Repurposing the antimycotic drug flucytosine for suppression of *Pseudomonas aeruginosa* pathogenicity

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Although antibiotic resistance represents a public health emergency, the pipeline of new antibiotics is running dry. Repurposing of old drugs for new clinical applications is an attractive strategy for drug development. We used the bacterial pathogen *Pseudomonas aeruginosa* as a target for the screening of antivirulence activity among marketed drugs. We found that the antimycotic agent flucytosine inhibits the expression of the iron-starvation σ-factor PvdS, thereby repressing the production of major *P. aeruginosa* virulence factors, namely pyoverdine, PrpL protease, and exotoxin A. Flucytosine administration at clinically meaningful dosing regimens suppressed *P. aeruginosa* pathogenicity in a mouse model of lung infection. The in vitro and in vivo activity of flucytosine against *P. aeruginosa,* combined with its desirable pharmacological properties, paves the way for clinical trials on the anti-*P. aeruginosa* efficacy of flucytosine in humans.

antivirulence drug | cystic fibrosis | drug repositioning | iron uptake | selective optimization of side activities (SOSA) approach

Only 70 y after the introduction of antibiotics in the clinical practice, the development and spread of resistance among pathogenic bacteria are limiting the therapeutic efficacy of these magic bullets. Inhibition of bacterial virulence, rather than growth, is an alternative approach to the development of new antimicrobials. Antivirulence drugs disarm rather than kill pathogens. In principle, they combat bacterial infections without exerting the strong selective pressure for resistance imposed by conventional antibiotics, with no predictable detrimental effect on the host microbiota (1). In the last decade, many antivirulence strategies have been proven effective in animal models of infection (reviewed in ref. 2), although no antivirulence compound has yet been tested in large-scale clinical trials.

A shortcut to the development of new drugs is searching for side activities in old drugs already approved for use in humans and for which safety issues have extensively been considered (3). This drug-repurposing strategy has a high probability of yielding safe and bioavailable hit compounds, which can move straightforward into clinical trials or be used as leads for drug optimization programs (3).

The Gram-negative bacterium *Pseudomonas aeruginosa* is one of the most dreaded nosocomial pathogens and the leading cause of chronic lung infection in patients with cystic fibrosis (CF) (4). Multidrug-resistant *P. aeruginosa* has become increasingly frequent in healthcare settings and poses a tremendous challenge to traditional antibiotic therapy (5). Because *P. aeruginosa* has a large armamentarium of virulence factors (6), inhibition of master regulatory networks controlling its pathogenicity, rather than individual virulence traits, is more likely to cause an overall attenuation of virulence (7).

The siderophore pyoverdine represents a promising target for antivirulence compounds. Pyoverdine is not only the primary iron carrier during *P. aeruginosa* infection and biofilm formation (8, 9) but also, a master signal molecule that controls virulence gene expression through a mechanism called surface signaling (10). Interaction of iron-loaded pyoverdine with its cognate outer membrane receptor FpvA triggers a signal through the inner membrane-spanning anti–σ-factor FpvR, leading to full activation of the alternative σ-factor PvdS, which is responsible for expression not only of pyoverdine genes but also, key virulence factors (i.e., exoproteases and exotoxin A) (10). Pyoverdine synthesis is stimulated by iron deficiency, a nutritional condition characterizing the biological fluids of infected mammals (11), whereas negative control of pyoverdine synthesis is exerted by the global regulator of bacterial iron homeostasis Fur, which represses *pvdS* transcription under high-iron conditions (12). Although the role of pyoverdine in pathogenicity has been known for years, this system has so far been ignored as a target for antivirulence drugs. Only recently, an enzymatic screening assay allowed the identification of two compounds inhibiting the in vitro activity of PvdQ, a periplasmic hydrolase that is required for pyoverdine maturation (13). However, the antipyoverdine activity of these inhibitors has not been tested in bacterial cultures or in vivo.

The aim of the present work was to apply a drug-repurposing approach to identify antipyoverdine compounds that could represent good candidates for in vivo use as antivirulence drugs against *P. aeruginosa*. By using a specific biosensor for pyoverdine inhibitors, we screened a chemical library of marketed drugs and identified a promising US Food and Drug Administration-approved compound that resulted effective in suppressing *P. aeruginosa* virulence in vitro and in an animal model of pulmonary infection.

Results and Discussion

Identification of a Pyoverdine Synthesis Inhibitor. A screening system for pyoverdine inhibitors, based on a *P. aeruginosa* PAO1 reporter strain carrying a transcriptional fusion between the PvdS-dependent *pvdE* promoter (P*pvdE*) and the *luxCDABE* operon inserted at a neutral chromosomal site, was constructed (Fig. S1). This system was used to screen a commercial library of 1,120 chemical compounds with known biological activities selected for their high chemical and pharmacological diversity and safety in humans (Prestwick Chemicals). Blind screening led to the identification of one compound that reproducibly reduced bioluminescence and pyoverdine production by the reporter strain under iron-depleted conditions. This compound was decoded as flucytosine [5-Fluorocytosine (5-FC)], a synthetic fluorinated pyrimidine used as an antimycotic drug with the brand name of Ancobon.

To confirm the antipyoverdine activity of 5-FC, the compound was purchased from a different supplier (Sigma-Aldrich) and used for additional investigation. Although 5-FC did not affect *P. aeruginosa* growth (Fig. 1*A*) (minimum inhibitory concentration >

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10 mM), it had a very high inhibitory activity on P*pvdE*-dependent bioluminescence emission and pyoverdine production (Fig. 1*B*), with IC_{50} values of 2 and 3 μM, respectively. 5-FC also showed a similar inhibitory effect on the transcription of other pyoverdine biosynthetic genes (Fig. S2*A*), suggesting that 5-FC negatively affects the expression of the entire pyoverdine biosynthesis machinery. As a control, no variation in the expression of the housekeeping gene *proC* was observed in the presence of 5-FC (Fig. S2*B*).

5-FC Inhibits Pyoverdine Synthesis in Diverse *P. aeruginosa* Strains. Because *P. aeruginosa* strains can produce one of three different pyoverdine types (I, II, or III), which are recognized by cognate FpvA receptor variants (14), we assessed the effect of 5-FC on prototypic *P. aeruginosa* strains producing different types of pyoverdine and carrying the P*pvdE*::*lux* reporter construct. The inhibitory activity of 5-FC on both pyoverdine production and *pvdE* transcription was similar among *P. aeruginosa* strains producing type I, II, or III pyoverdine (Fig. 1 *B* and *C*), indicating that the antipyoverdine activity of 5-FC is independent of the chemical nature of the pyoverdine molecule and the structure of the ferripyoverdine receptor.

5-FC–dependent pyoverdine inhibition was also tested on a small collection of *P. aeruginosa* CF isolates ($n = 20$), including clonal variants isolated from the same CF patients during a period of more than 15 y (15) (Table S1). The IC_{50} values of 5-FC for CF isolates were comparable with or even lower than the IC_{50} determined for the laboratory strain PAO1 (Fig. 1*D*), suggesting that susceptibility to 5-FC is conserved in CF isolates.

5-FC Inhibits *pvdS* Gene Expression. To investigate the effect of 5-FC on pyoverdine signaling, we determined the antipyoverdine activity of 5-FC on a set of *P. aeruginosa* mutants impaired in different steps of the pyoverdine signaling cascade (Fig. 2*A*). The FpvA- and PvdA-deficient mutants are impaired in pyoverdine uptake and synthesis, respectively, and they are, therefore, unable to activate the PvdS σ-factor through pyoverdine-mediated signaling (signaling-off mutants). In contrast, the FpvR-deficient mutant cannot suppress PvdS activity in the absence of pyoverdine signaling, thus resulting in signaling-insensitive up-regulation of PvdS-dependent genes (constitutive signaling-on mutant) (Fig. 2*A*). 5-FC inhibited pyoverdine production and *pvdE* gene expression in all mutants tested (Fig. 2*A*), indicating that pyoverdine signaling is not the target of 5-FC. However, 5-FC seemed to be slightly less effective against the *fpvR* mutant (Fig. 2*A*). Because the constitutively active state of pyoverdine signaling in the *fpvR* mutant results in maximal activation of the PvdS intracellular pool (16), the lower activity of 5-FC in the *fpvR* background suggests that intracellular levels and/or activity of PvdS are critical for the inhibitory activity of 5-FC.

The effect of 5-FC on *pvdS* gene expression was investigated in *P. aeruginosa* carrying a transcriptional fusion between the *pvdS* promoter and the β-gal gene. 5-FC reduced *pvdS* promoter activity in a dose-dependent manner (Fig. 2*B*), suggesting that 5-FC acts as an inhibitor of *pvdS* transcription and consequently, reduces PvdS intracellular levels, expression of pyoverdine genes, and ultimately, pyoverdine production. To verify this hypothesis, the effect of 5-FC on pyoverdine production was assessed in a *pvdS*-deficient *P. aeruginosa* mutant carrying the *pvdS* coding sequence on a multicopy plasmid under the control of a constitutive promoter. Although 5-FC inhibited pyoverdine production by the WT strain carrying the empty vector, it had no effect on the strain constitutively expressing PvdS (Fig. 2*C*). Interestingly, 5-FC also repressed the transcription of the Fur-Fe^{2+}–regulated gene *pchR* (Fig. 2*D*), encoding an AraC/XylS-like transcriptional regulator essential for production of the second *P. aeruginosa* siderophore pyochelin (17). Accordingly, the expression of the PchR-regulated gene *pchE* and production of pyochelin were also reduced in the presence of 5-FC (Fig. 2*D*). 5-FC also inhibited the transcription of additional iron-repressible genes, namely *feoA* and *foxA*

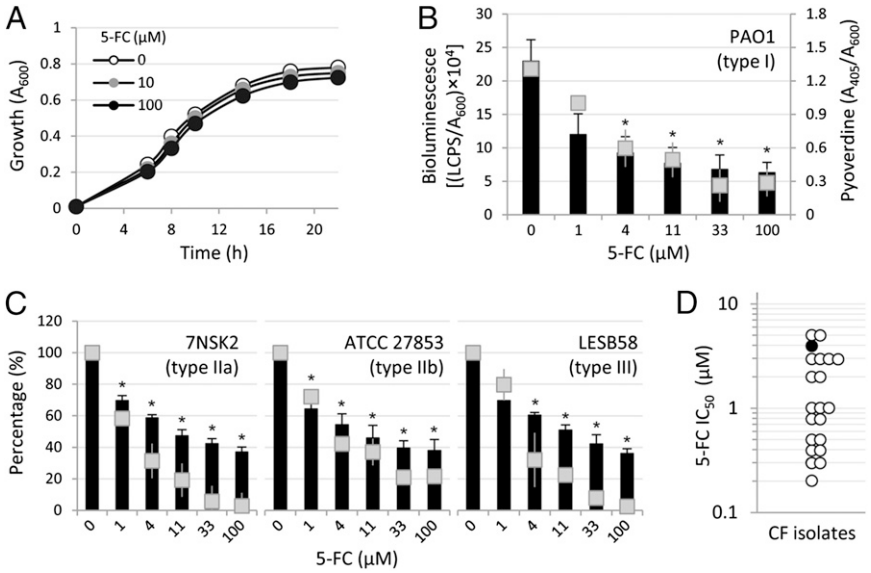

Fig. 1. 5-FC inhibits pyoverdine production in *P. aeruginosa*. (*A*) *P. aeruginosa* PAO1 growth curve in TSBD in the presence of different 5-FC concentrations (0–100 μM). (*B*) Dose–response effect of 5-FC (0–100 μM) on bioluminescence (black bars, left *y* axis) and pyoverdine production (gray squares, right *y* axis) by PAO1 P*pvdE*::*lux* at 14 h of growth in TSBD. (*C*) Dose–response effect of 5-FC (0–100 μM) on bioluminescence (black bars) and pyoverdine production (gray squares) at 14 h of growth in TSBD by *P. aeruginosa* strains producing different pyoverdine types and pyoverdine receptors (IIa, IIb, or III) and carrying the P*pvdE*::*lux* fusion. Values are normalized to A_{600} of bacterial cultures and expressed as percentage of the corresponding untreated control values. Values represent the mean (± SD) of at least three independent assays. The specific pyoverdine receptor type expressed by each strain is indicated in *B* and *C*. (*D*) IC_{50} values (micromolar) of 5-FC for pyoverdine production in 20 *P. aeruginosa* CF isolates (white circles) compared with the PAO1 reference strain (black circle). *Statistically significant differences ($P < 0.01$, ANOVA) in both bioluminescence and pyoverdine production with respect to the corresponding untreated controls.

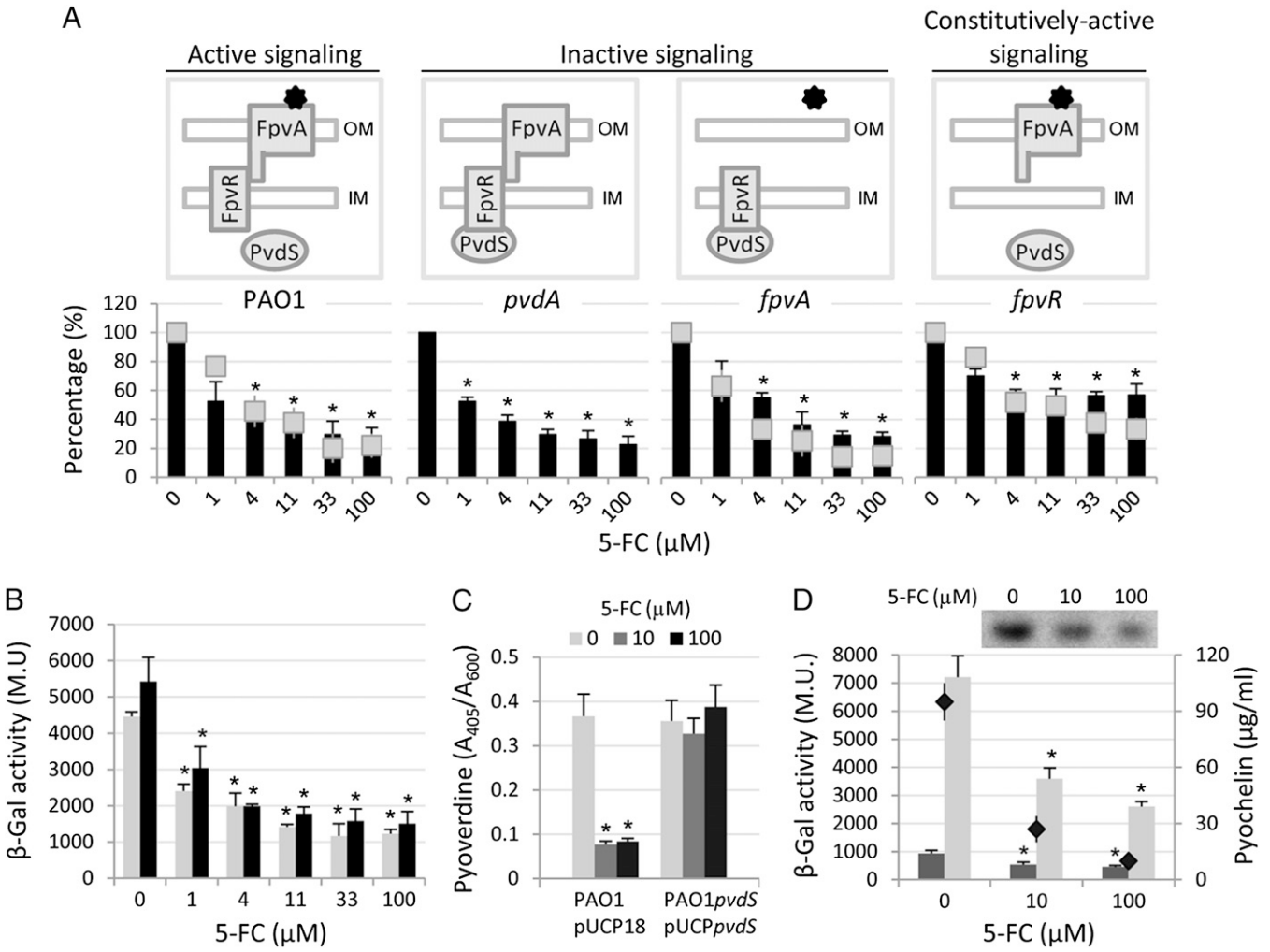

Fig. 2. 5-FC down-regulates *pvdS* transcription. (*A*) Dose–response effect of 5-FC (0–100 μM) on bioluminescence emission (black bars) and pyoverdine production (gray squares) at 14 h of growth in TSBD by *P. aeruginosa* WT and mutant strains defective in different steps of the pyoverdine signaling cascade and carrying the P*pvdE*::*lux* reporter fusion. Values are normalized to the cell density of the bacterial cultures and expressed as percentage of the corresponding untreated control values. The different behavior of mutants with respect to pyoverdine signaling is illustrated in *Upper* (black stars represent pyoverdine). (*B*) Dose–response effect of 5-FC (0–100 μM) on β-gal expression by PAO1 P*pvdS*::*lacZ* during exponential (gray bars) and stationary phase of growth in TSBD (black bars). (*C*) Effect of 5-FC (0–100 μM) on pyoverdine production at 8 h of growth in TSBD by *P. aeruginosa* PAO1 carrying the empty vector (pUCP18) and its isogenic *pvdS* mutant constitutively expressing PvdS (pUCP*pvdS*). (*D*) Effect of 5-FC (0–100 μM) on β-gal expression by *P. aeruginosa* PAO1 P*pchR*::*lacZ* (dark gray histograms, left *y* axis) and PAO1 P*pchE*::*lacZ* (light gray histograms, left *y* axis) and pyochelin production by PAO1 WT (black diamonds, right *y* axis) after 14 h of growth in TSBD. Values represent the mean (± SD) of three independent assays. *Inset* shows ferripyochelin yields following separation of PAO1 culture extracts on a representative TLC plate. *Statistically significant differences ($P < 0.01$, ANOVA) with respect to the corresponding untreated controls.

(Fig. S2 *C* and *D*), which are directly and indirectly controlled by Fur-Fe^{2+}, respectively (12). However, 5-FC–dependent suppression of pyoverdine production was also observed in a *P. aeruginosa* PAO1 *fur* mutant (Fig. S2*E*), suggesting that 5-FC could repress iron uptake genes through a Fur-independent mechanism.

5-FC Down-Regulates PvdS-Dependent Expression of Virulence Genes. The finding that 5-FC inhibits *pvdS* transcription implies that this compound could also affect the expression of PvdS-regulated virulence factors other than pyoverdine. To verify this hypothesis, we investigated the effect of 5-FC on the expression of two major virulence factors of *P. aeruginosa*, the endoprotease PrpL and exotoxin A, which are directly and indirectly regulated by PvdS, respectively (12). PrpL and exotoxin A were monitored at the expression level using *PprpL::lacZ* and *PtoxA::lacZ* fusions and at the protein level using antiexotoxin A Western blot analysis and a PrpL enzyme activity assay. As for pyoverdine, the expression of *toxA* and *prpL* genes was down-regulated in 5-FC–treated cultures with respect to untreated controls (Fig. 3*A*), consistent with the strongly reduced ToxA and PrpL levels in culture supernatants (Fig. 3 *B* and *C*).

5-FC Suppresses *P. aeruginosa* Pathogenicity in Vivo. The promising antivirulence activity of 5-FC in vitro led us to investigate the efficacy of 5-FC as an anti-*P. aeruginosa* drug in a mouse model of pulmonary infection. Mice were infected intratracheally with *ca.* 10^6 *P. aeruginosa* PAO1 cells embedded in agar beads and then treated two times daily with i.p. administration of either a therapeutic dose of 5-FC (30 mg/kg per day) or the placebo (saline). As a control, mice were also infected with an isogenic *pvdS* mutant and treated with saline. Although 75% of placebo-treated mice were killed within 4 d of PAO1 infection, 5-FC treatment almost completely protected mice from the *P. aeruginosa* lethal challenge (Fig. 4*A*). Notably, all mice infected with the *pvdS* mutant survived the challenge (Fig. 4*A*), highlighting the importance of PvdS as a major pathogenicity determinant in *P. aeruginosa* pulmonary

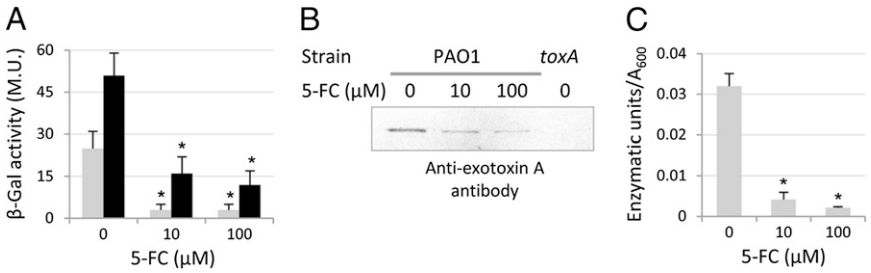

Fig. 3. 5-FC inhibits PvdS-dependent virulence gene expression. Effect of 5-FC (0–100 μM) on (*A*) β-gal expression by PAO1 P*toxA*::*lacZ* (gray bars) and PAO1 P*prpL*::lacZ (black bars) and (*B*) exotoxin A levels and (*C*) PrpL enzymatic activity in 10 μL PAO1 culture supernatants after 8 h of growth in TSBD. Values represent the mean (± SD) of three independent assays, whereas the Western blot is representative of two independent experiments giving similar results. *Statistically significant differences ($P < 0.01$, ANOVA) with respect to the corresponding untreated controls.

infection. After 6 d of infection, the bacterial load in lungs of surviving mice was comparable between mice infected with PAO1 and PAO1*pvdS* as well as between 5-FC–treated and -untreated mice (Fig. S3), confirming that 5-FC inhibits virulence rather than cell viability. Moreover, lung histopathology revealed that lesions and inflammation in bronchi and pulmonary parenchyma were similarly reduced in both 5-FC–treated and PAO1*pvdS*-infected mice compared with untreated mice infected with WT PAO1 (Fig. 4*B*).

Antivirulence Activity of 5-FC Requires Metabolic Conversion to 5-Fluorouracil. The antimycotic compound 5-FC is a prodrug that is taken up by fungi through one or more cytosine permeases, deaminated to 5-fluorouracil by a cytosine deaminase, and subsequently, converted to 5-fluoro-UMP and 5-fluoro-dUMP, ultimately causing perturbation of DNA and protein synthesis (18). Although 5-FC by itself is not toxic, 5-fluorouracil is highly cytotoxic. Therefore, the direct use of 5-fluorouracil in medicine is restricted to the treatment of solid tumors (19).

All *P. aeruginosa* genomes sequenced so far contain homologs of the *codA* and *codB* genes of *Escherichia coli* (www.pseudomonas.com), encoding a cytosine deaminase and a cytosine permease, respectively (20). To assess whether conversion to 5-fluorouracil is essential for the antipyoverdine activity of 5-FC, we tested 5-FC against individual *P. aeruginosa codA* and *codB* deletion mutants. Inhibition of pyoverdine production and *pvdS* gene expression by 5-FC was strongly reduced in the PAO1*codB* mutant and completely abrogated in the PAO1*codA* mutant, indicating that 5-FC uptake and conversion to 5-fluorouracil are essential for 5-FC activity in *P. aeruginosa* (Fig. 5). Interestingly, a very high 5-FC concentration (1 mM) retained some activity against the PAO1*codB* mutant (Fig. 5), suggesting that 5-FC can also enter *P. aeruginosa* cells through low-affinity secondary systems or by passive diffusion.

Conclusions

This work represents proof that the pyoverdine system is a suitable target for the development of antivirulence compounds against *P. aeruginosa*. We showed that the antimycotic drug 5-FC inhibits the production of critical virulence factors, like pyoverdine, exotoxin A, and protease PrpL, by down-regulating *pvdS* gene expression. 5-FC also suppressed *P. aeruginosa* pathogenicity in a mouse model of lung infection, consistent with the essential role played by PvdS during pulmonary infection (Fig. 4). The molecular mechanisms by which 5-FC inhibits *pvdS* transcription are unknown at the moment, although we showed that (*i*) 5-FC has an inhibitory effect on the expression of iron uptake genes and (*ii*) 5-FC uptake and metabolic conversion to 5-fluorouracil are essential steps for 5-FC activity. Cytosine deaminase is typically produced by microorganisms and has no counterpart in higher eukaryotes, including mammals. These features confer to 5-FC selective activity on those species capable of assimilating and activating the prodrug.

Our results gain additional relevance if the pharmacological properties of 5-FC are taken into account. 5-FC is currently used combined with other antifungal agents for the treatment of systemic mycoses and fungal pneumonias (21, 22). Orally administered 5-FC is almost completely adsorbed, reaches peak concentrations in serum within 1–2 h, and easily reaches most body sites (21). 5-FC is also well-tolerated and has very low toxicity as long as serum concentrations are maintained below 50 μg/mL (388 μM) (21, 23). This serum level is almost 40-fold higher than the 5-FC concentration (10 μM) able to exert the maximal inhibitory effect in vitro on *P. aeruginosa* virulence gene expression (Figs. 1, 2, and 3). 5-FC has also been successfully used to treat fungal infections in CF patients, including a case of pulmonary candidiasis, without causing side effects (24, 25). These issues raise the possibility that currently recommended 5-FC dosing regimens would also be effective as antivirulence therapy against *P. aeruginosa*. We hope that our findings will foster clinical investigations aimed at verifying the efficacy of 5-FC in the treatment of *P. aeruginosa* infections, offering the unique chance of assessing the clinical impact of an antivirulence drug.

Materials and Methods

Bacteria, Media, and Chemicals. Bacterial strains and plasmids used in this work are listed in Table S2. *P. aeruginosa* CF isolates are described in Table S1. Bacteria were grown in LB (26) for general genetic procedures, whereas they were grown in the low-iron media trypticase soy broth dialysate (TSBD) (27) or M9 minimal medium supplemented with succinate (26) for specific assays. 5-FC was purchased from Sigma-Aldrich. Exogenous pyoverdine was added as pyoverdine-conditioned medium (8).

General Genetic Procedures. *E. coli* was routinely used for recombinant DNA manipulations. The P*pvdE*::*lux* construct was generated by cloning in plasmid mini–CTX-lux (28) the SalI-HindIII DNA fragment encompassing the *pvdE* promoter region excised from pMP190::P*pvdE* (29). The P*pvdE*::*lux* construct was integrated into the genome of *P. aeruginosa* strains as described (30). The PAO1*pvdS* mutant was generated by replacement of the entire *pvdS* coding sequence with a Gm^R cassette using a previously described strategy (31). The in-frame deletion mutants PAO1*codA* and PAO1*codB* were generated using the suicide vector pDM4 as described (32). The complementing plasmids pUCP*codA* and pUCP*codB* were generated by cloning the *codA* and *codB* coding sequence, including their putative ribosome binding site, downstream to the *lac* promoter in the pUCP18 plasmid (Table S2). The P*pchR*::*lacZ* and P*feoA*::*lacZ* transcriptional fusions were generated by cloning a PCR-amplified DNA fragment encompassing the entire promoter region of *pchR* and *feoA* genes, respectively, into the promoter probe plasmid pMP220 (Table S2). Primers and restriction enzymes used for cloning of PCR products are listed in Table S3.

Screening for Pyoverdine Inhibitors. Overnight cultures of PAO1 P*pvdE*::*lux* were diluted to $A_{600} = 0.003$ in the iron-poor TSBD medium, and growth at 37 °C in microtiter plates in the presence or absence of 50 or 5 μg/mL each Prestwick compound (200 μL final volume) was monitored for up to 20 h. A_{600} and bioluminescence light counts per second (LCPS) were measured in

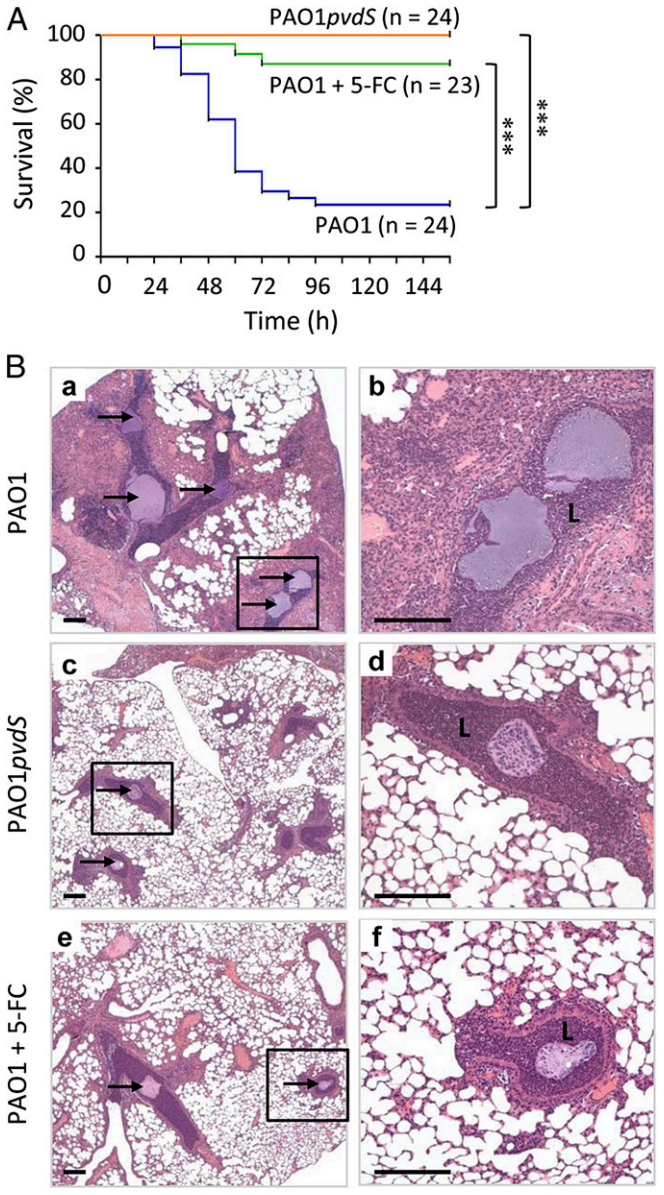

Fig. 4. 5-FC suppresses *P. aeruginosa* virulence in vivo. (*A*) Effect of 5-FC on *P. aeruginosa* PAO1 lethality in a mouse model of pulmonary infection. Mice were infected intratracheally with *P. aeruginosa* PAO1 embedded in agar beads and treated with i.p. administrations of 30 mg/kg per day 5-FC (green lines) or saline (blue lines). As control, mice infected with PAO1*pvdS* and treated with saline were used (orange lines). Data were pooled from two independent experiments (*n* indicates the total number of mice). ***$P <$ 0.0001 (Mantel–Cox test). (*B*) Murine lung histology. Four additional mice per group were infected with *P. aeruginosa* PAO1 or PAO1*pvdS* embedded in agar beads, treated with 5-FC or saline as described in *A*, and euthanized at day 2 postinfection (10). Lung sections were stained with H&E. PAO1-infected mice showed a massive bronchiolitis and huge interstitial/alveolar inflammation. In PAO1*pvdS*- and PAO1-infected mice treated with 5-FC (+5-FC), the inflammation was focal, and most of alveolar spaces were spared. Beads, indicated by arrows, are visible in the bronchial lumen (L), and *P. aeruginosa* macrocolonies can be observed into the beads. b, d, and f are enlargements of the boxed areas in a, c, and e. (Scale bars: 200 μm.)

a Victor^{3}V plate reader (Perkin-Elmer) as described (30). Pyoverdine fluorescence was assessed as emission at 460 nm after excitation at 405 nm (33). Luminescence and fluorescence values were normalized by the cell density and subtracted of untreated PAO1*pvdA* P*pvdE*::*lux* values. Criteria used for the selection of hit compounds were (*i*) ≥50% inhibition of normalized bioluminescence emission and/or pyoverdine-specific fluorescence and (*ii*) ≤20% alteration of growth relative to the untreated control. Criterion *ii* was aimed at avoiding any unspecific effect of altered growth on bioluminescence and/or pyoverdine production. For promising compounds, pyoverdine was also quantified in diluted cell-free culture supernatants (see below).

Miscellaneous Assays. Pyoverdine levels in culture supernatants were measured as A_{405} in 100 mM Tris·HCl (pH 8) and normalized by the cell density (A_{600}) of the bacterial cultures (33). Exotoxin A was detected in 10 μL culture supernatants by SDS/PAGE followed by Western blot with a polyclonal antiexotoxin A antibody (Sigma-Aldrich). PrpL and β-gal enzymatic activities were determined as previously described (34, 35). Pyochelin was isolated by ethyl acetate extraction of acidified culture supernatants, resuspended in methanol, and resolved by TLC on silica gel (36). Pyochelin was detected by spraying with 0.1 M $FeCl_3$ and quantified by A_{520} readings of ferripyochelin eluted with methanol from TLC plates (37). Anti-PvdA Western blot analysis was performed using the 3H6D12 monoclonal antibody as described (38).

Mouse Model of *P. aeruginosa* Lung Infection. C57BL/6 male mice (Charles River) were infected intratracheally with 10^6 *P. aeruginosa* viable cells embedded in agar beads as described (15), except for the use of TSBD agar instead of TSB agar for beads preparation. Mice were treated two times daily (starting 2 h postinfection) by i.p. administration of 50 μL 50 mM 5-FC in saline or 50 μL saline as control. Two 50-μL doses/d of 50 mM 5-FC correspond to a daily dosage of about 30 mg/kg (mouse weight was 20–22 g), which is within or below the dosage range recommended for humans; the dosage ranges for humans are 25–100 mg/kg per day for infants (<1 mo) and 50–150 mg/kg per day for children and adults (http://www.drugs.com/dosage/flucytosine.html). Mortality was monitored for a 6-d time period. Surviving mice were killed at day 6 postinfection, and lungs were excised, homogenized, and plated to determine the number of viable cells per lung. Four additional mice per group were infected with *P. aeruginosa* PAO1 or PAO1*pvdS* and treated with 5-FC or saline as described above, and they were euthanized at day 2 postinfection for lung histology. Lungs were removed *en bloc*, fixed in 4% (wt/vol) paraformaldehyde/PBS, and processed for paraffin

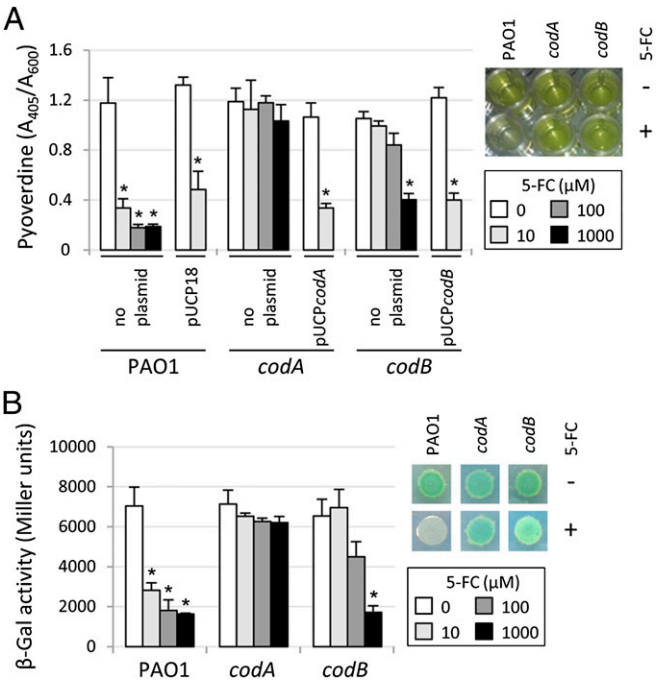

Fig. 5. Enzymatic conversion to 5-fluorouracil is essential for the anti-pyoverdine activity of 5-FC. Effect of 5-FC (0–1,000 μM) on (*A*) pyoverdine production by PAO1, PAO1*codA* (*codA*), and PAO1*codB* (*codB*) containing or not containing the plasmid pUCP18, pUCP*codA*, or pUCP*codB* as indicated and (*B*) β-gal activity by the same strains containing the P*pvdS::lacZ* fusion construct grown for 14 h in TSBD. *Insets* show (*A*) pyoverdine production in M9 medium (green fluorescence) and (*B*) β-gal activity (blue color) in M9 agar plates containing the chromogenic substrate X-gal after 14 h of growth in the absence (–) or presence (+) of 100 μM 5-FC. *Statistically significant differences ($P < 0.01$, ANOVA) with respect to the corresponding untreated controls.

embedding. Longitudinal sections of 5 μm taken at regular intervals were obtained using a microtome from the middle of the five lung lobes and stained with H&E. Animal studies were conducted according to protocols approved by the San Raffaele Scientific Institute Institutional Animal Care and Use Committee.

Statistical Analysis. Statistical analysis was performed with the software GraphPad Instat using one-way ANOVA. Survival curves for the mouse infection assay were analyzed using the log-rank Mantel–Cox test.

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