Inhibitors of bacterial virulence identified in a surrogate host model

Mohammed Benghezal,1* Eric Adam,1† Aurore Lucas,1 Christine Burn,1 Michael G. Orchard,1 Christine Deuschel,1 Emilio Valentino,1 Stéphanie Braillard,1 Jean-Pierre Paccaud1 and Pierre Cosson2
1Athelas SA, Chemin des Aulx 18, CH-1228 Plan-les-Ouates, Switzerland.
2Centre Médical Universitaire, Département de Physiologie Cellulaire et Métabolisme, rue Michel Servet 1, CH1211 Genève 4, Switzerland.

Summary

Antibiotic resistance continues to reduce the number of available antibiotics, increasing the need for novel antibacterial drugs. Since the seminal work of Sir Alexander Fleming, antibiotic identification has been based exclusively on the inhibition of bacterial growth in vitro. Recently, inhibitors of bacterial virulence which interfere with bacterial pathogenesis mechanisms have been proposed as an alternative to antibiotics, and a few were discovered using assays targeting specific virulence mechanisms. Here we designed a simple surrogate host model for the measurement of virulence and systematic discovery of anti-virulence molecules, based on the interaction of *Tetrahymena pyriformis* and *Klebsiella pneumoniae* cells. We screened a library of small molecules and identified several inhibitors of virulence. In a mouse pneumonia model we confirmed that an anti-virulence molecule displayed antibacterial activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, by reducing dramatically the bacterial load in the lungs. This molecule did not inhibit bacterial growth in vitro but prevented biosynthesis of the *Klebsiella* capsule and lipopolysaccharides, a key requirement for virulence. Our results demonstrate that anti-virulence molecules represent an alternative to antibiotics and those can be discovered using non-animal host models.

Introduction

Virulence is defined as the ability of a pathogen to cause damage to a host organism (Casadevall and Pirofski, 1999). Accordingly, bacterial virulence genes are essential to mount a harmful infection, but they are dispensable for growth of bacteria in vitro. A combination of bacterial genetics and animal experiments has led to the identification of many bacterial virulence genes involved, for example, in metabolic pathways, resistance to the host immune system, secretion of toxins, or cell–cell signalling (Saenz and Dehio, 2005). Distinct from conventional antibiotics, inhibitors of virulence targeting for example two-component signal systems (Roychoudhury et al., 1993; Hentzer et al., 2003; Giacometti et al., 2005; Wright et al., 2005), type III secretion (Kauppi et al., 2003; Nordfelth et al., 2005), protease activity (Panchal et al., 2004; Turk et al., 2004) or the transcriptional regulator ToxT (Hung et al., 2005) have been identified and several of them were reported to interfere with bacterial pathogenesis in animal models of infection (Hentzer et al., 2003; Giacometti et al., 2005; Hung et al., 2005; Wright et al., 2005). Hence, anti-virulence molecules may represent novel and effective antibacterial drugs. However, their discovery has been hampered mainly because of the difficulties in measuring and quantifying bacterial virulence. Consequently, anti-virulence molecules have seldom been systematically searched for and they may represent an untapped source of antibacterial compounds (Alksne and Projan, 2000). Recently, a number of alternative non-mammalian host models have been developed to study bacterial virulence (Pradel and Ewbank, 2004), allowing an easier assessment of bacterial virulence.

*Klebsiella pneumoniae* is a major agent of nosocomial infections in hospital settings and is responsible for life-threatening pneumonia and urinary tract infections (Podschun and Ullmann, 1998). The worldwide occurrence of multiresistant strains, including several outbreaks of strains expressing extended-spectrum β-lactamases, highlights the need for novel antibacterial drugs (Bratu et al., 2005). Previously, a host model system based on the amoeba *Dictyostelium discoideum* was successfully used to identify *K. pneumoniae* virulence genes (Benghezal et al., 2006). Unfortunately, this system was not amenable to high-throughput screening. In the present study, a similar host model system was designed using
the ciliate *Tetrahymena pyriformis* and optimized for high-throughput screening to identify inhibitors of *K. pneumoniae* virulence.

**Results and discussion**

*Tetrahymena pyriformis* was previously exploited in a semiquantitative bio-safety test to assess the presence of toxic bacterial metabolites (Schlimme *et al.*, 1999). Here, *T. pyriformis* was used to measure the virulence of the clinical strain of *K. pneumoniae*, Kp52145. Wild-type *K. pneumoniae* grew in the presence of *T. pyriformis* when the two organisms were cocultured (Fig. 1A), showing that it could overcome predation by *Tetrahymena*. In the presence of *Tetrahymena*, virulent bacteria grew almost as well (20% less bacterial growth), than in their absence. The isogenic *wbbM* mutant defective in lipopolysaccharide synthesis (Benghezal *et al.*, 2006) grew as well as the wild-type strain in the absence of *Tetrahymena*, but in the presence of *Tetrahymena* it was cleared from the culture after a few hours (Fig. 1A).

Two other *Klebsiella* mutants with cell surface defects, *waaQ* (Benghezal *et al.*, 2006) and *rmpA* (Arakawa *et al.*, 1991), gave similar results to *wbbM* (data not shown). The fact that *wbbM*, *waaQ* and *rmpA* *Klebsiella* mutants are non-virulent in mice (Benghezal *et al.*, 2006) suggests that the *Tetrahymena–Klebsiella* assay provides a relevant measure of the virulence of *K. pneumoniae* towards mammals.

Ninety-eight positive hits were obtained when a library of 15,000 synthetic chemical compounds was screened in the *Tetrahymena–Klebsiella* assay. Two of these compounds, D1 [3-(allylsulfanyl)-6-methyl-1,2,4-triazine-5-ol] and D41 [3-(allylsulfanyl)-6-(thiophen-2-yl)-1,2,4-triazine-5-ol], were structurally related triazines (Fig. 1B).

As a control, an inactive triazine derivative D0 [3-(methylsulfanyl)-6-methyl-1,2,4-triazine-5-amine] was synthesized by replacing a hydroxyl with an amino group (Fig. 1B and Appendix S1 in Supplementary material). The growth of *Klebsiella* cells was identical either with (unfilled square) or without (square) D1 at 20 μM. Coculture of *Tetrahymena* and *Klebsiella* (square) or D1-treated *Klebsiella* (unfilled square). D1-treated cells were unable to overcome *Tetrahymena* predation. Results represent the average of 15 trials and standard deviation is indicated by the error bars.
Prophylactic effect of D1 and D41 on experimental pneumonia

In a mouse pneumonia model of infection, treated or not with D1 or D41 were similar, indicating that these drugs did not affect dramatically the length of the lipopolysaccharides. However, D1 or D41 treatment led to a 10-fold reduction in the amount of lipopolysaccharides, as 10 times more bacterial extract was required to give the same signal on SDS-PAGE (Fig. 2D). A quantification of the capsule using K2-antisera showed that D1 and D41 induced a five- to sixfold reduction in the capsule amount (Table 3). Altogether, these results suggest that triazine derivatives inhibit Klebsiella virulence by interfering with the biosynthesis of capsule and of lipopolysaccharides.

Prior to testing the in vivo efficacy of D1 and D41, their cytotoxicity was analysed. D41 was toxic to cells at a concentration of 50 μM and above (data not shown) and was not investigated further. On the contrary, D1 did not show any cytotoxicity up to the highest concentration tested (200 μM). When D1 was administered intraperitoneally to mice (60 mg kg⁻¹), pharmacokinetics showed that its concentration in plasma reached 50–70 μg ml⁻¹ (271–380 μM) after 10–60 min, and then decreased to 10 μg ml⁻¹ (53 μM) after 6 h. The efficacy of D1 compound was then evaluated in a mouse pneumonia model of infection. Mice were challenged by intranasal instillation of K. pneumoniae and D1 treatment was initiated simultaneously. The bacterial load in the lungs of infected mice treated with different concentrations of D1 was measured 24 h later. In D1-treated mice, the bacterial load was significantly reduced in a dose-dependent manner compared with untreated mice (Fig. 3A). At high concentration, D1 decreased the bacterial load to the same extent as the antibiotic cefotaxime, i.e. below the initial infective dose (Fig. 3A). The reduction of the bacterial load could not be attributed to antibiotic action of D1 because the peak plasma concentration of D1 was sevenfold lower than its MIC. To test whether D1 could also inhibit the virulence of another Gram-negative bacterium, performed. First, the length of the lipopolysaccharides was tested by SDS-PAGE. As shown in Fig. 2D, the lipopolysaccharides patterns of K. pneumoniae cells treated or not with D1 or D41 were similar, indicating that these drugs did not affect dramatically the length of the lipopolysaccharides. However, D1 or D41 treatment led to a 10-fold reduction in the amount of lipopolysaccharides, as 10 times more bacterial extract was required to give the same signal on SDS-PAGE (Fig. 2D). A quantification of the capsule using K2-antisera showed that D1 and D41 induced a five- to sixfold reduction in the capsule amount (Table 3). Altogether, these results suggest that triazine derivatives inhibit Klebsiella virulence by interfering with the biosynthesis of capsule and of lipopolysaccharides.

### Table 1. Triazine derivatives anti-virulence (IC₅₀) and antibiotic (MIC) activities.

| Compound | IC₅₀ (μM) | MIC (mM) | MIC (μg ml⁻¹) |
|----------|----------|----------|--------------|
| D1       | 1.64 ± 0.19 | 2.8 | 512 |
| D41      | 0.49 ± 0.22 | > 2.4 | > 512 |
| Cefotaxime | NA  | 6.5 × 10⁻³ | 0.031 |

Anti-virulence activities (as described in the Experimental procedures) and IC₅₀ in the Tetrahymena–Klebsiella assay were determined from concentration–response curves derived with data average from four trials using PRISM software. Standard deviation is indicated. Antibiotic activities were performed as described in the Experimental procedures and MICs were determined.

MIC values obtained [512 μg ml⁻¹ (2.8 mM) for D1 and > 512 μg ml⁻¹ (2.4 mM) for D41] compared with the MIC of the cefotaxime antibiotic [31 ng ml⁻¹ (65 nM)], we concluded that neither the D1 nor the D41 compounds displayed significant antibiotic activity (Table 1). K. pneumoniae grown in the presence of D1 or D41, however, were eradicated by Tetrahymena (Fig. 1C for D1 and data not shown for D41), demonstrating that Klebsiella virulence was affected. The anti-virulence activity of these compounds was further quantified using a dose–response to determine their IC₅₀ values. Table 1 shows that D compounds displayed IC₅₀ values of 1.64 ± 0.19 μM and 0.49 ± 0.22 μM for D1 and D41 respectively. The D0 compound was inactive at all concentrations.

Interestingly, Klebsiella colonies lost their mucoid phenotype when Klebsiella were grown on agar Petri dishes containing either D1 or D41 but not the inactive D0 compound. These changes were quantified by evaluating bacterial mucoidy using the string test (Fang et al., 2004) (Table 2). D41 suppressed the mucoid phenotype at a concentration of 3 μM and higher, whereas D1 required a concentration of 10 μM (Table 2). No effect was seen following treatment with the inactive D0 compound (Table 2). These results correlated with the compounds’ anti-virulence activities (D41 > D1, D0 inactive) and suggested a selective mode of action for D compounds. Ultrastructural analysis indeed revealed that the bacterial capsule was affected by D1 and D41. Whereas a prominent capsule surrounded untreated bacteria (Fig. 2A), growth in the presence of D1 or D41 strongly reduced the thickness of the capsule and altered its morphology (Fig. 2B, D1 and D41 data not shown). The inactive D0 analogue did not affect the morphology of Klebsiella (data not shown). A similar altered capsule phenotype was observed for the wbbM mutant (Fig. 2C). D1 and D41 therefore interfere with Klebsiella mucoidy and capsule biosynthesis, leading to a phenotype similar to that of wbbM mutant bacteria. To further characterize the mode of action of D1 and D41 and to test whether these drugs affect either the biosynthesis of the capsule, the lipopolysaccharides or both, additional experiments were performed.
mice were challenged with *Pseudomonas aeruginosa* in a neutropenic mouse pulmonary model of infection. A dose-dependent reduction in bacterial load in the lungs was observed in D1-treated animals, 48 h after infection (Fig. 3B). The highest dose of D1 (135 mg kg⁻¹ day⁻¹) brought the bacterial load back to the initial infective dose (Fig. 3B). The decrease of the bacterial load could not be explained by antibiotic action of D1, because it had no inhibitory activity on *P. aeruginosa* growth in a standard MIC assay, even at the highest dose tested (2.8 mM). Hence, these results indicated that D1 displays no *in vitro* antibiotic activity but inhibits both *K. pneumoniae* and *P. aeruginosa* virulence in infected animals. As the mice were neutropenic in the *Pseudomonas* infection model and as D1 affected the bacterial cell surface (at least of *Klebsiella*), it is unlikely that D1 acts by stimulating the innate immune system or neutrophils in particular.

In summary, development of a surrogate host system enabled the measurement of *K. pneumoniae* virulence and the high-throughput identification of anti-virulence molecules. A triazine derivative displayed *in vivo* antibacterial activity against two Gram-negative bacteria species, demonstrating conservation of the virulence mechanism targeted by the small molecule. The mode of action of triazine derivatives involved a dramatic reduction in the amounts of capsule and lipopolysaccharides. As these compounds are also active against *Pseudomonas aeruginosa* and *K. pneumoniae* it is tempting to postulate that a transport/export machinery for lipopolysaccharides and/or the capsule is the target of these drugs. Our approach

**Table 3.** *Klebsiella pneumoniae* capsule quantification using K2-antiserum ELISA with whole bacteria cells.

| Treatment | Absorption at 405 nm |
|-----------|----------------------|
| None      | 1.7 ± 0.24           |
| DMSO      | 1.8 ± 0.19           |
| D1        | 0.3 ± 0.05           |
| D41       | 0.4 ± 0.04           |
opens the door to a systematic discovery of anti-virulence molecules and may lead to novel forms of therapy or combined anti-virulence/antibiotic therapy, which are badly needed to fight antibiotic-resistant bacteria and chronic infections in patients such as cystic fibrosis sufferers.

**Experimental procedures**

*Paramecium tetraurelia*–*Klebsiella pneumoniae* virulence assay was performed by coculture of the two organisms: Kp52145 bacteria or the indicated mutant were grown 18 h in Luria–Bertani (LB) to an optical density (600 nm) of about 2.5, diluted in 20 vols of SM medium (Charette and Cosson, 2004) mixed with 1 vol. of *Tetrahymena* (100 000 ml\(^{-1}\) in SM). Fifty microlitres of this cell suspension was transferred in each well of a 384 well plate using the mFill – Microplate Reagent Dispenser (Bio-Tek, USA). Compounds were added from a library of small molecules in DMSO at the indicated concentration (10 µM final concentration after a 500-fold dilution, unless otherwise indicated) using a 384 well pin-replicator (250393, Nunc). Plates were incubated overnight at 35°C. The absorbance of the suspension was measured at 450 nm every hour to follow the growth of bacteria. *Tetrahymena* cells only accounted for a negligible absorbance. During screenings only the value at the 20 h time point was measured. Working with small volumes required the use of a water-saturated atmosphere incubator to avoid evaporation during the incubation period. To assess the homogeneity of the assay within the plates, quality control plates were performed using DMSO only. Apart from some border effect (first and last rows and columns), the assay was found highly homogeneous throughout the plate. The day-to-day reproducibility of the assay was of more than 70%, meaning that more than 70% of the hits could be reconfirmed. Average reproducibility of high-throughput assays are usually of 50%. As the assay starts upon mixing the two types of cells, batches of 24 plates were performed to avoid any delay due to pin-replication of the compounds before incubation at 35°C. The percentage of anti-virulence activity was calculated using the formula activity = 100(1 – (ODt/ODref)) × ODref, ODt is the 450 nm optical density of the test well in presence of the compound and ODref in absence. IC\(_{50}\) values were determined from concentration–response curves derived with data average from four independent trials using PRISM software. It was found that the dose–response curves were almost superimposed, ruling out the need of more replicates to determine the IC\(_{50}\). Also, as mentioned above, the location in the 384 well plate was not important and provided the first and last rows or columns were avoided dose–response experiments could be performed throughout the plate. The antibiotic activity, i.e. the MIC of each compound was determined according to NCCLS standards (Ferraro, 2003). To evaluate compounds’ cytotoxicity, HepG2 cells were seeded in 96 well tissue culture plates (90 µl well\(^{-1}\)) and incubated at 37°C overnight. Ten microlitres of medium or of compound were added to each well, and the cells were incubated 48 h at 37°C. ATP levels were then measured using the ATP-lite kit (Perkins-Elmer Life Science), following manufacturer’s instructions. Pharmacokinetics of D1 was analysed following a single intraperitoneal injection of 150 µl of D1 at 60 mg kg\(^{-1}\) in PBS/DMSO. Blood was collected by retro-orbital bleeding from a group of nine mice at time points 5, 10, 15, 30, 60, 360 min post injection. Plasma concentrations of D1 were determined by LC-MS-MS.

For the capsule quantification and analysis of lipopolysaccharides, cells were grown overnight at 37°C on Tryptone Soya Agar (CM0131, Oxoid) Petri dishes, complemented with either D1, D41, DMSO or non-complemented. Analysis of the length of the lipopolysaccharides was performed by phenol extraction and SDS-PAGE as previously described (Izquierdo et al., 2002). Whole cells capsule quantification was performed using an ELISA and anti-K2 antiserum (Tomas et al., 1988).
A previously described mouse pneumonia model (Benghezal et al., 2006) was used to evaluate the effect of compounds on bacterial pathogenicity. This protocol was approved by the Swiss federal veterinary office (authorization 31.1.1083 2172 III). Briefly, 8-week-old female BALB/cJ mice were anaesthetized and infected by intraperitoneal instillation of 10^6 Klebsiella pneumoniae bacteria in 20 μL. Mice were sacrificed 24 h after infection, the lungs were collected, homogenized, and serial dilution plated on LB plates to determine the colony forming units.

For Pseudomonas infections the same procedure was followed, but neutropenic Swiss-albino mice were infected with 6 x 10^6 bacteria. Neutropenia was obtained by intraperitoneal injections of 75 mg kg^-1 of cyclophosphamide on days –4 and 0. Mice were sacrificed 48 h after infection. Treatment with cefotaxime at 150 mg kg^-1 was started, but neutropenic Swiss-albino mice were infected with 10^6 Pseudomonas aeruginosa strain causing primary liver abscess and septic metastatic complications. J Exp Med 199: 669–705. Ferraro, M.J. (2003) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Approved standard. M7-A6, 5th edn. Villanovan, PA: National Committee for Clinical Laboratory Standards (NCCLS).

To analyse the morphology of bacteria, they were fixed successively in phosphate buffer containing 2% glutaraldehyde (16 h, room temperature), then in 2% osmium tetroxide (4°C, 1 h). Fixed cells were dehydrated and embedded in Epon resin and processed for conventional electron microscopy as described previously (Orci et al., 1973). Grids were examined in a Tecnai transmission electron microscope (FEI, Eindhoven, the Netherlands).

Acknowledgements

This work was funded by a grant from the Swiss National Science Foundation (to P.C.). We thank the Pôle Facultaire de Microscopie Ultrastructurale (PFMU) at the University of Geneva Medical School for access to electron microscopy equipment. We thank Leigh Gebbie for critical reading of the manuscript. The first two authors contributed equally to this work.

References

Alksne, L.E., and Projan, S.J. (2000) Bacterial virulence as a target for antimicrobial chemotherapy. Curr Opin Biotechnol 11: 625–636.

Arakawa, Y., Ohta, M., Wacharotayankun, R., Mori, M., Kido, N., Ito, H., et al. (1991) Biosynthesis of Klebsiella K2 capsular polysaccharide in Escherichia coli HB101 requires the functions of rpmA and the chromosomal cps gene cluster of the virulent strain Klebsiella pneumoniae Chedid (O1:K2). Infect Immun 59: 2043–2050.

Benghezal, M., Fauvarque, M.O., Tournebize, R., Froquet, R., Marchetti, A., Bergeret, E., et al. (2006) Specific host genes required for the killing of Klebsiella bacteria by phagocytes. Cell Microbiol 8: 139–148.

Bratu, S., Mooty, M., Nichani, S., Landman, D., Gullans, C., Pettinato, B., et al. (2005) Emergence of KPC-possessing Klebsiella pneumoniae in Brooklyn, New York: epidemiology and recommendations for detection. Antimicrob Agents Chemother 49: 3018–3020.
Roychoudhury, S., Zielinski, N.A., Ninfa, A.J., Allen, N.E., Jungheim, L.N., Nicas, T.I., and Chakrabarty, A.M. (1993) Inhibitors of two-component signal transduction systems: inhibition of alginate gene activation in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **90**: 965–969.

Saenz, H.L., and Dehio, C. (2005) Signature-tagged mutagenesis: technical advances in a negative selection method for virulence gene identification. *Curr Opin Microbiol* **8**: 612–619.

Schlimme, W., Marchiani, M., Hanselmann, K., and Jenni, B. (1999) BACTOX, a rapid bioassay that uses protozoa to assess the toxicity of bacteria. *Appl Environ Microbiol* **65**: 2754–2757.

Tomas, J.M., Camprubi, S., and Williams, P. (1988) Surface exposure of the O-antigen in *Klebsiella pneumoniae* O1:K1 serotype strains. *Microb Pathog***5**: 141–147.

Turk, B.E., Wong, T.Y., Schwarzenbacher, R., Jarrell, E.T., Leplla, S.H., Collier, R.J., *et al.* (2004) The structural basis for substrate and inhibitor selectivity of the anthrax lethal factor. *Nat Struct Mol Biol* **11**: 60–66.

Wright, J.S., III, Jin, R., and Novick, R.P. (2005) Transient interference with staphylococcal quorum sensing blocks abscess formation. *Proc Natl Acad Sci USA* **102**: 1691–1696.

**Supplementary material**

The following supplementary material is available for this article online:

**Appendix S1.** Synthesis of compounds.

This material is available as part of the online article from [http://www.blackwell-synergy.com](http://www.blackwell-synergy.com)