC = 10 μM)	–	–
20	205572		17	++	–	–
23	666767		17	++	–	–
24	141672		5	+++ (EC = 10 μM)	–	–
25	108334		20	+++	–	–
27	172774		50	+++	–	–

Table 1 (*Continued*)

Compound	NSC ID	Structure	IC_{50} (μM)	Antivirulence[a]	Antibiotic[b]	Safety[b]
28	696924		9	+++ (EC = 10 μM)	–	–
34	641999		12	++	–	–
37	403447		n.d.	++	–	–

IC_{50}, half maximal inhibitory concentration; EC, effective concentration; n.d., not determined.

[a] + Allows only vegetative growth and aggregation; ++, allows elevation; +++, allows culmination. The EC was defined as the minimum concentration that allows the culmination of the social developmental of *D. discoideum* according to the antivirulence assays.

[b] Antibiotic and safety assays were performed as specified in Section 2 (more detailed experimental procedures can be found in the Supplementary data).

antivirulence activity in a surrogate host–microbe system (*Tetrahymena–Klebsiella*) [20]. Furthermore, this compound shows a favourable predicted ADMET profile (Supplementary Table S2), suggesting that it might be considered as a lead compound for the development of novel antivirulence agents.

Established screening systems which identify compounds that kill or inhibit the growth of bacteria can no longer be used. Consequently, antimicrobial screening using classical minimum inhibitory concentration (MIC) measurements will become obsolete in the future. In addition, a high percentage of antimicrobial agents fail when tested in cell-based assays or under in vivo conditions. High-throughput infection in Petri dishes using simple host models should reduce this ineffectiveness. Here we demonstrate two advantages of *D. discoideum* as a host–microbe model. First, the simplicity and reproducibility of the *Dictyostelium* system is better than those of other mammalian as well as non-mammalian systems. Specifically, we show that *D. discoideum* offered a robustness and ease of manipulation for an efficient host–pathogen model for the screening of antivirulence compounds. Second, the amoeba virulence assay in combination with developmental safety tests provided a better characterisation and screening tool for discovering novel antivirulence agents with a higher efficacy and safety profile. Therefore, in summary, we have designed a simple high-throughput developmental assay using *D. discoideum* and report the first in vivo antivirulence agents targeting *Pa*PPK1 without side effects to the host or its beneficial bacteria.

Finally, the identified *Pa*PPK1 inhibitors are candidates for the development of next-generation antivirulence therapeutics as (i) they do not affect bacterial cell viability or growth, (ii) they reduce *P. aeruginosa* virulence in a simple host–model and (iii) they do not disturb the host or its beneficial bacteria.

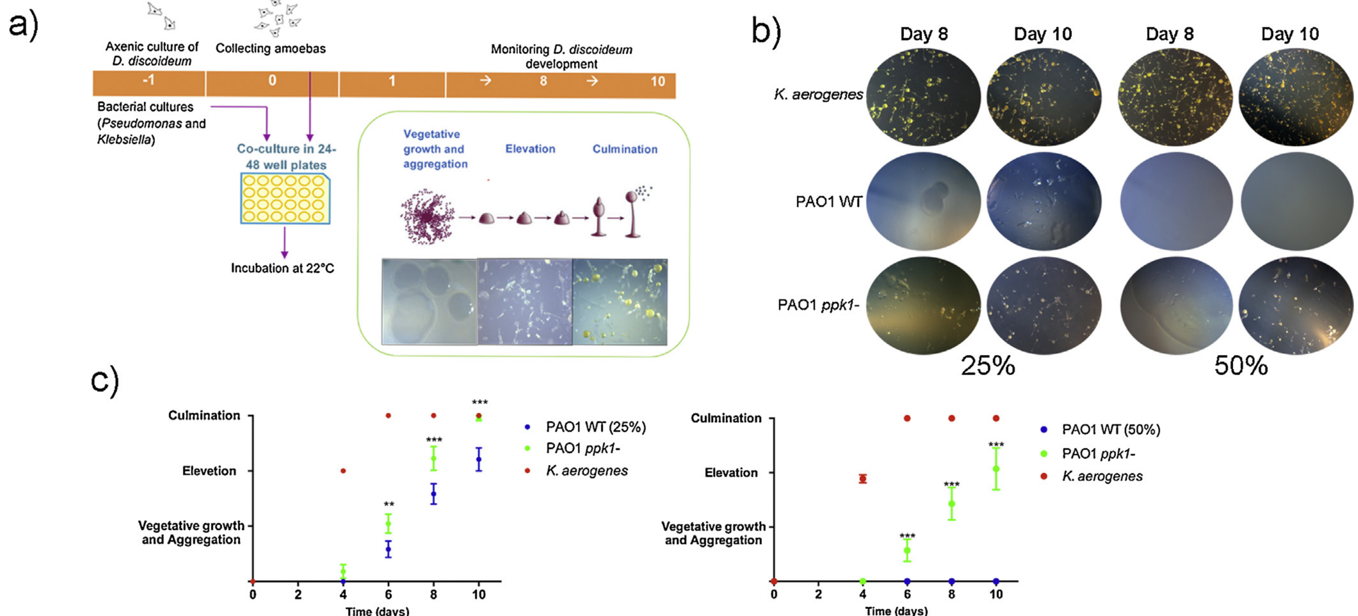


Fig. 2. *Dictyostelium discoideum* development assays as a tool for *Pseudomonas aeruginosa* virulence evaluation. (a) Co-culture of axenic *D. discoideum* cells and bacterial cells (*P. aeruginosa* PAO1 and *Klebsiella aerogenes*) in 24–48-well microplates. (b and c) Antivirulence assays with 25% and 50% *P. aeruginosa* in SM broth agar for 10 days. All phases of *D. discoideum* development (vegetative growth and aggregation, elevation and culmination) were monitored every 48 h for 10 days. WT, wild-type.

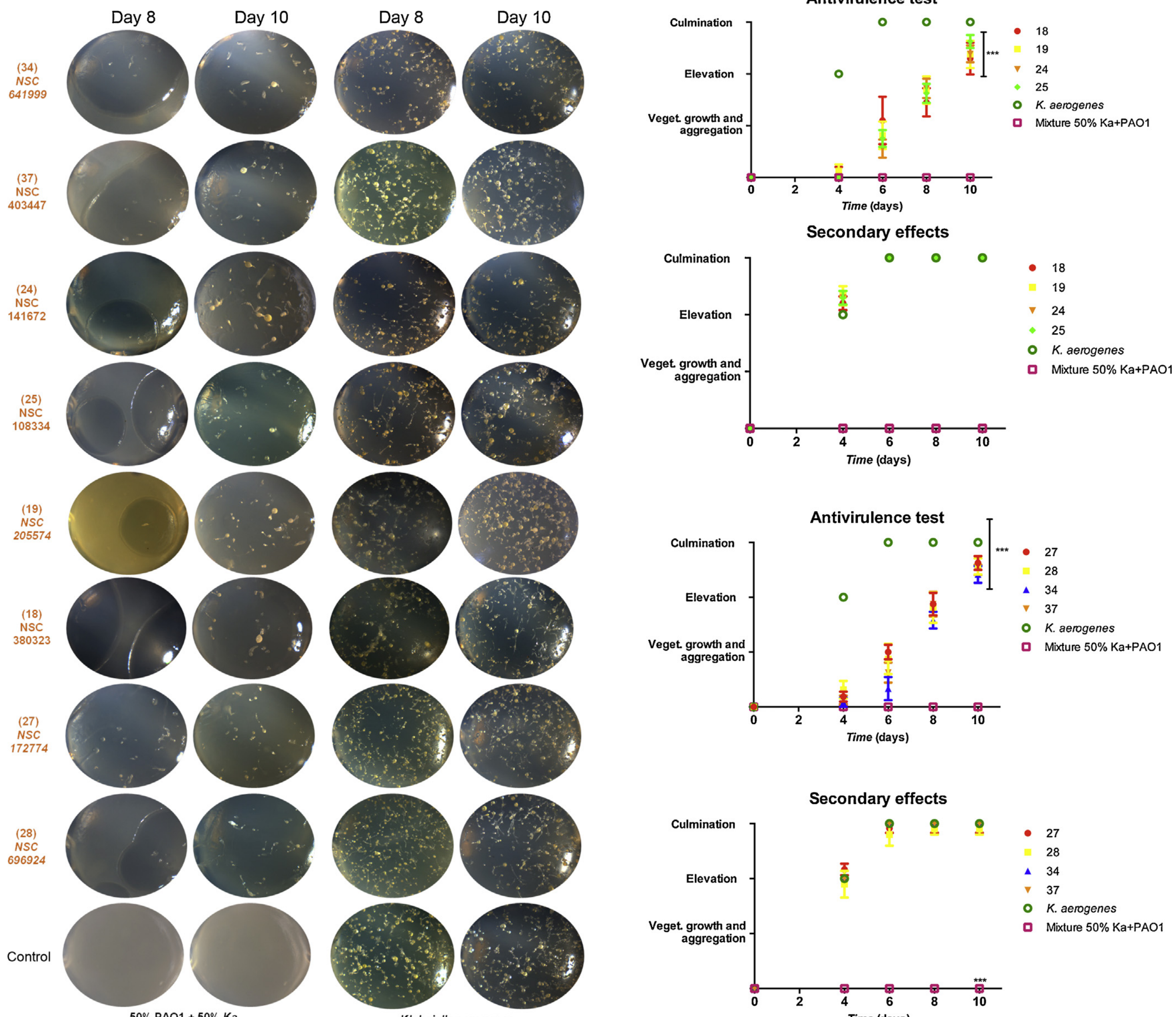


Fig. 3. In vivo evaluation of antivirulence compounds using a high-throughput *Dictyostelium discoideum* development plate assay. Antivirulence assays with 50% *Pseudomonas aeruginosa* and safety (secondary effects) tests were monitored every 48 h for 10 days. All phases of *D. discoideum* development (vegetative growth and aggregation, elevation and culmination) were monitored and results for the top eight antivirulence compounds are shown.

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Competing interests: None declared.

Ethical approval: All procedures complied with national guidelines of the Animal Use Ethics Committee of the University of Chile (Santiago, Chile) and the Bioethics Advisory Committee of Fondecyt-Conicyt (the funding agency for this work).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2016.02.005.

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