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

Short title: Essential serine protease for *Chlamydia*

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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. 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 *C. trachomatis* strain, JO146 was demonstrated to inhibit 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 phase and these findings provide proof of concept that proteases can be targetted for anti-microbial therapy for intracellular pathogens.

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 occular infecting serovars are the most common cause of preventable blindness worldwide (WHO, 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 & 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 for pathogenic bacteria (Chitlaru *et al.*, 2011, Hoy *et al.*, 2010). This protease has been described in *C. trachomatis* using *in vitro* and microscopy methods, and potentially functions as both a bacterial cell associated protease and as a secreted virulence factor (from 28 hours post infection) (Huston *et al.*, 2007, Huston *et al.*, 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 critical for viable infectious yield (Heuer *et al.*, 2009).

Other chlamydial virulence factors have also been uncovered using small molecule approaches, including by application of 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 used a CtHtrA protease inhibitor, JO146, to demonstrate an essential role for CtHtrA in developmental cycle progression during the chlamydial replicative phase. JO146 treatment resulted in chlamydial cell morphology loss, the diminishing of inclusion vacuoles and eventual loss and complete bacterial lethality by 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. Two inhibitors

were identified, JO146 and JCP83 (IC_{50}s: 12.5 μM, and 47.19 μM). Both are peptides with a C-terminal phosphonate 'warhead' which reacts irreversibly with the protease active site serine residue (Fig. 1A) (Oleksyszyn & Powers, 1991) (Jackson *et al.*, 1998), they differ only in the P3 position (Val/Ala). The both have valine as the P1 residue and P2 proline residues. The two inhibitors were quite selective towards CtHtrA when screened against a series of other serine and HtrA family proteases (Table 1). Inhibition was only observed for HTRA2 and elastase. Neither was cytotoxic for McCoy and HEp-2 cells, common cell lines used for *Chlamydia* culture. Specifically, no cell lysis or impaired metabolic turnover was detected 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* cultures at different time points throughout the developmental cycle (Fig. 1B) (lag ~8h, 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). The role of host cell protein synthesis during JO146 inhibition (16 h PI) was tested at an MOI of 0.3 in HEp-2 cells. The viability was determined at 44 h PI and the lethality of JO146 was maintained in the presence of cycloheximide (Fig. 2A).

C. 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* D at 100 μM doses in McCoy cells. These data support that JO146 activity is dependent on the amount of *Chlamydia* present, does not require host cell protein synthesis and is not dependent on the host cell type.

A JO146 activity based probe binds CtHtrA during cell culture

A JO146-Cy5 activity-based probe was used to monitor inhibitor protein binding throughout the *C. trachomatis* developmental cycle (Fig 3A). This probe was synthesised to retain the tri-peptide sequence and functional group which binds to the active site serine but has an additional Cy5 fluorophore. Three predominant bands were observed with one additional faint band. A dominant doublet of bands around ~48-50 kDa was observed to be bound by JO146-Cy5 (Fig 3A). These bands corresponded to the banding pattern of CtHtrA throughout the developmental cycle (western blot shown below, Fig. 3A). This CtHtrA doublet has also been observed previously by another group with their own antibodies (Wu et al., 2011). The

high levels of CtHtrA present at time 0 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 intense band was at approximately ~37kDA. Two additional, less intense bands were detected at approximately ~25 kDa and ~125 kDa (Fig. 3A).

Competitive binding assays were conducted 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 thirty minutes later JO146-Cy5 was added. The same bands previously observed in Fig. 3A were also observed (lane 1, Fig. 3B). The binding of JO146-Cy5 to all of these bands except the ~25 kDa band was competitively inhibited by JO146 pre-incubation (Fig. 3B). The ~25, ~37 and ~125 kDa bands 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 band corresponding to CtHtrA. CtHtrA JO146-Cy5 binding was competitively inhibited by the addition of JO146 to live unlysed cultures supporting that the compound enters the inclusion 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 bands of interest and confirm their identities by proteomics. The ability to pull down purified recombinant CtHtrA bound to JO146-Biotin using streptavidin beads was confirmed (Supporting data Fig. S1.). This approach was then used on chlamydial cell culture lysates. The experiment was conducted at 24 h PI to maximise the yield of chlamydial material present and included controls of HEp-2 lysates only and HEp-2 *C. trachomatis* lysate without JO146-Biotin. In the experiment where JO146-biotin was pulled down from a HEp-2 *C. trachomatis* lysate we observed clumping and aggregation. This may suggest that when we are pulling down

CtHtrA from a lysate we are actually also pulling down proteins (potentially specific or non-specific substrates) which are bound by CtHtrA. We have previously demonstrated that CtHtrA is a chaperone and protease which 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 in a chaperone like activity which may not necessarily be specific substrates. Accordingly, several bands were observed in the elution from the pull down which did not correspond to the size of the bands previously detected to be bound by JO146-Cy5 in Fig 3 (Fig. 4.). CtHtrA was identified as the band in this experiment which was also present at the same size as the predominant band bound by JO146-Cy5 (~48 kDa). Interestingly, we also identified the chlamydial protein MOMP (major outer membrane protein), 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.). However, in spite of these additional bands observed this experiment demonstrates that JO146-biotin does bind 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. In order to further investigate why the complete lethality was only observed within a specific time frame further analysis were

conducted by firstly, removing the compound during the culture experiments, and secondly 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 viability was determined at 44, 54, and 64 h PI. JO146 treatment at 16 h PI was completely lethal for chlamydial viability, even after extended culturing until 54 and 64 h P (consistent with the fig 1 data where viability was measured at 44 h PI) (Fig. 5A). JO146 treatment was still highly effective but not completely lethal when washed out after 4 and 8 hrs after treatment (20 and 24 h PI respectively) with 1-2 log reduction in viability detected compared to the controls (Fig. 5B & C). This loss of viability when the compound was washed out 4 and 8 h after addition was partially rescued by extended culture in the absence of the compound (to 54 and 64 h PI) although this was only by ~0.5 log (Fig. 5B & C). To further explore the developmental cycle time point dependency of JO146 lethality, treatments of either isolated chlamydial EBs or host cells prior to commencing the infection was tested. Neither of these treatments was completely lethal (consistent with Fig. 1 data), although some reduction in viability was observed with the host cell JO146 treatment prior to infection (~1 log) (Supporting data Fig. S2).

In order to establish if the compound is stable during the mid-developmental cycle conditions where lethality was observed an *in vitro* stability experiment using the JO146-Cy5 Activity-based probe was conducted. The JO146-Cy5 Activity-based probe was added to cell culture lysates (16 h PI) cultured under identical conditions as used for the experiment shown in Fig. 1. In this case the cell cultures were lysed prior to compound addition because the activity based probe version of JO146 is 539 Da larger than JO146 and not likely to be cell permeable. The cultures were then incubated for 4 and 8 hrs (37°C, 5% CO_2) under the standard culture conditions and the lysates were harvested into SDS-PAGE buffer and examined by SDS PAGE to monitor the presence of the JO146-Cy5 compound. As shown in

Fig. 5D, the JO146-Cy5 compound remains present and bound to the same bands during the time of this experiment implying that the critical nature of the timing of the compound addition relates specifically to a developmental cycle feature of *Chlamydia* rather than stability of the compound after addition.

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

JO146 was added to HEp-2 *C. trachomatis* cultures at 16 h PI and the progression of the inclusion vacuole size was monitored in real time using wide field microscopy. 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 visualised (Fig. 6A). This was quantified by measuring inclusion size and number of inclusions present confirming that the inclusions decrease in size and number during JO146 treatment (Fig. 6B-C). No significant difference was detected between DMSO and JO146 treatments for number of host cells present and the number of host cells which appear to die with and without an inclusion visible throughout the entire duration of the video (Supporting data, Fig. S3).

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, phalloidin (β-actin) and DAPI (nucleus) and examined at a series of time points after 100 μM 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 to the DMSO controls (Fig. 7F). There appear to be far fewer chlamydial cells within the inclusions, in some cases no discernable cell shapes are apparent at all compared to the regular and numerous circular cell shapes stained for in the DMSO treated controls. These observations were also consistent when the *Chlamydia* were stained using anti-HtrA antibodies and measured either by Deltavision or Confocal Microscopy (Supporting data, Fig. S4 and S5). Similar observations were made when the cultures were examined using super resolution microscopy (outlined in the supporting data results section and Fig. S6).

This observation of chlamydial inclusion development failure was further investigated by measuring EB formation in the presence of JO146 over time. 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 Actin levels over the same time course (Supporting data Fig. S7). 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. S8).

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. No recruitment of lysosomes, SQSTM1, or LAMP1 to the chlamydial inclusion was observed regardless of JO146 or DMSO treatment (Supporting data, Fig. S9).

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

The effectiveness of JO146 treatment *in vivo* was evaluated using the *C. muridarum* mouse model of genital tract infection. JO146 and JCP83 inhibited CmHtrA during *in vitro* assays, although the IC_{50s} were not as low as those for CtHtrA (Table 1). JO146 and JCP83 treatment of *C. muridarum* infections in mouse cell culture (McCoy cells) *in vitro* led to a ~2-~2.5 log reduction in viable infectious yield of elementary bodies, with JO146 slightly more effective (Fig. 8A-B). The *C. muridarum* developmental cycle is complete within 26-30 h, and again the most effective time for JO146 treatment was consistent with the replicative phase. Neither compound was completely lethal when administered during HEp-2 *C. muridarum* infections although JO146 was the most effective ($p<0.001$) (Supporting data, Fig. S10).

The impact of vaginal treatment with 50 mg/kg 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 synchronised female BALB/C mice were tested by treating the mice every second day of infection with vaginal administration of DMSO, or 50 mg/kg JO146. Vaginal swabs were collected every third day and the amount of viable *Chlamydia* shed from the genital tract was determined (Fig. 8C). There was a significant difference ($p<0.05$) in the total viable *Chlamydia* shed from 50 mg/kg JO146 treatments compared to DMSO control.

Discussion

A chemical approach to inhibit the serine protease CtHtrA during the chlamydial developmental cycle in human cell culture has demonstrated completely lethality for *C. trachomatis*. Specifically, treatment of cultures during the replicative phase with a CtHtrA protease inhibitor (JO146) led to complete 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, and 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 peptides with war-heads or functionally reactive groups which form a covalent bond with the active site serine. The screen identified two compounds (JO146 and JCP83) from two distinct synthetic sources both with very similar peptide sequences and the same reactive chemistries. When screened against a variety of proteases *in vitro* the compounds were quite selective towards CtHtrA. JO146 and JCP83 were demonstrated to be lethal against *Chlamydia* when added during the replicative phase of the chlamydial developmental cycle. JO146 has a lower IC_{50} value from the protease assays (compared to JCP83) and was also more effective on the *in vitro* cultures. Several observations supported that 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, 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 published to an important protease target for intracellular impairement of chlamydial growth. The authors used a caspase-1 inhibitor (WEHD-fmk) which 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 considerable doubt has been shed on the role of CPAF during cell culture due to the experimental methods utilised, in particular this more recent work identified that goglin-84 fragmentation may in fact be an artefact of 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 impacted on the effectiveness of JO146 treatment. JO146 was most effective when added at 16 h PI which is the middle of the chlamydial replicative phase of development. JO146 was completely lethal when added at 16 h PI at all concentrations above 10 μM. 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. This suggests that the CtHtrA function which is essential for *Chlamydia* is a replicative phase specific function. Whilst we know the chlamydial developmental cycle is asynchronous, the completion of replication by binary fission been described as quite rapid. Miyairi and co-workers comprehensively characterised 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 quite tightly defined as between 12 and 24 hr PI. The data presented here strongly supports that JO146 is only effective on those chlamydial cells which are actively replicating or transitioning to EBs, as prior treatment of EBs (Fig. S2) or treatment late during the developmental cycle was not effective. At 16 h PI, exactly mid-replicative phase the compound is most effective, 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 pre-treatment (Supplementary data Fig S2.). However, only the 16 h PI treatment was completely lethal suggesting that the major impact of JO146 is specific to *Chlamydia*. Interestingly, with extended cultures (54 and 64 h PI) after 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). However, extended culture did not restore any viability when JO146 was not removed from the cultures demonstrating that so long as the compound remains present throughout the replicative phase and during the transition to EB phase it is completely lethal for *Chlamydia*. Therefore, these data combined indicate that JO146 is inhibiting a replication phase specific function which is essential for *Chlamydia*. Whilst HtrA has been described as a general protein protection

protease and chaperone with broad roles in general protein maintenance and stress response in many bacteria it is also known for other bacteria that quite specific protein substrates of HtrA are essential for viability and pathogenesis of the organism. Perhaps the best described example of this is that the *Shigella* protein IcsA requires HtrA/DegP for it's correct assembly, in the absence of HtrA *Shigella* does not correctly present IcsA on the surface of the cell, and therefore the IcsA function of recruiting Actin to generate actin tails and invade new host cells is impaired in *Shigella htrA*$^{-}$ mutants leading to virulence attenuation (Purdy *et al.*, 2007). Therefore, in light of these known functions of other bacterial HtrAs and the data presented here it seems likely that JO146 is inhibiting a replication specific function of CtHtrA which is essential for *Chlamydia*.

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 complete 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 replicative phase of *C. trachomatis* disrupts the chlamydial developmental cycle, by impacting reticulate body cellular morphology, resulting in the inclusion vacuoles diminishing in size, and being ultimately lost from the cell without viable elementary body formation.

The use of an activity-based probe strategy enabled validation that the compound was selective to CtHtrA and only two other proteins *in vivo*. The targets of JO146 comprised a protein band corresponding to CtHtrA and two additional mammalian cell protein bands. Competition assays were used to confirm that the activity-based probe binding was consistent with JO146 binding. The band 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 a pull down experiment which further validated that JO146 binds to CtHtrA in the cell culture lysate. These experiments represent the first use of an activity-based probe strategy for any target within *Chlamydia* and provide key evidence that this compound is relatively specific for CtHtrA.

The observation of loss of chlamydial cell structure, diminishing inclusion sizes, and failure to progress to elementary body formation also supports that this inhibition impacts on replicative phase cellular properties. The loss of cell structure is consistent with our previous *in vitro* observation that CtHtrA is activated and forms chaperone-like oligomers using outer membrane protein assembly sequences (Huston et al., 2011).

Regardless of the mechanism of chlamydial death, JO146 was also shown to be 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 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* being successfully applied *in vivo* for *Chlamydia*, with all other published inhibitors not yet tested in the animal model.

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 war-head chemistry was screened against CtHtrA activity and hits tested against other proteases. The proteases tested included trypsin, chymotrypsin, elastase, and recombinant forms of HTRA1 (human), HTRA2 (human), *Escherichia coli* DegS and DegP, (using previously published assays (Merdanovic *et al.*, 2010, Wilken *et al.*, 2004)). The lead compounds from the screen were validated by mass spectrometry. JO146 [Boc-Val-Pro-Val^{P}(OPh_2)], JO146-Biotin and JCP83 [Boc-Ala-Pro-Val^{P}(OPh_2)] were synthesised commercially using standard protocols when additional stocks were required (VCare, China). The activity-based probe JO146-Cy5 was synthesised from JO146 by first acidic removal of the *tert*-butyloxycarbonyl group using a 1/1 mixture of dichloromethane and trifluoroacetic acid for 30 minutes at room temperature. After concentration *in vacuo*, the resulting free N-terminus was capped with 1 molar equivalent of Cy5-*N*-hydroxysuccinimide ester and 5 equivalents of *N*,*N*-diisopropylethylamine in DMSO for 1 hr 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 media, at 37°C 5% CO_2. *Chlamydia muridarum* strain Weiss was routinely cultured in McCoy cells 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 20000 host cells per well 24 hours prior to the *Chlamdyia* infection. Cycloheximide was not added to any experiments except that in Fig. 2*A* (1 μg/ml). 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 8 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. Quantitative analysis of viability and morphological properties was conducted using GraphPad Prism, statistical analysis was routinely conducted usign 2-way Anova and Bonferroni post-tests (typically relative to the DMSO control).

Microscopy

C. trachomatis cultures were examined using immunofluorescence using the Leica SP5 Confocal microscope with antibodies against CtHtrA, MOMP (Biodesign), LAMP-1 (AbChem), and SQSTM1 (AbChem), and secondary antibodies conjugated to Alexafluor dyes (Invitrogen) (Huston et al., 2008). Live cell imaging using a Leica AF6000 widefield microscope. The CellTracker concentration was optimised 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 mins 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 Inc, Issaquah, WA). 3D structured illumination microscopy (3D-SIM) of the *Chlamydia* inclusions was conducted using a Deltavision OMX OMX Imaging Sytem with Blaze module as previously described (Strauss *et al.*, 2012). Raw images were processed and reconstructed as previously described (Schermelleh *et al.*, 2008, Gustafsson *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 700nm. Activity-based probe binding was conducted on cultures from T25 flasks at different time points, whilst 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).

Pull-down and proteomics

JO146-biotin was used for pull-down experiments. Streptavidin Dynabeads (Invitrogen, Australia) were used to pull down from cell culture lysates in accordance with the manufacturers instructions. Cells were firstly harvested form the flasks using trypsin. Cells were then washed in PBS three times to remove the trypsin. The cells were suspended in RIPA buffer (Peirce) and incubated on a turn wheel at room temperature for one hour to lyse the cells. Debri and unlysed cells were removed from the suspension by centrifugation at 10

000 × g for 10 mins. JO146-Biotin or DMSO was added to the lysates and incubated on a turn wheel at room temperature for 30 mins prior to addition of streptavidin Dynabeads. The samples were harvested using a magnetic block to allow buffer changes, 3 PBS washes and 4 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 into smaller pieces. Gels were washed three times with 50% acetonitrile in 50 mM ammonium bicarbonate (NH_4HCO_3) and then dried. Samples were rehydrated with 100 ng of trypsin in 25 mM ammonium bicarbonate then covered with the minimum volume of ammonium bicarbonate. After an overnight digestion at 37 ℃, peptides were extracted twice with a solution containing 50% acetonitrile and 5% formic acid. The extracted digests were vacuum-concentrated, centrifuged then run on 5600. The Sample (10 µL) was injected onto a peptide trap (Michrome peptide Captrap) for pre-concentration and desalted with 0.1% formic acid, 2% ACN, at 5 µL/min for 10 minutes. The peptide trap was then switched into line with the analytical column. Peptides were eluted from the column using a linear solvent gradient from H_2O:CH_3CN (98:2; + 0.1% formic acid) to H_2O:CH_3CN (10:90; + 0.1% formic acid) at 600 nL/min over a 70 min period. The 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 second), with ten largest multiply charged ions (counts >150) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 100 milli-seconds (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 x 10^4 *C. muridarum* intravaginally. Methodology is described in the supplementary data.

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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 (bcasein)
CmHtrA	47 μM (± 7.19 μM)	~100 μM	93.69 μM (± 12.18 μM)	~400 μM (bcasein)
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 (bcasein)
DegS	NA	>500 μM	NA	>500 μM (RseA)
HTRA1	> 200 μM	>200 μM	>200 μM	~200 μM (bcasein)
HTRA2	NA	~75 μM	NA	~150 μM (bcasein)

[a] Peptide substrates were as follows; chymotypsin: AAF-pNA (SigmaAlrich), trypsin: benzyl DL-R-pNA (SigmaAldrich), elastase: Nmethylsucc – AAPV-pNA (SigmaAldrich), DegP: DPMFKLV-pNA, and HTRA1: (D-Arg)-(D-Arg)-E(EDANS)-GKASPVAFP-K(Dab)-(D-Arg)-(D-Arg).

[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.

Figure Legends

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. (C-F) Viable infectious yield of *C. trachomatis* at the conclusion of the developmental cycle (44 h post infection (h PI)). 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, gray: 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. Data are presented as mean ± S.E.M, * indicates $p<0.05$, ***$p<0.001$ (n = 6 from three independent experiments).

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). Data are presented as mean ± S.E.M, * indicates $p<0.05$, ***$p<0.001$ (n = 27, data from three independent experiments are included).

Fig. 3. Activity-based probe confirms JO146 binds *C. trachomatis* proteins including a band at the size corresponding to CtHtrA. (A) JO146-Cy5 binding throughout the developmental cycle. HtrA and MOMP western blots on the samples are shown below the Cy5 scanned gel. Lanes represent time (h PI). (B) 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. 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.

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 elutions 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 of the figure.

Fig. 5. JO146 treatment is most effective when maintained in the culture throughout the replicative and transition to EB developmental cycle phase. (A) SDS-page gel 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). (B) 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. (C*)* 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. (*D*) 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.

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. Data are presented as mean ± S.E.M, * indicates $p<0.05$, ***$p<0.001$

(n = 14 for the inclusion size, n = 4 for the inclusion numbers). An MOI of 1 was used for the experiment.

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 & D) and 8 h after treatment 24 h PI A & B) respectively. MOMP is stained green, DAPI (nucleus): blue, and phalloidin (β-actin): red. (F) Representation of the inclusion size (μm) of JO146-treated cells (black) and DMSO-treated cells (gray) at each time point. White bars are untreated cells. A minimum of 6 fields of view and 25 inclusions were measured at each time point. Data are presented as mean ± S.E.M, *** $p<0.001$ (n = 25). (G) Viable infectious yield at various time points PI. Light gray bars represent control cells (no DMSO and no JO146) dark gray bars represent DMSO-treated cells. Cells treated with 100 μM did not show any viable infection.

Fig. 8. Inhibitor treatment is effective *in vivo* using the mouse model of chlamydial infection. (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, gray: 0 μM (media only), red: 10 μM inhibitor, green: 50 μM inhibitor, blue: 100 μM inhibitor, and yellow: 150 μM inhibitor. (C) JO146 reduced shedding of viable *C. muridarum* from the vagina of infected mice when administered every second day during an infection. Data are presented as mean ± S.E.M (6 animals, triplicate swabs from each animal at each time point were analysed n = 18).