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Amoebae-Based Screening Reveals a Novel Family of Compounds Restricting Intracellular *Legionella pneumophila*

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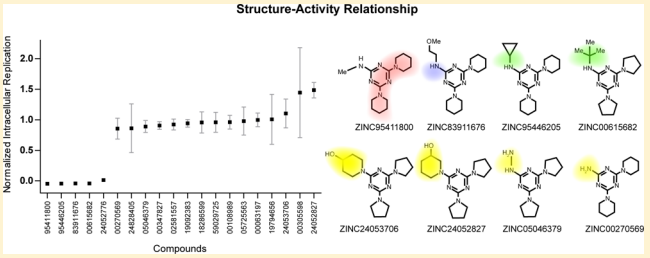
Supporting Information

ABSTRACT: The causative agent of Legionnaires' disease, *Legionella pneumophila*, grows in environmental amoebae and mammalian macrophages within a distinct compartment, the '*Legionella*-containing vacuole' (LCV). Intracellular bacteria are protected from many antibiotics, and thus are notoriously difficult to eradicate. To identify novel compounds that restrict intracellular bacterial replication, we previously developed an assay based on a coculture of amoebae and GFP-producing *L. pneumophila*. This assay was used to screen a pathway-based, highly diverse chemical library, referred to as the Sinergia library. In this work, we chose to focus on a group of 11 hit compounds, the majority of which originated from the query molecule CN585, a compound that targets the protein phosphatase calcineurin. Further studies on 78 related compound variants revealed crucial structural attributes, namely a triple-ring scaffold with a central triazine moiety, substituted in positions 3 and 5 by two piperidine or pyrrolidine rings, and in position 1 by an amine group bearing a single aliphatic chain moiety. The most effective compound, ZINC00615682, inhibited intracellular replication of *L. pneumophila* with an IC_{50} of approximately 20 nM in *Acanthamoeba castellanii* and slightly less efficiently in *Dictyostelium discoideum* or macrophages. Pharmacological and genetic attempts to implicate calcineurin in the intracellular replication of *L. pneumophila* failed. Taken together, these results show that the amoebae-based screen and structure−activity relationship analysis is suitable for the identification of novel inhibitors of the intracellular replication of *L. pneumophila*. The most potent compound identified in this study targets (an) as yet unidentified host factor(s).



KEYWORDS: *amoeba, antibiotics, antivirulence, calcineurin, intracellular replication, Legionella, macrophage, pathogen vacuole, screen, structure−activity relationship (SAR), type IV secretion*

Legionella pneumophila is a ubiquitous environmental bacterium found within a wide range of both natural and artificial freshwater sources.[1,2] *L. pneumophila* resists phagocytosis and degradation by predatory freshwater amoebae through the production of numerous "effector" proteins, which are injected into amoebae via a type IV secretion system (T4SS) and lead to the formation of the replication-permissive "*Legionella*-containing vacuole" (LCV).[3−5] As the majority of known effector protein targets are evolutionarily conserved between both protozoa and alveolar macrophages,[6] *L. pneumophila* can also infect human lungs, leading to a potentially fatal pneumonia known as Legionnaires' disease.[1,2]

The mortality rate for Legionnaires' disease ranges between 2 to 5%, depending on the outbreak.[7] Beyond its importance as a human pathogen, *L. pneumophila* also subverts host processes similar to other vacuolar bacteria such as *Mycobacterium tuberculosis*.[8] Thus, the development of alternative, more effective methods of treating *Legionella* infection may also lead to treatments for *Mycobacterium* infections, which themselves kill millions each year.[9] As an intracellular pathogen, *L. pneumophila* is protected from a number of common antibacterial treatments, exhibits increased resistance to antibiotics,[10] and shows significantly higher antibiotic resistance following release from phagocytic cells compared to broth-grown counterparts.[11] Beyond this, chemical decontamination of man-made water systems often fails to kill *L. pneumophila* bacteria that reside within free-living amoebae.[12,13]

The process of developing novel antibiotics usually involves compound screens, in which large numbers of chemicals are

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tested for an effect on specific bacterial targets. Unfortunately, the use of high-throughput screening in antibacterial development remains contentious: large-scale efforts to develop new broad-spectrum antibiotics met with very limited success, due to a combination of issues with cell permeability and target specificity.[14] Another disadvantage of antibiotic compounds is that selection pressure for the development of drug-resistance against these compounds is extremely high, essentially forcing companies to develop drugs that will rarely be used.[15] As a result of these factors, the concept of "antivirulence" compounds has emerged as an alternate, promising strategy. These compounds are aimed at specific pathogenicity mechanisms such as binding to target cells, uptake processes, signaling pathways, and the secretion of toxins, as opposed to directly targeting bacterial replication.[16−18]

The development of antivirulence compounds has begun, as seen for example in the injectable antibody Urtoxazumab, which binds to and neutralizes Shiga-like toxin 2 and is currently in clinical trials.[19] Other examples include salicylidine derivatives, several of which have been shown to inhibit the type III secretion system (T3SS) of *Salmonella enterica* Typhimurium,[20] and Virstatin, an inhibitor of the ToxT transcription regulator, which prevents the expression of several *Vibrio cholerae* virulence factors.[21] More recently, nonantibiotic inhibitors of the *Pseudomonas aeruginosa* MvfR-regulated quorum sensing system have been identified, which bind to MvfR, block the synthesis of 4-hydroxy-2-alkylquinolone-based signaling molecules, and thus inhibit acute bacterial pathogenesis and persistence.[22]

Targeting virulence-associated pathways is a complex undertaking, especially when compared to the simplicity of searching for compounds which prevent replication. This approach requires either knowledge of the pathogenic process to be targeted (which can then be assessed via an in vitro system) or a broader screen to analyze the interaction of the bacterium with its host cell. Previous work in our group had led to the development of a novel screening system utilizing *L. pneumophila* replication within a natural prokaryotic host, *Acanthamoeba castellanii*.[23] We used this system to screen a pathway-based highly diverse chemical library, called the Sinergia library, for chemicals which successfully inhibited intracellular replication. Here, we report on detailed "hit finding and chemical space exploration" studies performed on hits discovered in this process, resulting in the identification and characterization of a family of advanced hits compounds derived from inhibitors of the calcineurin protein phosphatase enzyme.

RESULTS

Screening for Inhibitors of *Legionella* Replication. To identify inhibitors of *L. pneumophila* intracellular replication, the Sinergia library was established by utilizing virtual screening techniques based on "query compounds" known to target certain pathogen pathways.[24] This targeted, highly diverse library contains 1278 compounds that are grouped and referred to according to their query compound (e.g., targeting the protein phosphatase calcineurin or the autophagy pathway) (manuscript related to the creation and characterization of the chemical library in preparation).

The Sinergia library was screened for activity against the intracellular replication of *L. pneumophila* in *A. castellanii* using a previously described assay.[23] The compounds were added at a concentration of 10 or 30 μM, and *L. pneumophila* replication was normalized to the range between 0 (kanamycin treatment) and 1 (vehicle controls). A replication level of 0.5 (i.e., 50% inhibition) was chosen as the cutoff value to determine any hits of interest. Using a concentration of 30 μM, 39 hits were initially identified (Figure 1A, Table S1). To control for compound cytotoxicity, we used Alamar Blue viability assays and tested the response of *A. castellanii* to these compounds. Compared to DMSO controls, 9 of the 39 most effective compounds caused a significant change in cell viability (Figure 1A). Taking into consideration the compounds below and very close to the cutoff, 32 noncytotoxic compounds were considered further.

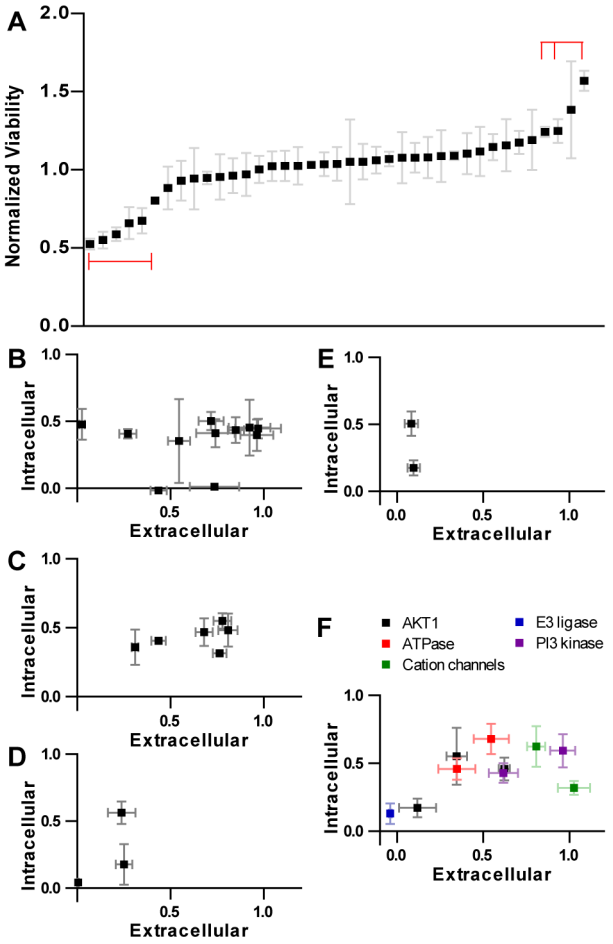

Figure 1. Effect of Sinergia library hit compounds on extra- and intracellular replication of *L. pneumophila*. (A) Toxicity of 39 hit compounds against *A. castellanii* as determined by Alamar Blue assay. Red bars indicate compounds causing statistically significant altered viability (*t* test, $p < 0.05$). (B−F) Effect of each Sinergia library hit compound on *L. pneumophila* replication either in AYE medium (extracellular) or in *A. castellanii* amoebae (intracellular). Hits were divided according to proposed target pathways: calcineurin (B), autophagy (C), tryptophan biosynthesis (D), proteasome inducers (E), and others (F). Graphs (A−F) indicate the mean and standard deviation (SD) of at least triplicate independent assays, with replication levels normalized to vehicle controls (<0.1% DMSO).

The hit compounds were then assessed for their ability to inhibit the intracellular and extracellular growth (in broth alone) of *L. pneumophila*, and the correlation between intra- and extracellular growth was visualized as a scatter plot (Figure 1). Hits chemically derived from compounds involved in host cell pathways such as calcineurin (Figure 1B), autophagy

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(Figure 1C), or other processes (Figure 1F) had predominantly minor effects on extracellular bacterial growth, whereas as expected, groups of hits chemically derived from compounds targeting bacterial systems, such as tryptophan biosynthesis (Figure 1D) or bacterial proteasomes (Figure 1E), tended to show high degrees of efficacy against extracellular *L. pneumophila*. As the calcineurin pathway compound group comprised both the majority of hits and appeared to act in a manner supporting an antivirulence effect, we elected to further characterize this group. However, the annotation of hits to calcineurin does not imply per se that the compounds directly affect this pathway (see below).

Characterization of Hits Chemically Derived from Compounds Targeting the Calcineurin Pathway. Following the initial screen, we retested 12 calcineurin compounds (11 hits and 1 borderline case) for efficacy based on the structural background. The compounds from the calcineurin pathway were derived from two distinct query molecules, the pyrazolopyrimidine moiety and CN585 compound (Figure 2A). A pyrazolopyrimidine derivative, compound NCI3, indirectly inhibits calcineurin overproduction,[25] and CN585 is a rationally designed derivative of 6-(3,4-dichloro-phenyl)-4-(*N*,*N*-dimethylaminoethylthio)-2-phenyl-pyrimidine, which has been reported to reversibly inhibit calcineurin activity with an IC_{50} of 3.8 μM.[26] The majority of effective hits (8 of 12) originated from the CN585 query molecule. However, among the hits identified, the degree of replication inhibition did not correlate with the underlying scaffold (pyrazolopyrimidine or CN585) (Figure 2B, Table S2).

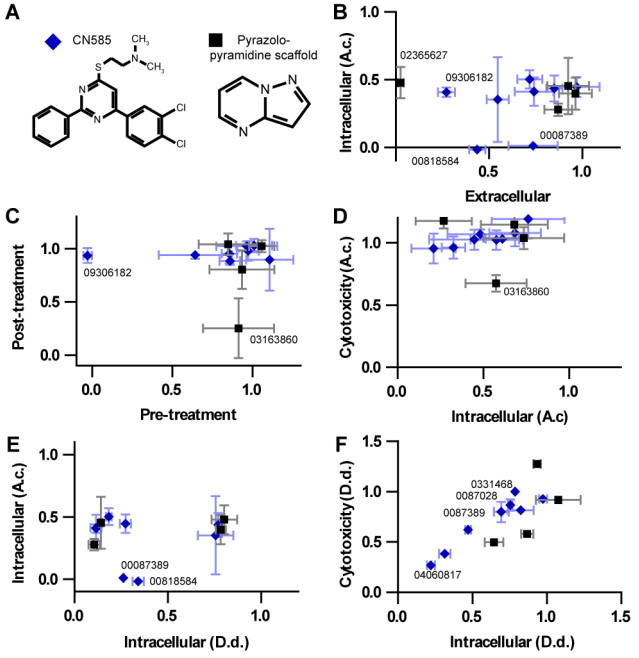

Figure 2. Characterization of the hits from the calcineurin pathway. (A) Hits within the calcineurin group were derived from two chemical queries, CN585 (blue diamonds) and pyrazolopyrimidine (black squares). (B) Comparison of compound efficacy (30 μM) against intracellular (*A. castellanii*) and extracellular *L. pneumophila*, segregated by the originating scaffold. (C) Comparison of compound efficacy (30 μM) when treating *A. castellanii* 30 min before or after infection with *L. pneumophila*. The majority of compounds needed to be present at the same time as infection occurs. (D) Comparison of efficacy versus toxicity (30 μM) in *A. castellanii* indicates that only one compound, ZINC03163860, was toxic to amoebae over the time scale of *L. pneumophila* infection. (E) Comparison of compound efficacy (30 μM) against intracellular replication in *A. castellanii* and *D. discoideum* reveals generally reduced efficacy in treating *D. discoideum*. (F) Comparison of efficacy versus toxicity (7.5 μM) in *D. discoideum* indicates that, unlike in *A. castellanii*, efficacy correlates with cytotoxicity. All graphs (B–F) indicate the mean and SD of at least three independent experiments.

In an attempt to further investigate the mode of action of these compounds, we examined the time dependence of compound treatment. The prior process of screening had involved the simultaneous addition of compounds and *L. pneumophila*; additional testing examined the effect of pretreating the amoebae with compound (for 30 min, infection then proceeded in media alone) and post-treatment (infection in media only, followed by compound addition after 30 min). The majority of compounds were effective only when present at the time of infection (Figure 2C). The sole compound active in post-treatment, ZINC03163860, was also observed to be slightly cytotoxic following further testing (Figure 2D), which may suggest off-target effects. A single compound, ZINC09306182, was an effective inhibitor of bacterial replication following pretreatment. The reason behind this is unclear, although it may relate to the significant difference in chemical structure compared to the other hits from the calcineurin pathway (see below).

In order to confirm the efficacy of these compounds, we cross-screened for those which inhibited the intracellular replication of *L. pneumophila* within the social amoeba *D. discoideum* (Figure 2E). The majority of compounds effective in *A. castellanii* also prevented replication in *D. discoideum*; however, a group of four compounds were relatively ineffective. To correlate the observed defects in the intracellular replication of *L. pneumophila* with altered vesicle trafficking, LCV formation, or pathogen vacuole composition, we attempted to determine alterations in the cellular localization of various small GTPases of the Rab family (Rab1, Rab5a, Rab7, Rab8a, Rab11b, and Rab14) following treatment with ZINC09306182 and *L. pneumophila* infection (Figure S1). However, compared to untreated cells, no significant differences in Rab localization could be observed in *D. discoideum* infected with *L. pneumophila* wild-type or a Δ*icmT* mutant strain.

Plotting the inhibition of intracellular replication versus cell viability indicated a fairly strong correlation for *D. discoideum* (Figure 2F), in stark contrast to *A. castellanii* (Figure 2D). This is potentially due to the extended time required for a full infection round in *D. discoideum* (7 days) as compared to *A. castellanii* (3 days), suggesting that long-term treatment with the compounds may be cytotoxic. In summary, the calcineurin compounds derived from two different query structures inhibited the intracellular growth of *L. pneumophila* in triazine when present at the initiation of infection and also impaired bacterial replication in *D. discoideum*.

Structure–Activity Relationship Analysis of the Hits Chemically Derived from Compounds Targeting the Calcineurin Pathway. For the 12 calcineurin hit compounds identified we performed structure–activity relationship (SAR) studies to develop an optimized set of molecules restricting *Legionella* infection. As mentioned above, the initial Sinergia library design utilized two different queries for compounds targeting the "calcineurin pathway (Figure 2A), which were further used as a basis to produce an extended set of 78 compounds for the subsequent rounds of screening (Figure

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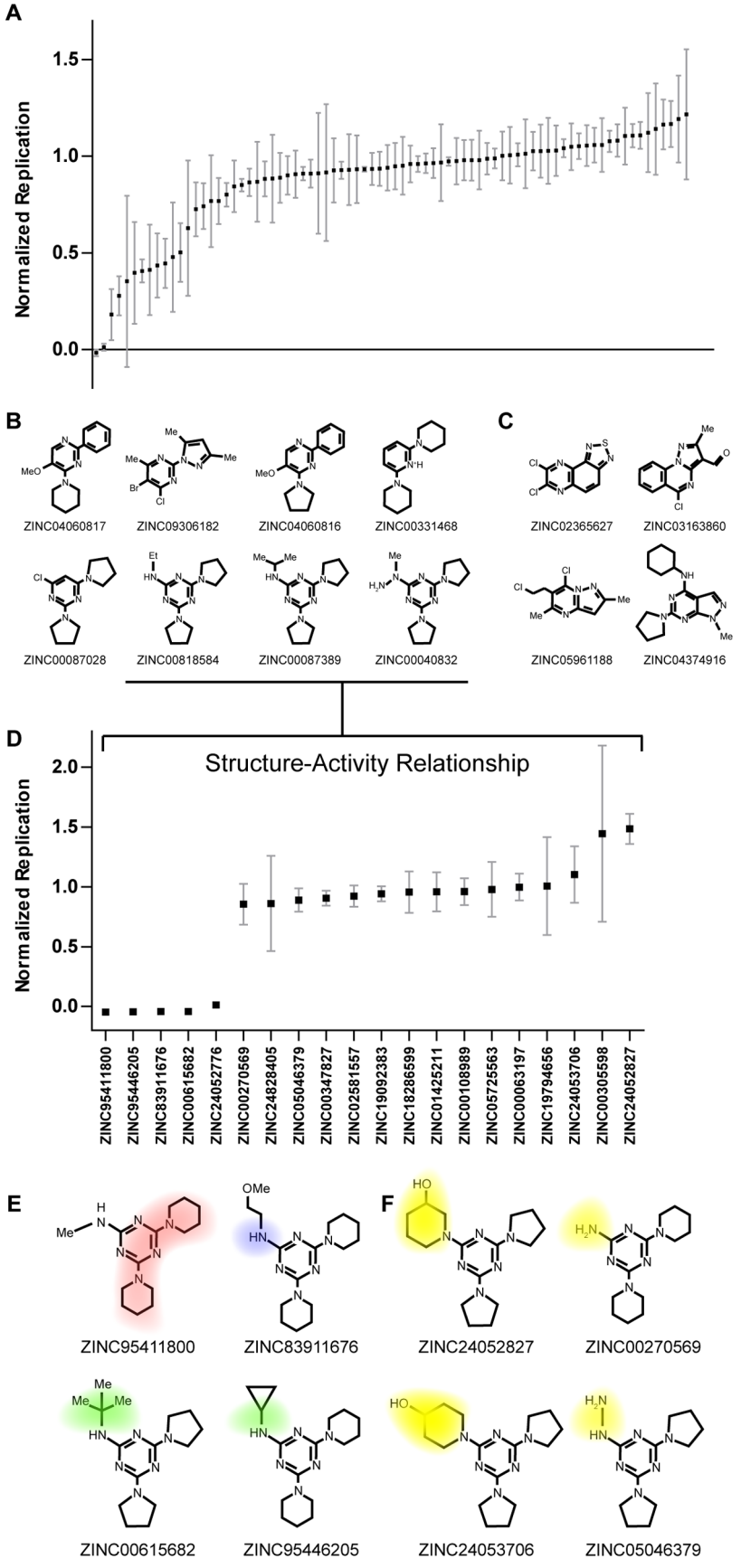

Figure 3. Structure–activity relationship analysis of hits from the calcineurin pathway. (A) Screening results of the initial 78 compounds derived from the 2 original query structures (CN585, pyrazolopyrimidine) indicate that the majority did not have an effect on replication. The graph shows the mean and SD of at least four separate experiments. (B, C) Structures of the 12 best hits derived from the CN585 or pyrazolopyrimidine queries,

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Figure 3. continued

respectively. (D) Regions of similarity for the compounds in (B) were determined, and compounds with minor chemical modifications were then tested for their effect on *L. pneumophila* replication within *A. castellanii*. (E) The most effective inhibitors of intracellular bacterial replication (advanced hits compounds) share several structural features: a triple-ring structure with a central triazine substituted at positions 3 and 5 by two piperidine or pyrrolidine heterocycles (red) and a nitrogen group at position 1 (blue), which is substituted by a hydrophobic aliphatic chain moiety (green). (F) Alterations to this pattern, such as the absence of substituents on the nitrogen atom, or its inclusion in a hydrazine group or in an aliphatic hydroxylated ring, render the compound unable to inhibit *L. pneumophila* replication.

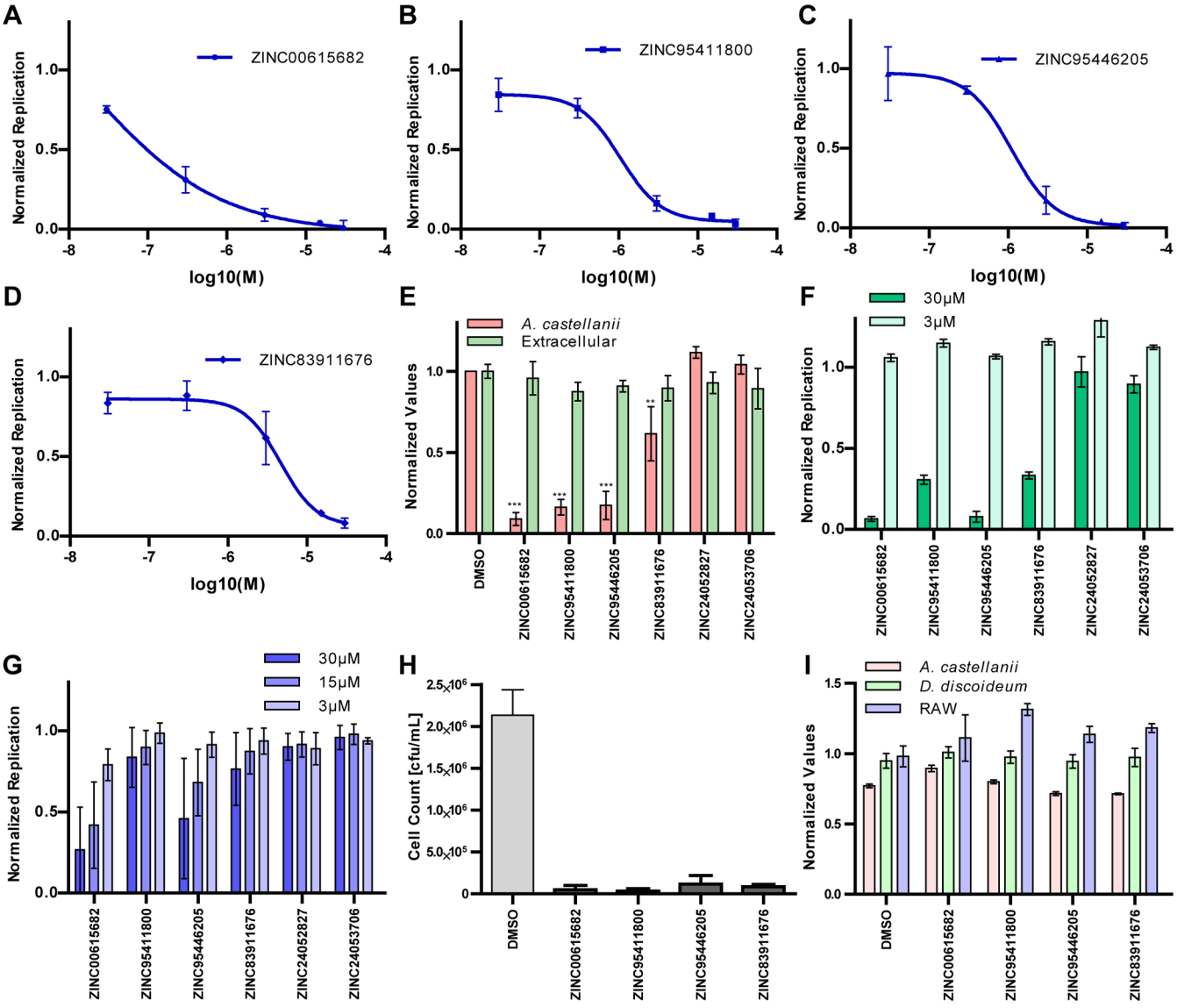

Figure 4. Characterization of advanced hits compounds. (A–D) Dose–response curves of advanced hits compounds for intracellular replication of *L. pneumophila* in *A. castellanii*. (E) Comparison of the efficacy of advanced hits and ineffective compounds (3 μM) on intracellular replication in *A. castellanii* (red) and extracellular replication (green). The graph indicates the mean and SD of at least four separate experiments (**$p < 0.01$, ***$p < 0.001$; Student's t test). (F) Efficacy of compounds on intracellular replication in *D. discoideum* at 30 or 3 μM, normalized to the vehicle. The graph indicates the mean and SD of three experiments. (G) Efficacy of compounds on intracellular replication in RAW 264.7 macrophages, at 30, 15, or 3 μM, normalized to vehicle control. The graph indicates the mean and SD of six separate experiments. (H) Effects of advanced hits compounds on the intracellular growth of *L. pneumophila* in *A. castellanii* at 30 °C, as determined by CFU 3 days postinfection. (I) Cytotoxicity determination of advanced hits compounds toward *A. castellanii*, *D. discoideum*, or RAW 264.7 macrophages after 6 h, using the Presto Blue cell viability assay. The graph indicates the mean and SD of (H) three samples or (I) six samples.

3A). The 12 most effective compounds revealed that hits derived from the CN585 query were more numerous and showed greater homology between chemical structures than those originating from the pyrazolopyrimidine scaffold (Figure 3B,C). For this reason we chose to focus on the CN585 variants.

A 2D chemical similarity search (similarity threshold >70%) led us to identify common features among the CN585 variants in the ZINC database, and 20 analogues with molecular

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variations were chosen for further testing (Figure S2). Results from intracellular growth assays with *A. castellanii* indicated that the compound efficacy of a subset of molecules derived from the 20 CN585 compound analogues increased significantly (Figure 3D). Integration of this information led to the determination of the ideal structural attributes comprising a triazine moiety, substituted in positions 3 and 5 by two piperidine or pyrrolidine rings, respectively, and in position 1 by an amine group bearing a single aliphatic chain substituent moiety. In particular, different substituents from piperidine or pyrrolidine rings at positions 3 and 5 of the triazine moiety remarkably affected the capability of compounds to inhibit the intracellular replication of *L. pneumophila* (Figure 3E). Further analysis revealed the importance of the substitution patterns on the nitrogen atom in position 1 of the triazine ring. Indeed, compounds bearing a single lipophilic aliphatic chain as a substituent on the position 1 nitrogen displayed a considerably higher activity than those having a free nitrogen, a nitrogen substituted with hydrophilic groups (amine, amide, and hydroxyl ethyl), or tertiary nitrogen atoms (closed within the ring or differently substituted) (Figure 3F). Taken together, SAR analysis identified molecules and their structural determinants, which inhibited the intracellular replication of *L. pneumophila* much more efficiently than the original hit compounds. Four effective and two ineffective compounds were then taken as advanced hits and control compounds, respectively, for further characterization.

Characterization of Advanced Hits Compounds. Using the four advanced hits compounds (Figure 3E) and two control compounds (Figure 3F), we set out to characterize these molecules, which had been developed through structural modifications of CN585. To this end, we determined dose−response relationships of the four advanced hits compounds for the intracellular replication of *L. pneumophila* in *A. castellanii* (Figure 4A−D, Table S3). Three of them exhibited IC_{50} values of between 1 and 5 μM, and ZINC00615682 was an extremely efficient inhibitor with an IC_{50} of approximately 20 nM (Table 1). At 3 μM, we observed that the advanced hits compounds were neither toxic to *A. castellanii* nor able to inhibit the extracellular growth of *L. pneumophila* but did strongly inhibit intracellular replication (Figure 4E). Comparative testing in *D. discoideum* (Figure 4F) or RAW 264.7 macrophages (Figure 4G) indicated that intracellular bacterial replication in these host cells was less sensitive to compound treatment because, with the exception of the highly efficacious ZINC00615682, a decrease in replication was seen at 30 μM but not at 3 μM. These findings are similar to previously observed differences in the efficacy of anti-*Legionella* compounds in different host models.[23]

Table 1. IC_{50} Values for Inhibition of *L. pneumophila* Replication in *A. castellanii* by Advanced Hits and Control Compounds

compound	IC_{50} (M)	95% confidence intervals (M)
ZINC00615682	1.97×10^{-8}	3×10^{-12}−1.3×10^{-4}
ZINC95411800	1.05×10^{-6}	6.5×10^{-7}−1.6×10^{-6}
ZINC95446205	1.1×10^{-6}	6.3×10^{-7}−1.9×10^{-6}
ZINC83911676	4.63×10^{-6}	1.8×10^{-6}−1.2×10^{-5}
ZINC24053706	no effect	no effect
ZINC24052827	no effect	no effect

In order to correlate the production of GFP as a measure of bacterial growth with a more direct assay, we determined colony-forming units (CFU) of *L. pneumophila* growing intracellularly in *A. castellanii* upon exposure to the advanced hits compounds after 3 days (Figure 4H). This approach revealed that the four most potent advanced hits compounds (shown in Figure 3E) prevented intracellular bacterial growth almost completely. Thus, the production of GFP indeed correlates very well with bacterial replication as measured by CFU. Moreover, we also assessed the cytotoxicity of these compounds against *A. castellanii*, *D. discoideum*, and RAW 264.7 macrophages (Figure 4I). Compared to the vehicle control, the advanced hits compounds were not significantly cytotoxic to any of the phagocytes. In summary, the dose−response analysis performed for the four advanced hits compounds revealed that ZINC00615682 is a very effective inhibitor of *L. pneumophila* intracellular replication, preventing growth in *A. castellanii* with a low IC_{50} of ∼20 nM.

Calcineurin Does Not Play a Significant Role in *L. pneumophila* Replication. A number of the highly effective inhibitors of *L. pneumophila* replication characterized above are based on the molecule CN585, a noncompetitive inhibitor of calcineurin.[26] Thus, we presumed that our advanced hits compounds would be exerting a similar effect, and we attempted to determine whether calcineurin indeed played a role in their mechanism of action. Calcineurin is a heterodimeric protein phosphatase consisting of a calmodulin-binding catalytic subunit (calcineurin A) and a Ca^{2+}-binding regulatory subunit (calcineurin B).

We first analyzed the effect of known inhibitors and activators of calcineurin to determine any relationship to *L. pneumophila* intracellular replication. The calcineurin inhibitor cyclosporin A[27] did not have a major effect on *L. pneumophila* replication within *A. castellanii* or *D. discoideum* (showing approximately 20% inhibition at 10 μM) (Figure 5A). However, at a relatively high IC_{50} (∼30 μM), cyclosporin A reduced the replication of *L. pneumophila* in RAW 264.7 macrophages, when present at the time of infection. Furthermore, cyclosporin A did exert a relatively strong inhibitory effect on the extracellular growth of *L. pneumophila* (IC_{50} 2 μM, 95%CI: 1.4−3.1 μM), suggesting that it was acting as an off-target antibiotic.

The calcineurin inhibitor quercetin[28] inhibited the replication of *L. pneumophila* in *A. castellanii* only at high concentrations (IC_{50} ∼100 μM, 95%CI: 30−300 μM) with limited or no effects on infected *D. discoideum* or macrophages and on extracellular bacteria (Figure 5B). Moreover, the calcineurin activator chlorogenic acid[29] had no significant effect on intra- or extracellular replication of *L. pneumophila* (Figure 5C). Together, pharmacological experiments suggested that the protein phosphatase calcineurin does not play a significant role in the intracellular replication of *L. pneumophila*.

To further explore whether calcineurin might play a role in the intracellular growth of *L. pneumophila*, we depleted the enzyme in human A549 cells using RNA interference. To this end, the two individual subunits of calcineurin encoded by the *PPP3CB* and *PPP3R1* genes were depleted separately, after which the intracellular replication of *L. pneumophila* was assessed (Figure 5D). However, no effect on the intracellular replication of *L. pneumophila* was observed upon depletion of the individual calcineurin subunits. Western blot analysis of the depletion efficiency of the calcineurin catalytic subunit PPP3CB revealed a reduction of approximately 60% under the

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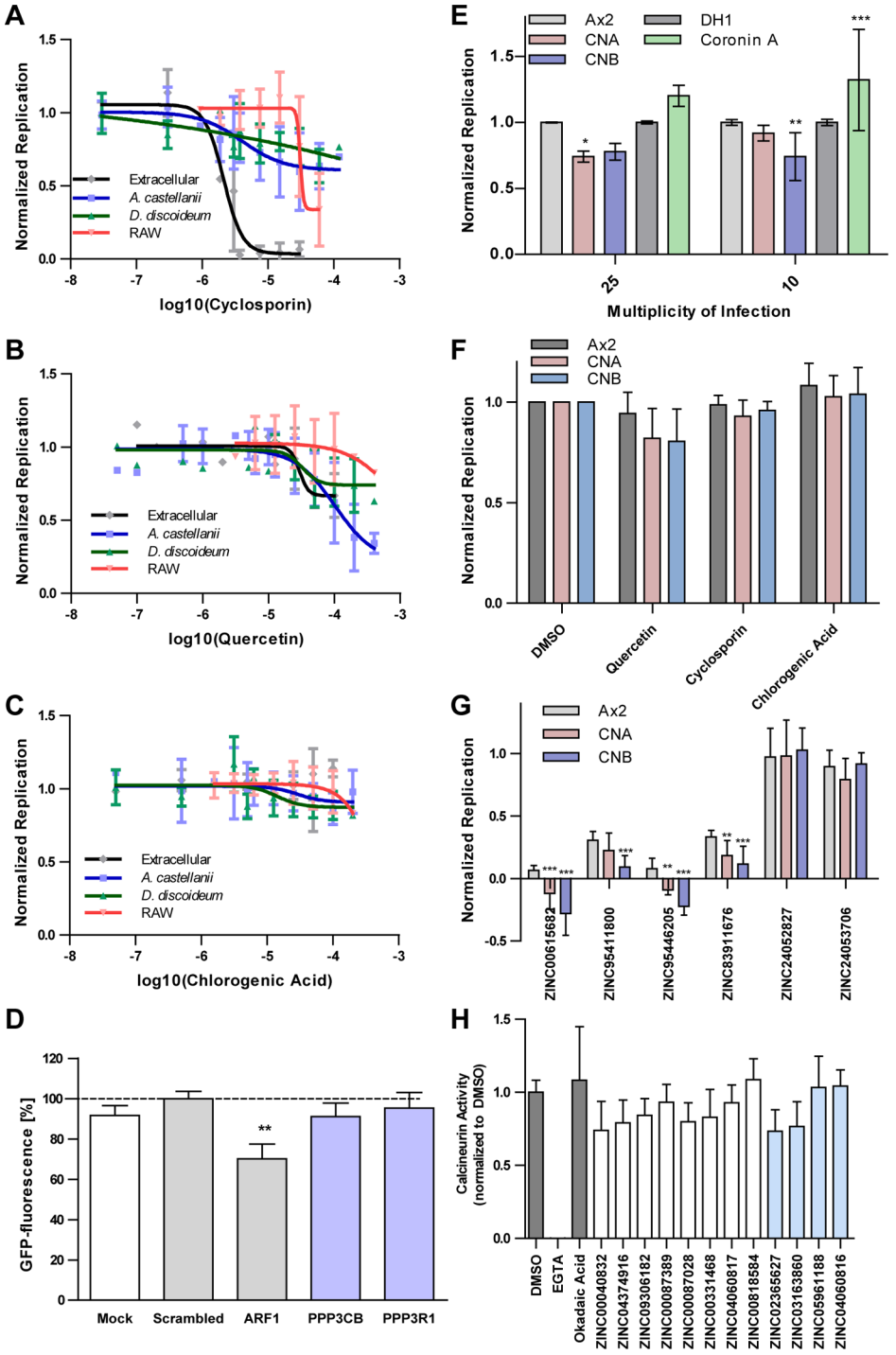

Figure 5. Calcineurin is not involved in *L. pneumophila* replication. (A–C) Dose–response curves plot (mean and SD of at least seven separate experiments) of *L. pneumophila* replication versus vehicle control (<0.5% DMSO), in media (extracellular), amoebae (*A. castellanii* or *D. discoideum*), or RAW 264.7 macrophages. (A) The calcineurin inhibitor cyclosporin A exhibits an antibiotic effect against *L. pneumophila* (IC_{50} 2 μM, 95% CI: 1.4–3.1 μM) and, at higher concentrations, inhibits replication within RAW 264.7 macrophages (IC_{50} ~30 μM). (B) The calcineurin inhibitor quercetin shows the inhibition of intracellular replication of *L. pneumophila* in *A. castellanii* at relatively high concentrations (IC_{50} ~100 μM). (C) The calcineurin activator chlorogenic acid has no effect on *L. pneumophila* replication. (D) Replication of *L. pneumophila* in A549 epithelial cells after depletion by RNAi of individual calcineurin subunits (PP3CB, PP3R1) or the small GTPase Arf1. The graph shows the mean and SD of triplicate experiments (**p < 0.01; Student's t test). (E) Replication of *L. pneumophila* in *D. discoideum* lacking coronin A (green) or depleted by RNAi for calcineurin A (red, CNA) or calcineurin B (blue, CNB) subunits, as normalized to the respective control strains (gray). The graph indicates the mean and 95% CI of at least triplicate experiments (*p < 0.05, **p < 0.01, ***p < 0.001; Student's t test). (F, G) Replication of *L. pneumophila* in *D. discoideum* wild type or strains depleted for calcineurin subunits by siRNA following treatment with (F) quercetin, cyclosporin A, or chlorogenic acid (15 μM) or (G) CN585-derived advanced hits or control compounds (3 μM). Replication was normalized to the vehicle control and compared to Ax2 wild-type amoebae (**p < 0.01, ***p < 0.001). (H) The calcineurin compounds (CN585, white; pyrazolopyrimidine moiety, blue; controls, gray) do not significantly affect calcineurin activity in vitro at 30 μM concentration. The graph shows the mean and SD of at least three replicate experiments (statistics: one way ANOVA, Gaussian approximation).

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conditions used (Figure S3). As a positive control, we depleted the small GTPase ARF1, which promotes the intracellular growth of *L. pneumophila*.[30] As expected, the depletion of ARF1 led to a reduction in replication efficiency.

We also assessed intracellular bacterial replication in *D. discoideum* strains with the RNAi-mediated knock-down of calcineurin subunits A and B. Here, the replication efficiency of *L. pneumophila* was slightly but significantly reduced (Figure 5E). Finally, *D. discoideum* strains lacking the upstream calcineurin activator coronin exhibited a small but significant increase in *L. pneumophila* replication (Figure 5E), in line with previous studies that have also observed increased replication of *L. pneumophila* within coronin-knockout *D. discoideum*.[31,32] Taken together, these results suggest that the absence of (active) calcineurin does not cause a major change in the intracellular replication of *L. pneumophila*.

Furthermore, we used *D. discoideum* strains depleted of calcineurin subunits to analyze the effects on the intracellular replication of *L. pneumophila* of compounds known or presumed to target calcineurin. As previously seen in wild-type *D. discoideum*, no obvious changes in intracellular bacterial replication were seen following treatment of the amoebae with cyclosporin A, quercetin, or chlorogenic acid (Figure 5F). Likewise, the advanced hits compounds developed previously (Figures 3E and 4) but not the control compounds (Figures 3F and 4) still blocked intracellular replication in *D. discoideum* depleted of calcineurin (Figure 5G). These findings indicate that the advanced hits compounds target factors in the cell, which are not implicated in the calcineurin pathway. Interestingly, drug treatment of *D. discoideum* depleted of calcineurin led to a further (synergistic) reduction of *L. pneumophila* replication, as compared to wild-type amoebae. Again, these results are in agreement with the notion that the advanced hits compounds target an alternative factor or factors to calcineurin.

Thermophoresis assays were then carried out in an attempt to evaluate the capability of the initially identified hits to bind to the human calcineurin−calmodulin complex. However, out of 12 compounds tested, only 2 showed a potential binding with a K_d value at high micromolar concentration range, indicative of weak, possibly nonspecific interactions (Table S4). Finally, to determine the effects of the advanced hits compounds (Figures 3E and 4) on the activity of mammalian calcineurin in vitro, recombinant human calcineurin was assayed using a phosphate release kit (Figure 5H). To this end, calcineurin-mediated phosphate release from an enzyme-specific peptide was measured and normalized to vehicle (full activity) and EGTA (no activity). Addition of the advanced hits compounds only slightly decreased calcineurin activity in vitro, and the changes were not statistically significant. In summary, the above results indicate that the advanced hits compounds identified in this study do not affect calcineurin but target another, as yet unidentified, factor or factors.

DISCUSSION

This study presents a hit finding and chemical space exploration analysis of compounds identified as inhibitors of intracellular replication of *L. pneumophila*, using *A. castellanii* as a model host system. We used hits identified in the Sinergia library, comprising more than 1200 compounds that were assembled based on a virtual screening with query compounds known to target distinct pathogen pathways. The rationale behind the design of this library was to increase the hit rate in a low-throughput screen. Among the hits identified, we chose to focus on a family of compounds sharing chemical similarities with inhibitors of the calcineurin protein phosphatase enzyme (Figures 1B and 2−4).

The majority of hits and corresponding advanced hits compounds were originally derived from CN585, a small molecule which specifically binds to and inhibits the catalytic calcineurin A subunit, sharing a binding region with cyclosporin/Cyp18.[26] Thus, we sought to determine whether calcineurin indeed plays a role in their mechanism of action. To this end, we used several established calcineurin modulators such cyclosporin A, developed as an immuno-suppressant drug due to the crucial role that calcineurin plays in T-cell activation (Figure 5).[33] Cyclosporin A is a cyclic nonribosomal peptide originally isolated from a soil fungus.[34] The compound has been shown to inhibit calcineurin activity by forming a complex with the phosphatase and an activating protein, cyclophilin.[35]

Treatment of *D. discoideum* with cyclosporin A or FK506 strongly inhibited cAMP-induced stalk cell formation and reduced the expression of prestalk- and prespore-specific transcripts, whereas the compounds had little effect on the growth and aggregation of the amoebae.[36] Thus, *D. discoideum* responds to cyclosporin A, and calcineurin is likely involved in the development of the amoebae. On the other hand, cyclosporin A did not affect the intracellular replication of *L. pneumophila* in amoebae (Figure 5A), indicating that calcineurin is not involved in this process. Moreover, the relatively high IC_{50} of cyclosporine A seen for the inhibition of growth in macrophages (~30 μM), as well as the antibiotic activity observed both in this study (Figure 5A) and previously published for *Mycobacterium avium*,[37] also suggests that this is not the case. Most likely, cyclosporin A acts as an antibiotic compound, rather than calcineurin activity affecting *L. pneumophila* replication.

The calcineurin inhibitors quercetin and chlorogenic acid did not affect the intracellular replication of *L. pneumophila*; therefore, calcineurin is not involved in the process and cannot be the target of the compounds identified in this study (Figure 5B,C). Quercetin is a plant-derived flavonoid molecule, which has been reported to bind to and inhibit calcineurin,[28] and chlorogenic acid is a phenol found in green coffee-bean extract and has been reported to activate calcineurin in a calmodulin-dependent manner.[38] Finally, calcineurin depletion in A549 cells (Figure 5D) or *D. discoideum* (Figure 5E) barely affected the intracellular replication of *L. pneumophila*. In summary, several highly effective compounds were developed by virtual screening and optimization starting from compounds targeting calcineurin. However, to the best of our knowledge, they do not appear to affect calcineurin, and thus, their precise mechanism of action remains to be elucidated.[39,40]

A commonly used drug, FK506 (also known as tacrolimus), indirectly inhibits calcineurin activity by binding to a separate protein, FKBP, together forming a potent antagonist.[27] Interestingly, the *Legionella* protein Mip (macrophage infectivity potentiator) shares structural similarity to the FKBP protein family, and Mip proteins are also known to bind FK506.[41] It has been shown that *L. pneumophila* Mip deletion mutant strains exhibit significantly decreased intracellular replication and survival from the earliest stages of uptake.[42,43] One possibility is that the compounds, ostensibly developed for calcineurin, are instead interfering with the activity of interacting proteins such as eukaryotic FKBP or, potentially, bacterial Mip.

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The majority of our hit compounds appeared most effective when present during the initial stages of infection, as opposed to 15−30 min after infection, suggesting that they play a role in phagocytic uptake and the initial establishment of the LCV. Because the advanced hits compounds do not seem to alter the LCV composition dramatically (Figure S1), the uptake process itself might be targeted by the drugs. It is known that local concentrations of calcium play an important role in the initiation of phagocytosis, an important factor in *L. pneumophila* uptake.[44] It has also been observed that calcium-binding proteins calnexin and calreticulin are localized to phagocytic cups during *L. pneumophila* uptake, after which they are actively recruited to the LCV membrane.[30,31]

We utilized *A. castellanii* as a model host cell due to the robust reproducibility of results and its role as a natural reservoir of *L. pneumophila*. One potential caveat of this model is that compounds effective in amoebae may not have the same efficacy in human macrophages. However, similar screens with *Burkholderia pseudomallei* in *D. discoideum* indicated that results transfer well to mammalian experiments.[45] Analogously, we have previously shown that amoebae allow the discovery of novel antibiotic compounds with high efficacy in macrophages.[23,46] Our results reported in this study also indicated that antivirulence compounds highly efficacious in amoebae were also effective in macrophage cell lines, further supporting the use of amoebae as a model system. Previously, murine J774 macrophages have been used to screen for antivirulence compounds, blocking the translocation of effector proteins through the T4SS of *L. pneumophila*.[47] In this study, the screening of a collection of more than 2500 annotated compounds (Biomol, NINDS, or Prestwick library) revealed that a number of host cell factors involved in phagocytosis promote translocation through the T4SS.

Antivirulence compounds are usually identified by searching for inhibitors of a specific, well-characterized virulence system. This type of strategy led to the discovery of compounds that inhibit the expression of the autoinducer synthase gene *pqsA*,[22] block the T3SS of *Salmonella enterica* typhimurium,[48] or prevent *E. coli* capsule production.[49] An alternative approach, as used in this study, is to conduct a wide-ranging screen against complex host cell−pathogen interactions, thus identifying active compounds showing efficacy against the target pathogen in this setting. Using a library of 640 FDA-approved compounds with known targets, a similar approach has been employed to determine antimicrobial drugs with efficacy against a range of intracellular bacteria replicating in human THP-1 macrophage-like cells.[50] This study identified several inhibitors of cholesterol homeostasis that interfere with the intracellular replication of *Coxiella burnetti* and, to a lesser extent, also with *L. pneumophila*. Moreover, compounds targeting cellular (G protein-coupled receptor-dependent) signaling pathways, calcium fluxes, or sterol homeostasis also had a strong effect on *C. burnetti* intracellular replication. Thus, repurposing approved drugs appears to be a promising strategy to identify novel antibacterial therapeutics.

In summary, using a pathway-based virtually designed library we performed a wide-ranging screen for antivirulence compounds against *L. pneumophila* replicating in *A. castellanii* amoebae. This study presents a hit finding and chemical space exploration analysis of compounds identified as inhibitors of intracellular bacterial replication. Following the identification of a cluster of hits tentatively targeting the host cell protein phosphatase calcineurin, we developed several advanced hits compounds with a strong antivirulence effect. Notably, one advanced hits compound, ZINC00615682, inhibited the intracellular replication of *L. pneumophila* in *A. castellanii* with a low IC_{50} of approximately 20 nM. In contrast to original expectations, the compounds characterized did not appear to target calcineurin. Future experiments are aimed at the identification of the direct targets of these compounds.

METHODS

Bacteria and Reagents. *L. pneumophila* wild-type JR32[51] or the isogenic mutant Δ*icmT* lacking a functional Icm/Dot T4SS,[52] constitutively producing GFP,[53] were grown on charcoal yeast extract (CYE) plates or in ACES yeast extract (AYE) medium,[54] with 10 μg/mL chloramphenicol. For infections, the bacteria were grown overnight in AYE at 37 °C to a final OD_{600} of 3.0, at which stage they have reached the transmissive, infectious phase. Murine RAW 264.7 macrophages (ATCC Tib-71, lab collection) and human A549 lung carcinoma epithelial cells (ATCC CCL-185, lab collection) were maintained in supplemented RPMI medium, *A. castellanii* (ATCC 30234) in PYG medium, and *Dictyostelium discoideum* in HL5 medium; all were passaged twice weekly. The *D. discoideum* strains used were Ax2 wild type, and derivatives were depleted for calcineurin A[39] or calcineurin B[40] or DH1 wild type and an isogenic coronin A knockout strain,[55] respectively. Compounds of the Sinergia library were purchased from various chemical suppliers (Table S5) and generally stored as 30 mM stock solutions in DMSO.

***Legionella* Extracellular Growth Assay.** Extracellular replication of *L. pneumophila* in the presence of compounds was determined as follows. *L. pneumophila* were taken from CYE plates after 3 days of growth and resuspended in 3 mL of AYE at a final OD_{600} of 0.02, matching the bacterial concentration used for intracellular assays (see below). Compounds of interest or vehicle controls (<0.5% DMSO, which did not interfere with the assays) were diluted into these cultures, which were then incubated on a rotating wheel overnight at 37 °C. OD_{600} measurements were then taken, and the values were normalized to the vehicle controls.

***Legionella* Intracellular Replication Assays.** Intracellular replication of *L. pneumophila* in phagocytes (*A. castellanii*, *D. discoideum*, and macrophages) was assessed using previously published protocols.[23] In brief, the phagocytes were seeded into 96-well plates and infected (MOI 20) with GFP-producing *L. pneumophila*. Infected cells were incubated at 25 °C (*D. discoideum*), 30 °C (*A. castellanii*), or 37 °C (macrophages), and the progress of replication was followed by the increase in GFP fluorescence over several days. Because the culture media used for the eukaryotic host cells do not support the growth of *L. pneumophila*, GFP fluorescence accurately reflects intracellular replication. Moreover, the correlation between GFP fluorescence and bacterial growth in *A. castellanii* at 30 °C was confirmed by determining CFU after 3 days.

The small-molecule compounds to be tested were added simultaneously, 30 min postinfection, or 30 min preinfection. For "pretreatment" experiments, the cells were incubated with the drugs for 30 min and washed three times with media after the drugs were taken off, followed by the addition of the bacteria.

Cytotoxicity Assay and Fluorescence Microscopy. The cytotoxicity of compounds to eukaryotic host cells (*A. castellanii*, *D. discoideum*, and RAW 264.7 macrophages) was determined using the Alamar Blue or Presto Blue reagent (Life

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Technologies). To mimic the conditions found in the intracellular replication assay, 96-well plates were set up as described above, and uninfected triplicate wells were treated with compound in 100 μL of LoFlo medium (ForMedium). Plates were incubated for 6 to 24 h at 30 °C (*A. castellanii*), 23 °C (*D. discoideum*) or 37 °C (macrophages), after which 10 μL of Alamar Blue reagent was added, and plates were incubated for another 3 to 4 h. The fluorescence at 595 nm was measured, and data were normalized between 1 (treatment with LoFlo alone) and 0 (SDS, total lysis of the cells). Means from each individual experiment were then combined for analysis. *D. discoideum* strains were prepared and imaged by confocal laser fluorescence microscopy as previously described using a Leica SP5 confocal laser scanning microscope.[56−58]

Calcineurin Activity Assay. In vitro calcineurin activity was assayed with kits from Enzo Life Sciences (BML-AK804) and used according to the manufacturer's instructions. Briefly, wells of a 96-well plate contained a mixture of calcineurin (40 U), calmodulin, RII phosphopeptide (ALVPIPGRFARRVpS-VAAN, 0.75 mM), and compound (30 μM) or vehicle control (<0.1% DMSO, which did not interfere with the assay). Calcineurin activity was determined by phosphate release after 15 min at 30 °C via the change in color of malachite green (BioMol-Green) reagent.

Thermophoresis Assay. Thermophoresis assays were carried out by using the microscale thermophoresis technology. Compounds were tested from 200 μM to 25 nM in buffer (10 mM HEPES, pH 8.0, 2 mM $CaCl_2$, 100 mM NaCl, 5 mM DTT, 1% glycerol, 5% DMSO, 0.05% Tween 20, 25 nM calcineurin, and 25 nM calmodulin), where calmodulin was labeled with NT-647-HNS (Nanotemper). Compounds were screened using the following process: 5 μL of 50 nM calmodulin was added to assay tubes, followed by 5 μL of 800 μM compound. Solutions were mixed, and serial dilutions were made by adding 5 μL to the following tube. Each assay tube then had 5 μL of 50 nM calcineurin added. Samples were centrifuged for 5 min at 10 000*g*, and the fluorescence within the tubes was measured at 80% LED and 40−80% laser power. Measurements were performed in at least triplicate experiments. Secondary analysis screened for the interaction of 50 nM calmodulin with 500 nM−15 pM calcineurin, reported to have a K_d of 45 (± 10) nM.

RNA Interference. The calcineurin subunit genes (*PPP3CB* or *PPP3R1*) were depleted in A549 epithelial cells using the siRNA oligonucleotides (Qiagen) indicated (Table S6) as previously published.[59,60] Briefly, A549 cells were seeded in 96-well plates and treated for 2 days with a final concentration of 10 nM siRNA oligonucleotides. After oligonucleotide transfection, the cells were infected with GFP-producing *L. pneumophila* (MOI 10) grown for 21 h in AYE, centrifuged, and incubated for 1 h. The infected cells were washed three times with prewarmed RPMI medium, and replication was determined by GFP fluorescence 48 h postinfection using a plate reader. Experiments were performed in triplicate, and replication values were normalized to that of cells treated with scrambled RNA (Allstars siRNA; Qiagen).

The calcineurin depletion efficiency was analyzed by Western blot. To this end, A549 cells were incubated with siRNA oligonucleotides for 48 h, trypsinized, washed, and subjected to SDS-PAGE and Western blot. The nitrocellulose membranes were blocked with BSA or fat-free milk, and antibodies against the calcineurin catalytic subunit (PPP3CB, ThermoFischer Scientific; 1:1000) or regulatory subunit (PPP3R1, ThermoFischer Scientific; 1:1000−1:100), followed by an HRPO-conjugated donkey antirabbit IgG antibody (GE Healthcare), were used. GAPDH (Cell Signaling) served as a loading control, and Qiagen AllStars oligonucleotides were used as a negative control.

Design of a Pathways-Based Highly Diverse Chemical Library (Sinergia Library). To design the Sinergia library, several pathways involved in host−pathogen interactions, in particular, those with a significant pathogen/host selectivity ratio, were targeted (manuscript in preparation). Eighteen different host- and pathogen-related pathways were examined as potential pharmacological targets. Ligands/metabolites known to interfere within these pathways were collected and used as queries or search templates for launching a campaign of ligand-based virtual screening (VS) from the ZINC database[61] by means of ROCS, a tool from the OpenEye software package.[24] Active ligands/metabolites served as query molecules to prepare pharmacophores for the ligand-based VS. The following workflow was applied: (i) the ZINC leadlike database composed of 2.5×10^6 compounds was screened, saving the 25 000 best hits for each query; (ii) hits were ranked according to the ROCS TanimotoCombo score; (iii) the first VS hit was selected, followed by the next one if structurally dissimilar to previous selections, using the Lingo method present in the OpenEye VIDA program to increase the chemical diversity and exploit the maximal chemical space of the ZINC leadlike database; (iv) at least two analogs were chosen per series from each screened pathway, saving 100 selected compounds to the pool of potential antibacterial hits composing the physical library for the experimental screen. Of the ∼1800 compounds selected by the VS procedure (∼100 per each pathway), 1258 could be purchased and made up the final Sinergia library.

Hits-Based Analogs Selection. Compounds resulting in decreased intracellular *L. pneumophila* replication by over 50% at the screening concentration (30 μM) were defined as hit candidates. The chemical space around the specific scaffolds characterizing the hit candidates was explored, searching for compounds with a chemical similarity, calculated by Tanimoto's metrics, of at least 70% as compared to hit candidates in the ZINC database. Small libraries of hit-based analogs were designed by visually inspecting and selecting compounds from providers, using medicinal chemistry hypotheses such as substitution patterns and isosteric replacements to define the structure−activity relationships and to guide the improvement of the compounds series.

Data Analysis. Data analysis was performed using Microsoft Excel and GraphPad Prism 5. To compare the effect of compound treatment on intracellular replication, fluorescence values were taken from the first time point following entry into the stationary phase. The results were then normalized such that media-only wells (no bacteria) were 0 whereas vehicle-treated wells were 1 (normal replication). The average of the replicate wells (minimum of 3 per plate) was then plotted as dose−response curves such that each individual point represented the average of a single experiment. Compound treatments were repeated a minimum of 3 times to control for the increased variability of bacteria−host cell interactions. Lines of best fit and associated IC_{50} values were calculated for each dose−response curve using the nonlinear fit (log inhibitor versus response, variable slope) function of Prism 5.

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■ ASSOCIATED CONTENT

Supporting Information

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Confocal imaging of *L. pneumophila*-infected *D. discoideum* treated with ZINC09306182. Two-dimensional structures of CN585 analogs. Western blot analysis of target protein depletion in A549 cells. Replication effects and toxicity of primary hit compounds. Replication effects of lead compounds on *L. pneumophila* extra- and intracellular growth in *A. castellanii*, *D. discoideum*, or RAW 264.7 macrophages. Calculated K_d values from thermophoresis. Structures and sources of compounds used in this study. Oligonucleotides used for RNA interference. (PDF)

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Notes

The authors declare no competing financial interest.

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