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Identification of a serine protease inhibitor which causes inclusion vacuole reduction and is lethal to *Chlamydia trachomatis*

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Summary

The mechanistic details of the pathogenesis of *Chlamydia*, an obligate intracellular pathogen of global importance, have eluded scientists due to the scarcity of traditional molecular genetic tools to investigate this organism. Here we report a chemical biology strategy that has uncovered the first essential protease for this organism. Identification and application of a unique CtHtrA inhibitor (JO146) to cultures of *Chlamydia* resulted in a complete loss of viable elementary body formation. JO146 treatment during the replicative phase of development resulted in a loss of *Chlamydia* cell morphology, diminishing inclusion size, and ultimate loss of inclusions from the host cells. This completely prevented the formation of viable *Chlamydia* elementary bodies. In addition to its effect on the human *Chlamydia trachomatis* strain, JO146 inhibited the viability of the mouse strain, *Chlamydia muridarum*, both *in vitro* and *in vivo*. Thus, we report a chemical biology approach to establish an essential role for *Chlamydia* CtHtrA. The function of CtHtrA for *Chlamydia* appears to be essential for maintenance of cell morphology during replicative the phase and these findings provide proof of concept that proteases can be targeted for antimicrobial therapy for intracellular pathogens.

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Introduction

Chlamydia are obligate intracellular bacterial pathogens with significant clinical importance. *Chlamydia* (*C.*) *trachomatis* is the most common sexually transmitted bacterial infection world wide and the ocular infecting serovars are the most common cause of preventable blindness worldwide (World Health Organization, 2011). In spite of the substantial health burden due to the *Chlamydia*, comparatively little is known about the organism's functional pathogenesis and disease mechanisms.

Members of the chlamydiae family are distinguished by an unusual bi-phasic developmental cycle that consists of a small, tough, spore-like, non-replicative extracellular form [termed the elementary body (EB)] and the intracellular replicative, metabolically active, non-infectious form [called the reticulate body (RB)]. The intracellular phase occurs exclusively in a unique vacuole known as the inclusion vacuole (reviewed Abelrahman and Belland, 2005). This developmental cycle and the obligate intracellular nature of the *Chlamydia* have hampered efforts to develop traditional techniques to genetically manipulate the organism. Although there have been recent developments, including a chemical mutation approach to generate chromosomal mutants (Kari *et al.*, 2011) and

successful transformation of the organism with its own plasmid (Wang *et al*., 2011), biological understanding of this organism remains limited relative to its economic and health impact.

The highly conserved protease HtrA (*Chlamydia* HtrA; CtHtrA) has been described as having a number of virulence functions in pathogenic bacteria (Hoy *et al*., 2010; Chitlaru *et al*., 2011). This protease has also been described in *C. trachomatis* using *in vitro* and microscopy methods, and in *Chlamydi*a it potentially functions both as a bacterial cell-associated protease and as a secreted virulence factor (from 28 h post infection) (Huston *et al*., 2007; 2008; Wu *et al*., 2011). Our previous investigations into the biochemical mechanism of activation implicated outer membrane protein sequences with activation of the chaperone form, suggesting a potential role in surface protein assembly (Huston *et al*., 2011). Recently the protease that has been considered to be the major chlamydial pathogenesis factor, CPAF, has been the focus of controversy as it appears that many of the functions attributed to this protease may have been detected as an artefact of the sample preparation (Chen *et al*., 2012), including a key function thought to be critical for viable infectious yield (Heuer *et al*., 2009).

Other chlamydial virulence factors have also been uncovered using small molecule approaches, including the bacterial type III protein secretion inhibitor (Wolf *et al*., 2006), and a peptidomimetic to the type III secretion ATPase protein interaction domain (Stone *et al*., 2011). These studies support the fact that chemical approaches are a powerful strategy to investigate the function of proteins within this unique organism. Here we screened for and found a novel selective inhibitor against a CtHtrA. Using this protease inhibitor, JO146, we demonstrated an essential role for CtHtrA for chlamydial developmental cycle progression during the replicative phase. JO146 treatment resulted in chlamydial cell morphology loss, the reduction in inclusion vacuoles and eventual complete bacterial lethality as the result of failure to develop viable infectious progeny (EBs).

Results

Selective and specific phosphonate inhibitors of CtHtrA are lethal to C. trachomatis *when added during the replicative phase of the developmental cycle*

A library of 1090 serine protease inhibitors [previously described (Arastu-Kapur *et al*., 2008; Hall *et al*., 2011)] was screened against CtHtrA *in vitro* protease activity (detailed in *Experimental procedures*). An initial screen identified two inhibitors, JO146 and JCP83 (IC_{50}s: 12.5 μM and 47.19 μM, Fig. 1A). Both are peptides with a C-terminal phosphonate 'warhead' electrophile which reacts irreversibly with the protease active-site serine residue (Oleksyszyn and Powers, 1991) (Jackson *et al*., 1998), and differ only in the P3 position (Val/Ala). Both inhibitors have valine at the P1 position and proline at P2. The two inhibitors were quite selective towards CtHtrA when screened against a series of other serine and HtrA family proteases (Table 1, Fig. S1). Inhibition was only observed for HTRA2 and elastase. Neither compound was cytotoxic to McCoy and HEp-2 cells, both common cell lines used for *Chlamydia* culture. Specifically, we detected no cell lysis or impaired metabolic turnover when the cells were incubated with JO146 or JCP83 for 8 h (Table S1, as described in the supporting data).

The two inhibitors were added to *C. trachomatis* HEp-2 cultures at different time points throughout the developmental cycle [Fig. 1B: lag ~ 8 h, replication at ~ 2.4 h per division, EB formation from ~ 24 h post infection (h PI) (Miyairi *et al*., 2006)]. Viable infectious elementary bodies (inclusion forming units) at completion of the developmental cycle after each of these independent treatments was then determined. As shown in Fig. 1C–F, addition of JO146 or JCP83 resulted in complete or significant ($P < 0.001$) loss of viable elementary body formation when added during the replicative phase. The activity was most effective at higher doses; however, at 16 h PI even 10 μM JO146 had a significant impact on viability with a lower *Chlamydia* multiplicity of infection (moi) (Fig. 1C, $P < 0.001$). The host cell numbers were the same under each condition so these data indicate that the amount of *Chlamydia* present (moi) associates with the effectiveness of the compounds.

The lethality of JO146 for C. trachomatis *at 16 h PI does not require host cell protein synthesis, and is not influenced by the type of host cell*

Chlamydia viable yield from cell culture improves when a host cell protein synthesis inhibitor (cycloheximide) is used (Thomas *et al*., 1977). We tested the role of host cell protein synthesis during JO146 inhibition (16 h PI) at an moi of 0.3 in HEp-2 cells. The lethality of JO146 was determined at 44 h PI and was found to be maintained in the presence of cycloheximide (Fig. 2A).

Chlamydia trachomatis is capable of infection and replication within a variety of epithelial cells. The effectiveness of JO146 against *C. trachomatis* when added at 16 h PI was evaluated in a variety of host cell types with viability determined at 44 h PI (moi 0.3). There were different yields of *C. trachomatis* depending on the host cell type infected which was expected (Fig. 2B). There were significantly ($P < 0.001$) higher yields of viable *Chlamydia* when cultured in McCoy cells; however, JO146 was still lethal for *C. trachomatis* at a concentration of 100 μM in McCoy cells. These data suggest that JO146 activity is

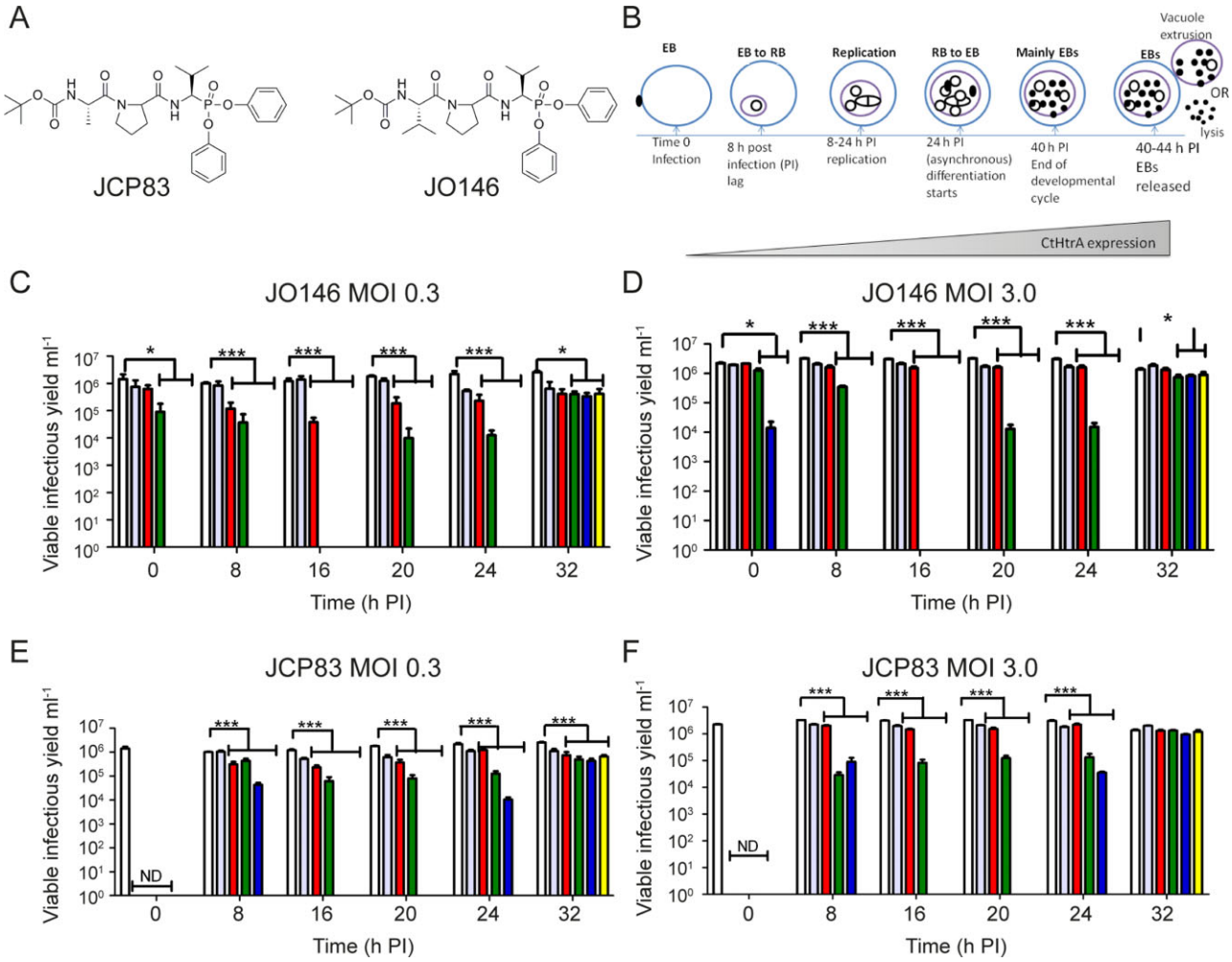

Fig. 1. JCP83 and JO146 are lethal to *Chlamydia* at the replicative phase of the developmental cycle.
A. CtHtrA inhibitors JCP83 [Boc-Ala-Pro-Val^{P}(OPh_2)] and JO146 [Boc-Val-Pro-Val^{P}(OPh_2)] identified during library screen.
B. The chlamydial developmental cycle and CtHtrA expression (Belland *et al*., 2003).
C–F. Viable infectious yield of *C. trachomatis* at the conclusion of the developmental cycle [44 h post infection (h PI)] is shown. HtrA inhibitors were added at different time points following the initial infection. (C) JO146 multiplicity of infection (moi) 0.3, (D) JO146 moi 3.0, (E) JCP83 moi 0.3 and (F) JCP83 moi 3.0. The bars represent from left to right; white: DMSO control, grey: 0 μM (media only), red: 10 μM inhibitor, green: 50 μM inhibitor, blue: 100 μM inhibitor, and yellow: 150 μM inhibitor. ND: Note that the time 0 compound treatments with JCP83 were not conducted due to limited supply of this compound. The bars represent the mean from three independent experiments, error bars represent the SEM ($n = 6$); *$P < 0.05$, ***$P < 0.001$.

dependent on the amount of *Chlamydia* present, that it does not require host cell protein synthesis and that inhibitor activity is not dependent on the host cell type.

A JO146 activity-based probe labels CtHtrA

We synthesized an activity-based probe based on the JO146 structure in which a Cy5 dye was attached in place of the Boc protecting group on the parent compound. We then monitored labelling of targets throughout the *C. trachomatis* developmental cycle (Fig. 3A and B). Three predominant labelled proteins were observed with one additional weakly labelled species. A dominant doublet of proteins around 48–50 kDa were labelled by JO146-Cy5 throughout the developmental cycle (Fig. 3B). These labelled proteins are consistent with the banding pattern and molecular weight of CtHtrA as observed by Western blot (Fig. 3B), and as consistent with previous reports using alternative antibodies (Wu *et al*., 2011). The high levels of CtHtrA present at time zero, or in the elementary bodies, do not show corresponding high levels of binding of JO146 as it is unlikely that the compound can enter this cellular form of *Chlamydia*. The next most intensely labelled band was at approximately 37 kDa. Two additional, less intensely labelled bands were detected at approximately 25 kDa and 125 kDa (Fig. 3B).

Table 1. Specificity of inhibitor compounds, IC_{50} of compounds against a range of proteases.

	JO146		JCP83	
	Substrate			
Protease	Peptide[a,b]	Protein[c]	Peptide	Protein
CtHtrA	12.5 μM (± 2.94 μM)	~ 200 μM	47.19 μM (± 7.37 μM)	~ 500 μM
CmHtrA	47 μM (± 7.19 μM)	~ 100 μM	93.69 μM (± 12.18 μM)	~ 400 μM
Chymotrypsin	> 500 μM	NA	> 500 μM	NA
Trypsin	> 500 μM	NA	> 500 μM	NA
Elastase	2.24 μM (± 0.12 μM)	NA	0.310 μM (± 0.22 μM)	NA
DegP	> 500 μM	> 800 μM	> 500 μM	> 800 μM
DegS	NA	> 500 μM	NA	> 500 μM
HTRA1	> 200 μM	> 200 μM	> 200 μM	~ 200 μM
HTRA2	NA	~ 75 μM	NA	~ 150 μM

a. Peptide substrates were as follows: chymotrypsin: AAF-pNA (SigmaAldrich), trypsin: benzyl DL-R-pNA (SigmaAldrich), elastase: Nmethylsucc – AAPV-pNA (SigmaAldrich M4765), DegP: DPMFKLV-pNA, and HTRA1: (D-Arg)-(D-Arg)-E(EDANS)-GKASPVAFP-K(Dab)-(D-Arg)-(D-Arg), CtHtrA/CmHtrA: MCA-ENLHLPLPIIF-DNP.
b. IC_{50}s for peptide substrates were determined by FRET or pNA assays with a range of concentrations of compounds. Data analysis conducted using GraphPad.
c. Protein-based substrate IC_{50}s were estimated from analysing the amount of protein substrate remaining after the assay using Coomassie-stained PAGE. Protein substrates used were CtHtrA: β-casein, CmHtrA: β-casein, DegP: β-casein, DegS: RseA (an activator peptide FFF-boc was included in this assay as required), HTRA1: Tau, HTRA2: β-casein.
NA: no substrate of this format is available for this protease.

We conducted competitive binding assays to confirm that the JO146-Cy5 activity-based probe bound to the same targets as JO146. *C. trachomatis* infected and uninfected HEp-2 cells were harvested at 22 h PI and a titration of JO146 was added to either lysed or unlysed cell cultures. JO146-Cy5 was added 30 min later. The same proteins previously observed in Fig. 3A were also observed (lane 1, Fig. 3C). The binding of JO146-Cy5 to all of the proteins except the ~ 25 kDa species was competitively inhibited by JO146 pre-incubation (Fig. 3C). The ~ 25, ~ 37 and ~ 125 kDa proteins were also present in the HEp-2 only cultures. Thus, JO146 appears to bind to two mammalian proteins (~ 125 kDa and ~ 37 kDa) and to a doublet of proteins corresponding to CtHtrA. CtHtrA JO146-Cy5 binding was competitively inhibited by the addition of JO146 to live, unlysed cultures supporting the compound entering the inclusion vacuole as CtHtrA is only detected inside the inclusion at this time point (Wu *et al.*, 2011).

A JO146-biotin activity-based probe was used to isolate the labelled proteins and confirm their identities by proteomics. Purified recombinant CtHtrA was incubated with the JO146-biotin activity-based probed and streptavidin-magnetic bead binding was used to confirm that CtHtrA can be isolated using this methodology, and therefore that this activity-based probe binds to CtHtrA (Supporting data Fig. S2). We then applied this approach to chlamydial cell culture lysates to identify proteins labelled by JO146-biotin. The experiment was conducted at 24 h PI to maximize the yield of chlamydial material present and included controls of HEp-2 lysates only and *C. trachomatis* infected HEp-2 lysate without JO146-biotin. In the experiment where JO146-biotin was isolated from a *C. trachomatis* infected HEp-2 lysate we observed clumping and aggregation. This may suggest that when we are binding and isolating CtHtrA from a lysate, proteins which are bound by CtHtrA are also isolated (potentially specific or non-specific substrates). We have previously demonstrated that CtHtrA is a chaperone and protease that can form large oligomeric cages to chaperone substrates (Huston *et al.*, 2011). Therefore, it is likely that, under cell lysis conditions, CtHtrA binds to many proteins which may not necessarily be specific substrates. Accordingly, several proteins were observed to be isolated by the JO146-biotin activity-based probe streptavidin isolation which did not correspond to the proteins previously detected to be bound by JO146-Cy5 in Fig. 3 (see Fig. 4). CtHtrA was identified by mass spectrometry (as described in *Experimental procedures*) (~ 48 kDa) as the only band which corresponds to this molecular mass with two peptides identified which correspond to CtHtrA (Mascot Score 104, significance threshold $P < 0.0005$). Interestingly, we also identified the chlamydial protein MOMP (major outer membrane protein) by mass spectrometry as the 37 kDa band (Mascot score 166, five peptides, significance threshold $P < 0.0005$); however, it is possible that this is a post-lysis artefact of CtHtrA binding rather than a genuine CtHtrA substrate or JO146-biotin target. Two host cell proteins (Myosin-9 and DHX9) were also present in the JO146-biotin pull-down (and not the negative controls); however, as suggested for MOMP it is also possible that these are pulled down by CtHtrA (possible artefact given the protein binding capacity of CtHtrA) rather than being direct JO146-biotin or CtHtrA substrates (Fig. 4). Regard-

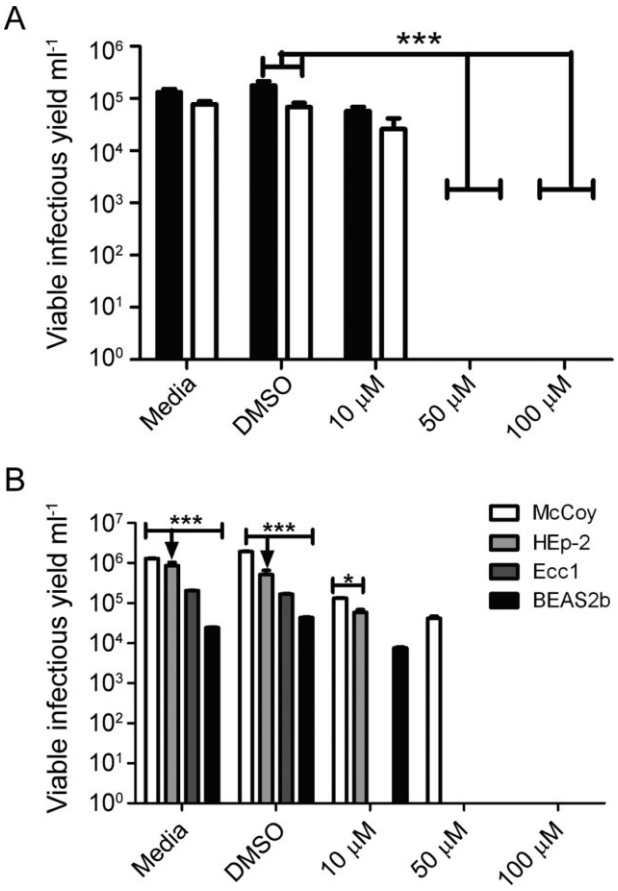

Fig. 2. Host cell protein synthesis and host cell type do not influence the effectiveness of inhibition.
A. Viable infectious yield of *C. trachomatis* at 44 h PI following cycloheximide (black bars) addition to cultures prior to JO146 addition at 16 h PI (moi 0.3). The white bars represent a control with no cycloheximide.
B. Viable infectious yield at 44 h PI of *C. trachomatis* cultured with different host cells treated with JO146 at 16 h PI. The bars represent the cell types McCoy, HEp-2, Ecc1, Beasb2b cells (left to right; moi 0.3).
The mean from three independent experiments with the standard error of the mean as error bars are shown on the graph ($n = 27$); $^{*}P < 0.05$, $^{***}P < 0.001$.

less, these results confirm that JO146-biotin binds to CtHtrA in a cell culture lysate.

The lethality of JO146 treatment relates to the timing of the chlamydial developmental cycle timing

The observed requirement of JO146 addition to be at 16 h PI for complete lethality may be a consequence of the phase of chlamydial growth (i.e. replicative phase), or alternatively may reflect a short term stability of the compound. Hence, we conducted further investigations by, first, removing the compound during the culture experiments, and second, extended culturing prior to determining viable infectious yield. JO146 was added to cultures at 16 h PI in an identical experiment to that shown in Fig. 1. JO146 was removed from the cultures by extensive washing at 20 and 24 h PI and chlamydial viability was determined at 44, 54 and 64 h PI. JO146 treatment at 16 h PI was lethal for chlamydial viability, even after extended culturing until 54 and 64 h PI (consistent with the Fig. 1 data where viability was measured at 44 h PI; see Fig. 5A). JO146 treatment was still highly effective but not completely lethal when washed out after 4 and 8 h after treatment (20 and 24 h PI respectively) with 1–2 log reduction in viability (Fig. 5B and C). The loss of viability when the compound was washed out 4 and 8 h after addition was partially rescued (~ 0.5 log) by extended culture in the absence of the compound (to 54 and 64 h PI) (Fig. 5B and C). To further explore the developmental cycle time point dependence of JO146 lethality, treatments of either isolated chlamydial EBs or host cells prior to commencing the infection was tested. In these experiments, some reduction in viability was observed with the host cell JO146 treatment prior to infection (~ 1 log; Supporting data Fig. S3).

We tested the compound's stability during the mid-developmental cycle conditions where lethality was observed, by monitoring the *in vitro* stability of the JO146-Cy5 activity-based probe. The probe was added to cell culture lysates (16 h PI) cultured under identical conditions as used for the experiment shown in Fig. 1. The cultures were then incubated for 4 and 8 h (37°C, 5% CO_2) under the standard culture conditions and the lysates were harvested and examined by SDS-PAGE to monitor JO146-Cy5 labelling. The JO146-Cy5 was stable throughout this experiment (Fig. 5D), suggesting that the critical nature of the timing of compound addition for maximum effectiveness related specifically to a developmental cycle feature of *Chlamydia* rather than compound stability.

JO146 treatment results in diminishing chlamydial inclusion vacuole size and eventual loss of inclusions over time in cell culture

We monitored inclusion vacuole size in real time using wide field microscopy after JO146 was added to HEp-2 *C. trachomatis* cultures at 16 h PI. The inclusions appear as non-stained or dark areas inside the cells which in control cultures (DMSO or media)increased in size over time (Fig. 6, and Video S1). In contrast, during JO146 treatment, the inclusions appeared to diminish in size and eventually could not be visualized (Fig. 6A). This was quantified by measuring inclusion vacuole size and number of inclusion vacuoles present confirming that the inclusions decrease in size and number during JO146 treatment (Fig. 6B and C). No significant difference was detected between DMSO and JO146 treatments for

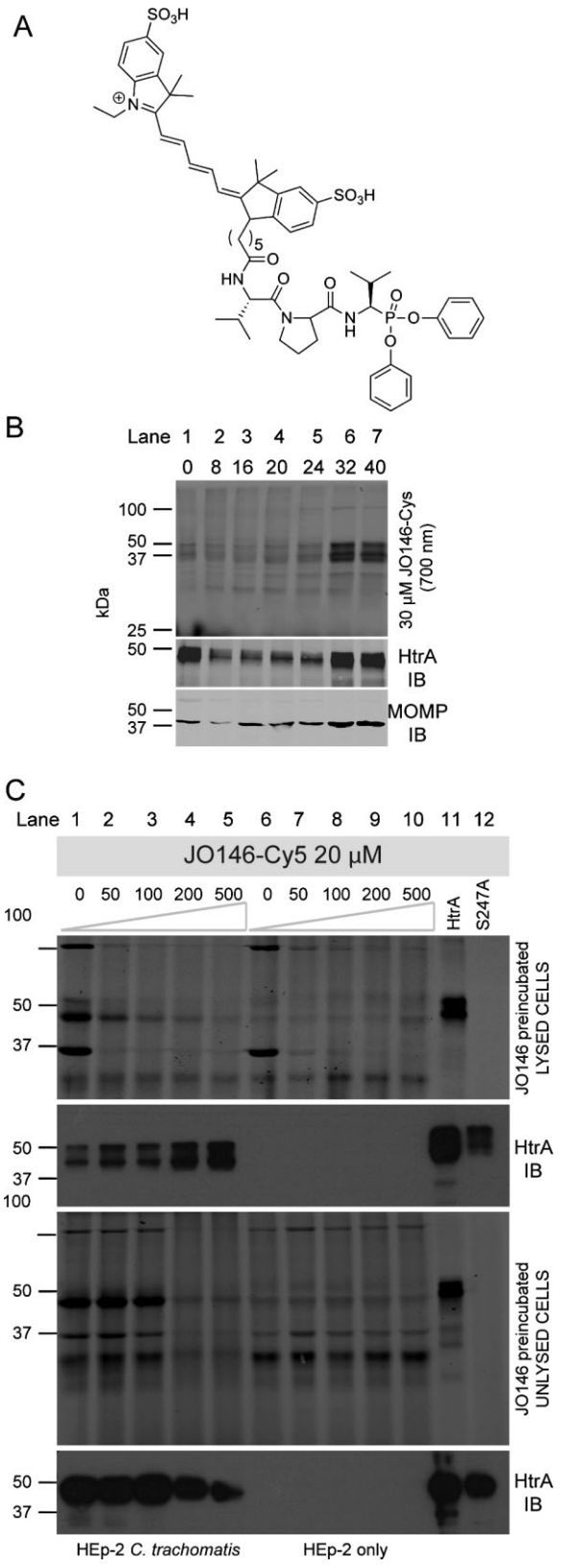

Fig. 3. Activity-based probe confirms JO146 binds *C. trachomatis* proteins including a band at the size corresponding to CtHtrA.
A. JO146-Cy5 structure.
B. JO146-Cy5 (20 μM) binding throughout the developmental cycle. CtHtrA and MOMP Western blots on the samples are shown below the Cy5 scanned gel. Lanes represent time (h PI).
C. JO146 (concentrations indicated above in μM) was added to lysed or unlysed cultures (upper and lower gels respectively) prior to lysis and binding with JO146-Cy5 (20 μM). Lanes represent *C. trachomatis* infected HEp-2 cells treated with increasing concentrations of JO146 (1–5), uninfected HEp-2 cells with the same concentrations (6–10), purified recombinant CtHtrA (11), and purified recombinant S247A CtHtrA (active-site serine mutant) (12). Corresponding CtHtrA immunoblots are shown below each gel.

number of host cells present or lost throughout the entire duration of the video (Supporting data, Fig. S4).

Confocal microscopy shows that JO146 treatment results in decreasing inclusion size and loss of chlamydial cellular morphology

The apparent diminishing size and eventual loss of inclusions from the cultures observed by real-time microscopy was further examined using immunocytochemistry and confocal laser scanning microscopy. Cultures (moi 0.3) were fixed and labelled for MOMP, β-actin (phalloidin) and nucleus (DAPI) and examined at a series of time points following JO146 addition at 16 h PI. Representative images are shown in Fig. 7A–E. The inclusions are much smaller at 24 h PI when treated with JO146 compared with the DMSO controls (Fig. 7F), and there are fewer chlamydial cells within the inclusions. In some cases no discernable cell shapes are apparent compared with the robust inclusion observed in the DMSO treated controls. The same observations were made when the *Chlamydia*

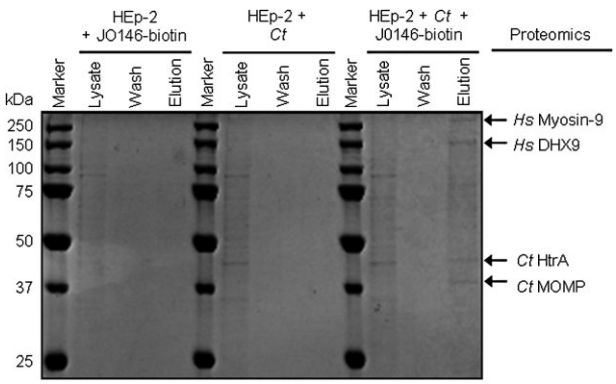

Fig. 4. JO146-biotin activity-based probe confirms JO146 is bound to CtHtrA. Samples from a dynabead-streptavidin pull-down of JO146-biotin from cell culture lysates are shown. The gel represents three individual pull-down experiments, indicated above, where the initial lysate, final wash and elution are shown on the Coomassie-stained 12% SDS-PAGE. Molecular weight marker sizes are indicated to the left. The proteomic identification of the excised bands from the *C. trachomatis* infected HEp-2 JO146-biotin pull-down lane is indicated to the right.

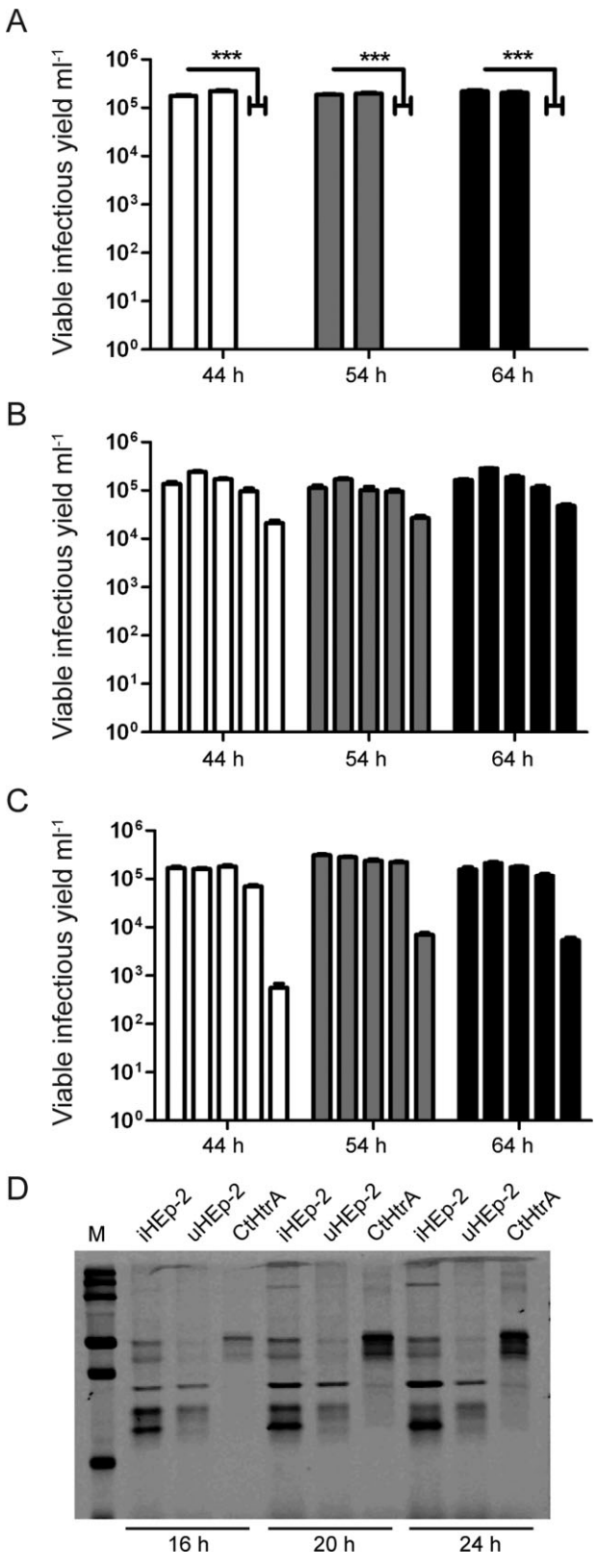

Fig. 5. JO146 treatment is most effective when maintained in the culture throughout the replicative and transition to EB developmental cycle phase.
A. Viable infectious yield of *Chlamydia* after JO146 treatments 16 h PI (compound not removed during the culture). The bars represent from left media only, DMSO and 100 μM JO146 treatment (not visible, i.e. completely lethal) when harvested at 44, 54 and 64 h PI. $^{***}P < 0.001$.
B. Viable infectious yield of *Chlamydia* after JO146 treatments 16 h PI when JO146 was washed out at 20 h PI. The cultures where harvested and viability determined at 44, 54 and 64 h PI. The bars represent (left–right): DMSO, media only, 10 μM, 50 μM, 100 μM JO146.
C. Viable infectious yield of *Chlamydia* after JO146 treatments 16 h PI when JO146 was washed out at 24 h PI. The cultures where harvested and viability determined at 44, 54 and 64 h PI. The bars represent (left–right): DMSO, media only, 10 μM, 50 μM, 100 μM JO146. The mean from three independent experiments are represented in the bar graphs and the error bars represent the standard error of the mean ($n = 27$).
D. SDS-PAGE of the JO146-Cy5 bound proteins in a lysate incubated under cell culture conditions for 4 (20 h PI) and 8 h (24 h PI). An *in vitro* stability assay was carried out where the JO146-Cy5 activity-based probe was incubated with lysates of infected (iHEp-2), uninfected HEp-2 (Hep-2) and purified recombinant CtHtrA (CtHtrA) for 4 and 8 h (20 h and 24 h indicated below the gel).

were stained using anti-HtrA antibodies and measured either by Deltavision or by Confocal Microscopy (Supporting data, Figs S5 and S6). Similar observations were made when the cultures were examined using super resolution microscopy (outlined in the supporting data results section and Fig. S7).

This observation of chlamydial inclusion development failure was further investigated by measuring EB formation over time in the presence of JO146. JO146 treatment at 16 h PI completely prevented the development of viable elementary bodies at all time points at which EBs were detected in the controls ($P < 0.001$; Fig. 7G). This was also consistent with an observed loss of the chlamydial proteins MOMP and CtHtrA without any impact on host β-actin levels over the same time-course (Supporting data Fig. S8). Viability of the host cells was monitored using a Live/Dead fixable flow cytometry assay for the same time points and there was no significant difference in numbers of dead host cells between JO146 (100 μM) treated and DMSO controls (Supporting data, Table S2 and Fig. S9).

Immunofluorescence for MOMP with LAMP1 (late endosome), or SQSTM1 (sequestosome) was conducted at 20 and 24 h PI after JO146 addition (at 16 h PI) to determine if either of the host proteins interacted directly with the JO146-treated inclusions. Additionally live monitoring of lysosome staining using lysotracker was also conducted at 20 and 24 h PI after JO146 addition (at 16 h PI). We observed no recruitment of lysosomes, or the makers SQSTM1 or LAMP1 to the chlamydial inclusion regardless of treatment conditions (JO146 or DMSO) (Supporting data, Fig. S10).

JO146 is effective in vivo *using the mouse* Chlamydia muridarum *model of disease*

The effectiveness of JO146 treatment *in vivo* was evaluated using the *Chlamydia muridarum* mouse model of genital tract infection. JO146 and JCP83 inhibited CmHtrA during *in vitro* assays, although with reduced potency compared with CtHtrA (Table 1). JO146 and JCP83 treatment of *C. muridarum* infections in *in vitro* mouse cell culture (McCoy cells) led to a ~ 2–~ 2.5 log reduction in viable infectious yield of elementary bodies, with JO146 being slightly more effective (Fig. 8A and B). The *C. muridarum* developmental cycle is complete within 26–30 h, and again the most effective time (12 h PI) for JO146 treatment was consistent with the replicative phase. Similar reduced viability was observed against *C. muridarum* when the compounds were tested using a different host cell (HEp-2) ($P < 0.001$; Supporting data, Fig. S11).

The impact of vaginal treatment with 50 mg kg^{-1} of JO146 every second day for 14 days on uninfected mice and on the progression of a vaginal *C. muridarum* infection was investigated. No toxicity was detected from JO146 treatment of uninfected mice (described in the supporting items). *C. muridarum* genital infections of progesterone synchronized female BALB/C mice were tested by treating the mice every second day of infection with vaginal administration of DMSO, or 50 mg kg^{-1} JO146. Vaginal swabs were collected every third day and the amount of viable *Chlamydia* shed from the genital tract was determined (Fig. 8C). In this experiment which involved only six mice, there was a small but statistically significant reduction ($P < 0.05$) in the total viable *Chlamydia* shed from 50 mg kg^{-1} JO146 treatments compared with DMSO control.

Discussion

A chemical approach to inhibit the serine protease CtHtrA during the chlamydial developmental cycle in human cell culture has demonstrated complete lethality for *C. trachomatis*. Specifically, treatment of cultures during the replicative phase with a CtHtrA protease inhibitor (JO146) led to lethality with no viable elementary bodies detected. This coincided with a loss of chlamydial cell morphology, diminishing inclusion size and eventual loss of detectable inclusions in the cultures. The lethality occurred independently of the host cell type, host cell protein synthesis, in the absence of any host cell toxicity or death, or any activation of the major pathogen protection pathways (lysosome or autophagy).

The compounds were identified by screening a library of serine protease inhibitor compounds using our previously established CtHtrA protease assay (Huston *et al.*, 2011). The library consisted of a collection of various electrophillic molecules that form a covalent bond with the active-site serine of serine proteases and hydrolyases. The screen identified two compounds (JO146 and JCP83) from two distinct synthetic sources with similar peptide sequences and identical primary electrophiles. When screened against a variety of proteases *in vitro*, the compounds were found to be selective towards CtHtrA. It is important to note that the activity of JCP83 and JO146 *in vitro* against protein substrates was not as effective as the *in vivo* activity observed against *Chlamydia*. It is not clear why this may be the case, although the *in vitro* substrates used were model proteins and not known substrates. JO146 and JCP83 were lethal against *Chlamydia* when added during the replicative phase of the chlamydial developmental cycle. JO146 has a lower IC_{50} value compared with JCP83 from the protease assays and was also more effective on the *in vitro* cultures. Several observations suggested a chlamydial target protein was required for the lethality of this compound. The lethal impact of JO146 was not associated with any host cellular toxicity or cell death. This lethality was impacted directly by the multiplicity of infection (amount of *Chlamydia* present in the cultures), did not require active host cell protein synthesis, and was independent of the host cell type. Although other studies have resulted in several log reductions in viability (Christian *et al.*, 2011; Stone *et al.*, 2011), this is the first time that any small molecule or inhibitor strategy has resulted in complete lethality to *Chlamydia*. There is another chlamydial protease (CPAF, chlamydial secreted protease activity factor) that may also be a critical factor for chlamydial growth. CPAF has been identified as an important protease target for intracellular impairment of chlamydial growth. The authors used a caspase-1 inhibitor (WEHD-fmk) that had previously been shown to inhibit the *in vitro* CPAF protease activity and demonstrated a 10-fold reduction in viable chlamydial yield when this compound was added at 24 h PI (Christian *et al.*, 2011). The validation of this inhibitor acting directly via binding to CPAF was the presence of golgin-84 cleavage (Christian *et al.*, 2011). However, subsequent to this publication, concerns have been raised about the role of CPAF during cell culture due to the experimental methods utilized. In particular, recent work determined that goglin-84 fragmentation may be an artefact of the chosen experimental design (Chen *et al.*, 2012). Therefore, it remains unclear if CPAF is an important protease for *Chlamydia*.

The timing of compound addition during the chlamydial developmental cycle had a significant impact on the effectiveness of JO146 treatment. JO146 was most effective when added at 16 h PI, consistent with an effect on the middle of the chlamydial replicative phase of development. JO146 was lethal when added at 16 h PI at all

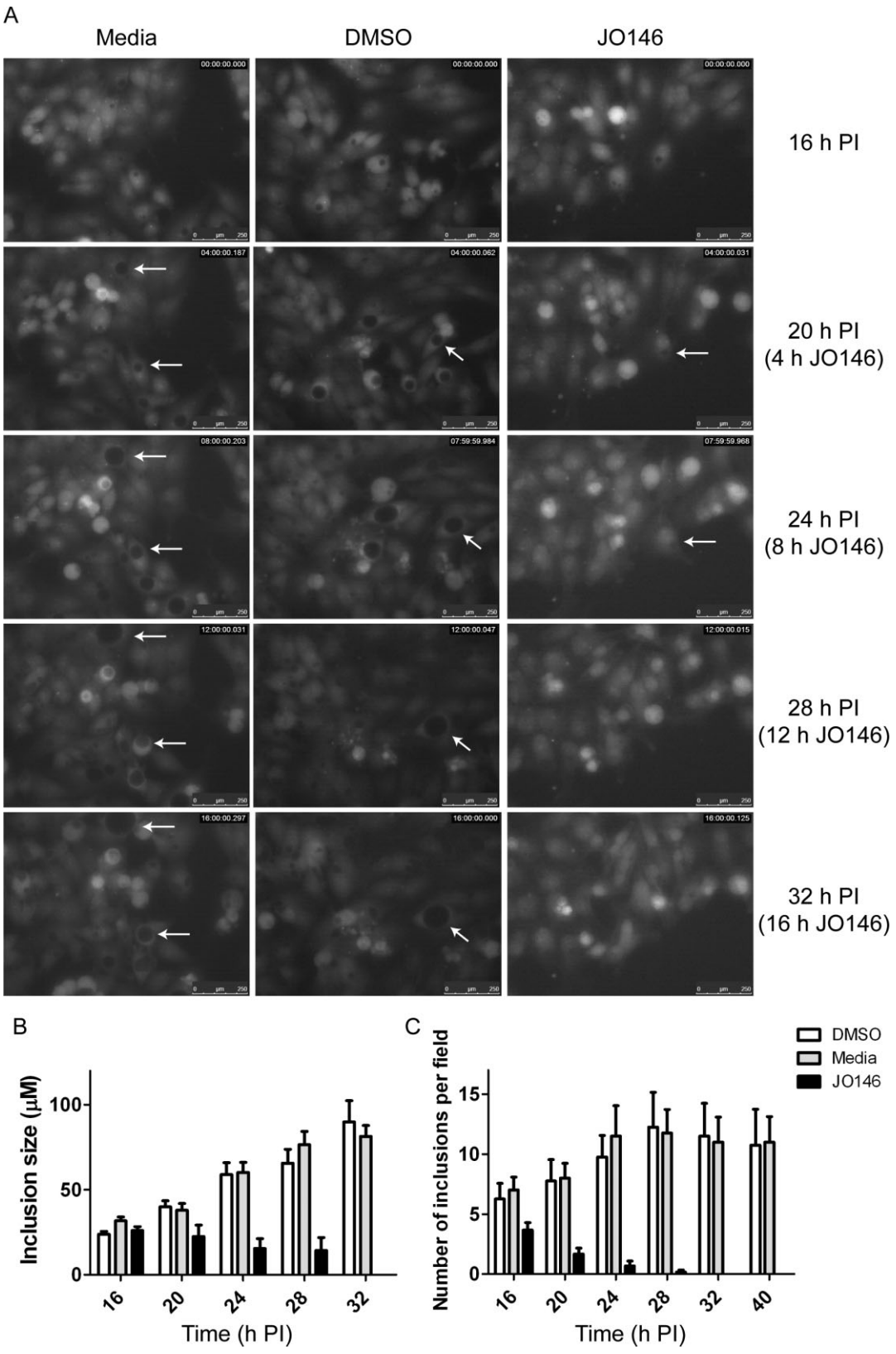
A
Media
DMSO
JO146
16 h PI
20 h PI
(4 h JO146)
24 h PI
(8 h JO146)
28 h PI
(12 h JO146)
32 h PI
(16 h JO146)
B
Inclusion size (μM)
100
50
0
16
20
24
28
32
Time (h PI)
C
Number of inclusions per field
15
10
5
0
16
20
24
28
32
40
Time (h PI)
DMSO
Media
JO146

Fig. 6. JO146 treatment leads to diminishing chlamydial inclusion vacuoles.
A. Representative images of the same location in a slide culture of a *C. trachomatis* infection of HEp-2 cells labelled with CellTracker Blue. Treatment conditions are indicated above (JO146 100 μM) and time to the right. Arrows indicate one example inclusion vacuole for each condition. The figures have had contrast adjustment which was conducted on the whole image for each figure in the series. Representative videos are provided as Video S1.
B. Analysis of real-time microscopy of JO146 treatment-impact on *C. trachomatis* inclusion size.
C. The number of visible inclusions in each field of view for each condition over time.
The mean from three independent experiments have been calculated and are presented in the graph, the error bars represent the standard error of the mean ($n = 14$ for the inclusion size, $n = 4$ for the inclusion numbers). An moi of 1 was used for the experiment.

concentrations above 10 μM. A dose of 50 μM resulted in a 1–2 log reduction in yield when added at 6, 20 or 24 h PI, but was completely lethal at 16 h PI. While we know the chlamydial developmental cycle is asynchronous, the completion of replication by binary fission is quite rapid. Miyairi and co-workers comprehensively characterized the parameters of replication and EB formation for a number of serovars and found that for serovar D, logarithmic replication occurs from approximately 12–24 h PI with a marked halt of replication from approximately 24 h PI onwards (Miyairi *et al.*, 2006). EB formation could be detected from approximately 20 h PI onwards and gradually increased until approximately 40 h PI (Miyairi *et al.*, 2006). Therefore, EB formation is highly asynchronous; however, the replicative phase is tightly defined as between 12 and 24 h PI. The data presented here strongly suggests that JO146 is only effective on chlamydial cells that are actively replicating or transitioning to EBs, as prior treatment of EBs (Fig. S2) or treatment late during the developmental cycle was not effective. The compound was most effective at 16 h PI, exactly mid-replicative phase, at 12 h PI not all cells will be replicating and after 24 h PI a significant proportion of the cells will be beginning to transition back to elementary bodies. The removal of JO146 at 24 h PI (8 h after administered) showed a 2.5 log reduction in viability indicating that the most effective phase of inhibition was throughout the replicative phase until EB formation. There may be some other 'off-target' impacts on the host cell which could explain the 0.5–1 log reductions in viability observed when JO146 was added early during the developmental cycle (8 h PI) or when some loss of viability was observed during host cell pretreatment (Supplementary data Fig. S2). However, only the 16 h PI treatment was lethal suggesting that the major impact of JO146 is specific to *Chlamydia*. Therefore, these data indicate that JO146 is inhibiting a specific function involved in replication that is essential for *Chlamydia*. Interestingly, with extended cultures (54 and 64 h PI) and following removal of the compound at 24 h PI, there was some rescue of viability indicating that the compound may be partially inducing chlamydial persistence (Fig. 5). HtrA has been described as a protease and chaperone with broad roles in protein maintenance and stress response in many bacteria (Clausen *et al.*, 2011). For some bacteria, substrates of HtrA are essential for viability and pathogenesis of the organism. Perhaps the best described example of this is the *Shigella* protein IcsA, which requires HtrA/DegP for its correct assembly. In the absence of HtrA *Shigella* does not correctly present IcsA on the surface of the cell, resulting in the inability to generate actin tails and virulence attenuation (Purdy *et al.*, 2007). The mechanism of chlamydial death observed during this study was unique, with loss of chlamydial cell structure within the inclusion as well as diminishing the chlamydial inclusion size with eventual loss of any detectable inclusions. This correlated with a loss of viable elementary bodies. This chlamydial death and inclusion loss appeared to relate directly to the observed *Chlamydia* defects and not a host mediated mechanism. Thus, it appears that addition of a CtHtrA inhibitor during the replicative phase of *C. trachomatis* disrupts the chlamydial developmental cycle, by impacting reticulate body cellular morphology, resulting in the reduction in inclusion vacuole size, and ultimate loss of inclusions from the host cell without viable elementary body formation.

The use of an activity-based probe strategy enabled direct assessment of compound selectivity. The targets of JO146 comprised a protein species corresponding to CtHtrA and two additional mammalian cell proteins. The labelled protein corresponding to CtHtrA was competitively inhibited by prior binding of JO146 during live cell culture, supporting that the compound is accessing the chlamydial inclusion as CtHtrA has only been detected inside the inclusion vacuole at this time point (Wu *et al.*, 2011). A biotin activity-based probe was used in an affinity purification experiment which further validated that JO146 binds to CtHtrA. These experiments represent the first use of an activity-based probe strategy for any protein within *Chlamydia* and provide key evidence that our lead compound JO146 preferentially binds CtHtrA.

Regardless of the mechanism of chlamydial death, JO146 was also effective *in vivo*. JO146 vaginal administration during female mice genital tract infection significantly reduced the viability of *C. muridarum*. This *in vivo* effectiveness is an exciting finding supporting the concept that chemical strategies can be applied both to

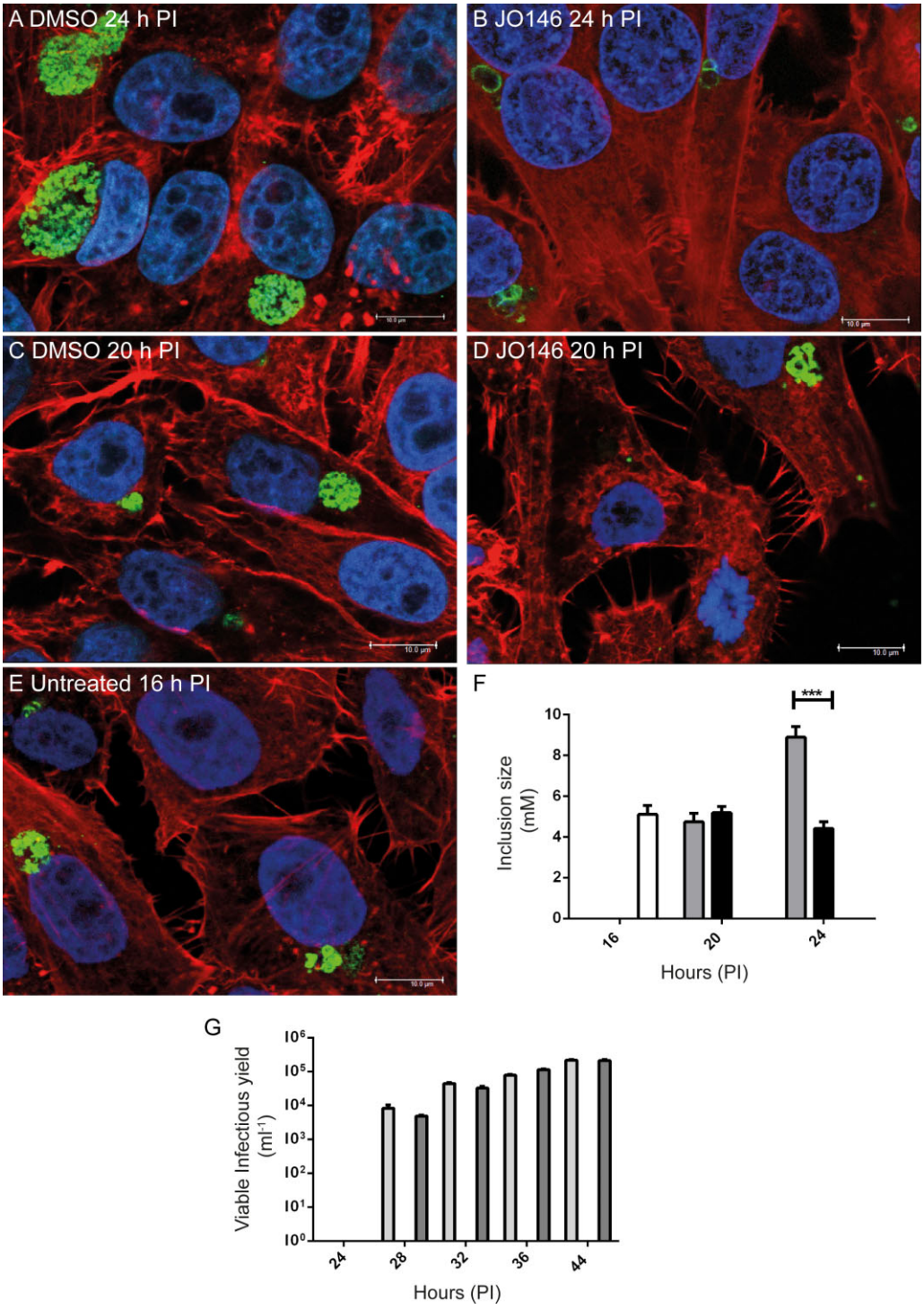

Fig. 7. Confocal microscopy analysis of *C. trachomatis* infected HEp-2 cultures indicates inclusion size diminishes after JO146 treatment (16 h PI).

A–E. Representative images from the time of treatment (16 h PI, E), 4 h after treatment (20 h PI, C and D) and 8 h after treatment 24 h PI, A and B) respectively. MOMP is stained green, cell nucleus DAPI (blue) and β-actin phalloidin (red).

F. Representation of the inclusion size (μm) of JO146-treated cells (black) and DMSO-treated cells (grey) at each time point, the white bar represented the untreated cells at 16 h PI. A minimum of 6 fields of view and 25 inclusions were measured at each time point and the mean of these data are indicated by the bars, the error bars indicate the standard error of the mean ($n = 25$); $^{***}P < 0.001$.

G. Viable infectious yield at various times (h PI). Light grey bars represent control cells (no DMSO and no JO146) dark grey bars represent DMSO-treated cells. Cells treated with 100 μM did not show any viable infection. The bars represent the mean of three independent experiments with the standard error of the mean indicated by the error bars ($n = 27$).

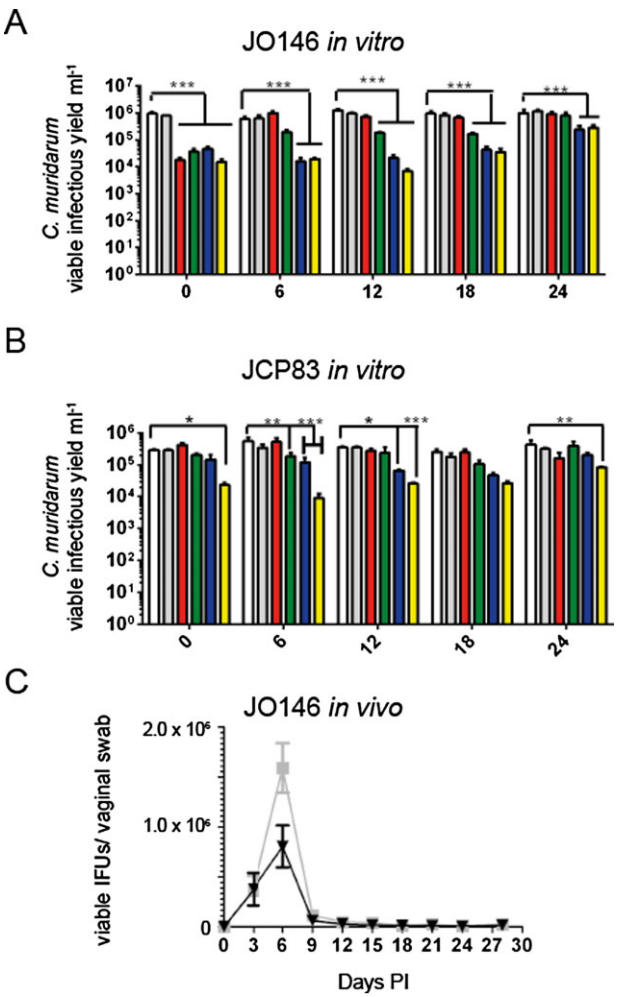

Fig. 8. Inhibitor treatment is effective *in vivo* using the mouse model of chlamydial infection.
A and B. (A) JO146 and (B) JCP83 treatment of *C. muridarum* McCoy cell cultures resulted in a significant loss of viable *Chlamydia*. The bars represent from left to right; white: DMSO control, grey: 0 μM (media only), red: 10 μM inhibitor, green: 50 μM inhibitor, blue: 100 μM inhibitor, and yellow: 150 μM inhibitor.
$^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.
C. JO146 reduced shedding of viable *C. muridarum* from the vagina of infected mice when administered every second day during an infection. The bars on the graph represent the mean from a single experiment with the error bars indicating the standard error of the mean (six animals, triplicate swabs from each animal at each time point were analysed $n = 18$).

investigate the functional role of proteases in the unique biology of this organism, but can also be applied to demonstrate *in vivo* significance. This is the first report of an inhibitor or small molecule for *Chlamydia* that has been successfully applied *in vivo* to treat *Chlamydia* infection. Together, these findings demonstrate that CtHtrA is essential for *Chlamydia* replication and provides proof of concept that CtHtrA is a suitable candidate for future drug development.

Experimental procedures

Protease activity, inhibitor screening and synthesis

Serine protease activity for CtHtrA was monitored using a previously described *in vitro* assay with the substrate (MCA-ENLHLPLPIIF-DNP) (Huston *et al*., 2011). A library of serine protease inhibitor compounds including isocoumarins, and peptides with various electrophiles was screened against CtHtrA activity and hits tested against other proteases. The screen was initially conducted using all compounds at a 500 μM concentration, compounds which showed a complete loss of activity were selected and further screened at a series of concentrations to identify those most effective at lower concentrations *in vitro*. The top hits were validated for purity and structural integrity using mass spectrometry, this led to the selection of JO146 and JCP83 as the most effective compounds which were structurally intact and pure. The proteases tested included trypsin, chymotrypsin, elastase, and recombinant forms of HTRA1 (human), HTRA2 (human), *Escherichia coli* DegS and DegP [using previously published assays (Wilken *et al*., 2004; Merdanovic *et al*., 2010)]. JO146 [Boc-Val-Pro-Val^{P}(OPh_2)], JO146-biotin and JCP83 [Boc-Ala-Pro-Val^{P}(OPh_2)] were synthesized commercially using standard protocols when additional stocks were required (VCare, China). The activity-based probe JO146-Cy5 was synthesized from JO146 by first acidic removal of the *tert*-butyloxycarbonyl group using a 1/1 mixture of dichloromethane and trifluoroacetic acid for 30 min at room temperature. After concentration *in vacuo*, the resulting free N-terminus was capped with 1 molar equivalent of Cy5-*N*-hydroxysuccinimide ester and five equivalents of *N*,*N*-diisopropylethylamine in DMSO for 1 h to give JO146-Cy5 after HPLC purification. MS (ESI): *m/z* 570.9 [½(2M+H)]$^{+}$, 1140.8 [M]$^{+}$.

Chlamydia *culture*

Chlamydia trachomatis serovar D/UW-3/Cx was routinely cultured in HEp-2 cells on DMEM, 10% Fetal calf serum (heat-inactivated serum was used) media, at 37°C 5% CO_2. *C. muridarum* strain Weiss was routinely cultured in McCoy cells (McCoy B) on DMEM, 10% fetal calf serum, at 37°C, 5% CO_2. Ecc1 (an endometrial cancer cell line) and BEAS2b (a human lung epithelial cell line) were cultured on the DMEM, 10% FCS, at 37°C 5% CO_2. Inhibitor experiments were routinely conducted in 48-well plates seeded with 20 000 host cells per well 24 h prior to the *Chlamdyia* infection. Cycloheximide was not added to any experiments except that in Fig. 2A (1 μg ml^{-1}). The viable infectious yield was determined from cultures harvested at the completion of the developmental cycle during which inhibitor treatment was conducted (time of harvest is indicated on the figure). The cultures harvested in SPG were serially diluted and cultured in fresh HEp-2 monolayers at 30 h PI the cultures were fixed and stained for microscopy. The number of inclusions visible at 30 h PI was then determined by counting inclusions from at least eight representative fields of view in triplicate wells for each serial dilution (with less than 80% infected host cells only considered valid) and extrapolating the field of view size to the size of wells to calculate the total number of inclusions

in the well, dilutions and volumes added to the wells were then accounted for to give the viable IFU ml^{-1}. Quantitative analysis of viability and morphological properties was conducted using GraphPad Prism, statistical analysis was routinely conducted using two-way ANOVA and Bonferroni post-tests (typically relative to the DMSO control).

Microscopy

Chlamydia trachomatis cultures were examined using immunofluorescence using the Leica SP5 Confocal microscope with antibodies against CtHtrA, MOMP (Biodesign), LAMP-1 (AbChem) and SQSTM1 (AbCam), and secondary antibodies conjugated to Alexafluor dyes (Invitrogen) (Huston *et al.*, 2008). Live cell imaging was performed using a Leica AF6000 widefield microscope. The CellTracker concentration was optimized so that it did not penetrate the chlamydial inclusion. 1 μM CellTracker (Invitrogen) was added to cultures grown in glass-bottomed, chamber-welled slides 45 min before JO146 addition. Images were constructed using the Leica application suite. Where indicated immunofluorescence was also monitored using a Deltavision (personal DV deconvolution microscope) (Applied Precision, Issaquah, WA). 3D structured illumination microscopy (3D-SIM) of the *Chlamydia* inclusions was conducted using a Deltavision OMX OMX Imaging System with Blaze module as previously described (Strauss *et al.*, 2012). Raw images were processed and reconstructed as previously described (Gustafsson *et al.*, 2008; Schermelleh *et al.*, 2008).

Activity gels, immunoblots and PAGE

Activity-based probe binding activity in cell culture and cell lysates was monitored using polyacrylamide gel electrophoresis and scanning of the gels using the Li-Cor Odyssey at 700 nm. Activity-based probe binding was conducted on cultures from T25 flasks at different time points, while the competitive binding assays were conducted on cultures from T80 flasks harvested at 22 h PI. Western blots for CtHtrA and MOMP were conducted as previously described (Huston *et al.*, 2008).

Affinity purification and proteomics

JO146-biotin was used for affinity purification experiments. Streptavidin Dynabeads (Invitrogen, Australia) were used to affinity purify JO146-biotin from cell culture lysates in accordance with the manufacturer's instructions. Cells were harvested, washed and then suspended in RIPA buffer (Peirce, Australia) and incubated on a turn wheel at room temperature for 1 h to lyse the cells. Debri and unlysed cells were removed from the suspension by centrifugation at 10 000 *g* for 10 min. JO146-biotin or DMSO was added to the lysates and incubated on a turn wheel at room temperature for 30 min prior to addition of streptavidin Dynabeads. The samples were harvested using a magnetic block to allow buffer changes, three PBS washes and four PBST washes were conducted prior to elution of bound products from the beads using 0.1% SDS and boiling. Samples were analysed by SDS-PAGE prior to gel excision for proteomics.

Gel excised bands were then analysed by the Australian Proteomics Analytical Facility. Gel slices were cut up, washed and dried prior to rehydration with 100 ng of trypsin in 25 mM ammonium bicarbonate. After an overnight digestion at 37°C, peptides were extracted twice with a solution containing 50% acetonitrile and 5% formic acid. The sample was then concentrated on a peptide trap column, prior to LC and in-line mass spectrometry. LC eluent was subject to positive ion nanoflow electrospray MS analysis in an information dependant acquisition mode (IDA). In the IDA mode a TOFMS survey scan was acquired (m/z 350–1200, 0.5 s), with 10 largest multiply charged ions (counts > 150) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 100 ms (m/z 100–1500) with rolling collision energy (ESI-QUAD-TOF). The peak lists of the LC/MS/MS data were generated using Analyst 2.0 MASCOT script and searched by Mascot against Human and Bacteria databases using MS/MS Ion search. Significance threshold for Human samples was ($P < 0.01$), Bacterial samples ($P < 0.0005$). This work was undertaken at APAF the infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS).

Animal model

All animal work must have been conducted according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, which has been embodied in the Queensland Animal Care and Protection Act 2001. The purpose of the Code is to ensure the humane care of animals used for scientific purposes, including teaching. QUT is accredited to conduct these activities. Animal ethics approval was granted from the QUT Animal Research Ethics Committee Approval number 1100000607. Female BALB/c mice provided by the Animal Resource Centre (Australia) were infected with 5×10^4 *C. muridarum* intravaginally. Methodology is described in the supplementary data.

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Supporting information

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