In vitro susceptibility of recent *Chlamydia trachomatis* clinical isolates to the CtHtrA inhibitor JO146

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Abstract

The present study aimed to establish if a previously identified *Chlamydia trachomatis* HtrA (CtHtrA) inhibitor, JO146, is effective against currently circulating clinical isolates to validate if CtHtrA is a clinically relevant target for future therapeutic development. Inhibition of CtHtrA during the middle of the chlamydial replicative cycle until the completion of the cycle resulted in loss of infectious progeny for six unique clinical isolates representing different serovars. This supports the potential for CtHtrA to be a clinically relevant target for development of new therapeutics and suggests the importance of further investigation of JO146 as a lead compound.

1.   Introduction

Chlamydia (C.) trachomatis is an obligate intracellular bacterial pathogen and is a commonly reported bacterial sexually transmitted pathogen worldwide. In the United States, over 1.4 million cases of *C. trachomatis* infection were reported in 2012, the highest number of cases ever reported to CDC for any condition [1].

Currently, the recommended first line of treatment for uncomplicated genital *C. trachomatis* infections is a single 1.0 g oral dose of the macrolide antibiotic azithromycin [2]. However, a number of recently published studies challenge the efficacy of azithromycin therapy for chlamydial infections [3-6]. Batteiger and co-workers [6] conducted a cohort

study among adolescent women and used a classification algorithm to characterize treatment outcomes among the study subjects after directly observed azithromycin treatment. Among women with paired episodes of chlamydial infections, 8% were probable treatment failures. A partner treatment study conducted by Golden and co-workers [7] reported that 8% (22 of 289 originally treated for *Chlamydia*) of cases treated reported no sexual intercourse after treatment and were classified as treatment failures. These studies suggest the possible future need for improved anti-chlamydial therapies.

Our group identified a serine protease inhibitor, JO146, against *C. trachomatis* High Temperature Requirement A (CtHtrA) [8-11]. JO146 was found to be lethal to *C. trachomatis* D when added at the mid-replicative stage of the chlamydial developmental cycle [8]. The addition of JO146 was lethal during reversion or recovery from penicillin persistence and during heat stress [12].

Laboratory strains of *C. trachomatis* that are commonly used for biological experiments may not reflect the isolates currently infecting men and women [13]. Differences in genome dynamics, and virulence attributes and infectivity [14-16] may result in varying sensitivities to JO146 between recent clinical isolates and the type strains of *C. trachomatis* used for investigations to date. Therefore, we aimed to validate that CtHtrA is a clinically relevant target for potential future therapeutic development by testing the efficacy of the inhibitor JO146 against recent clinical isolates from women.

## 2.	Materials and Methods

### 2.1.	Clinical isolates, Chlamydia culture and J0146 treatment conditions

Six *C. trachomatis* clinical isolates were obtained and cultured from separate women enrolled in the Australian *Chlamydia* Treatment Study (ACTS) [17] (The Alfred Human Research Ethics Approval number 223/12). The isolates were designated as: 1-017(13) (serovar K), 1-079(1) (serovar G), 1-019(1) (serovar D), 1-048(1) (serovar E), 1-028(1) (serovar E), and 1-020(1) (serovar D). The isolates were cultured in McCoy B cells grown in Dulbecco's minimal essential medium (DMEM, Life Technologies) supplemented with 5% fetal calf serum (Lonza), 10 μg ml^{-1} gentamicin (Invitrogen), 100 μg ml^{-1} streptomycin sulphate (Sigma), incubated at 37°C, 5% CO_2. For the purposes of this study, JO146 (chemical formula: $C_{31}H_{44}N_3O_7P$) was commercially synthesised [18]. The compound was synthesised, HPLC purified, and confirmed by MALDI-MS by GL Biochem (Shanghai, China).

The impact of JO146 on *Chlamydia* was determined in McCoy B cells infected at a multiplicity of infection (MOI) of 0.3 by centrifugation for 30 minutes at 500 × g at 28°C. At 16 hours post infection (h PI), the cells were treated with JO146 (0, 10, 50 and 100 μM) and DMSO (solvent) control (all experiments were done in triplicate, at least two separate experiments, with one representative experiment shown). At the completion of experiment (44 h PI unless otherwise stated) *Chlamydia* was harvested into storage medium (sucrose phosphate glutamate (SPG): 10 mM sodium phosphate (Na_2HPO_4), 250 mM sucrose ($C_{12}H_{22}O_{11}$), 5 mM L-glutamine ($C_5H_{10}N_2O_3$)) for subsequent determination of infectious yield (IFU ml^{-1} as described below).

The duration of treatment required for JO146 effect was determined by removal of the compound from the cultures after 8 h treatment. At 16 h PI *C. trachomatis* cultures were treated with either 0 µM (media only), 100 µM JO146, or DMSO. At 24 h PI (i.e. 8 hours after treatment) treatments were removed by three sequential washes with pre-warmed media and the cultures continued until harvest into SPG media at 44 and 68 h PI.

Inclusion forming units (IFU ml^{-1}) were determined by subsequent passage culture on McCoy B cells. The SPG harvested cultures were lysed by sonication (Ultrasonic, Microson) and serially diluted onto McCoy B cultures. At 30 h PI, cultures were fixed and stained using immunocytochemistry for host nucleus and chlamydial inclusions, and examined by microscopy to enumerate inclusion forming units [18].

2.2 Confocal microscopy

McCoy B cells infected with *C. trachomatis* cultured on coverslips were used for confocal microscopy. At nominated time points the cells were fixed and immunocytochemistry conducted as previously described [12]. Confocal images were obtained using an Olympus FV1200 confocal laser scanning microscope (FluoView$^{®}$ FV1200, Olympus Corporation, Japan). Sizes of 30 independent inclusions for each treatment and time point were measured manually through the use of NIS-Elements Basic Research 3.2 software.

2.3   Western blot

Cultures of T25 flasks of McCoy B cells infected with *C. trachomatis* were harvested for western blot analysis of the Major Outer Membrane protein (MOMP) and host β-actin after JO146 treatment at 16 h PI and harvested at 24 h PI. Western blots were conducted as previously described [22].

2.4 Statistical analysis

Statistical analysis was performed using PRISM (GraphPad Software Inc., V7.0). Statistical significance was defined as $p < 0.05$. Statistical tests used and number of samples are indicated with each figure.

3.   Results

3.1. *HtrA inhibition using JO146 during McCoy B culture of chlamydial isolates prevents chlamydial inclusion vacuole size development*

Our previous work using HEp-2 cells on *C. trachomatis* D (UW-3/Cx) a long term passaged isolate (referred to here as CtD) demonstrated that the inclusions decreased in size and were lost from the host cells [18]. Therefore, here we examined the morphology of the McCoy B cultures of the clinical isolates at 20, 24, and 40 h PI after JO146 treatment (16 h PI). JO146 treatment resulted in smaller inclusion vacuole sizes than the DMSO treated control cultures (Supplementary Fig. S1). .

Inclusions sizes were measured to quantify these observations. This difference in inclusion size was apparent at 24 h PI although only four clinical isolates (1-079(1), 1-079(13), 1-019(1), and 1-028(1)) showed statistically significant differences ($p < 0.01$) in the size of inclusions compared to DMSO control at this time point (Fig. 1). At 40 h PI, *Chlamydia* inclusions in the presence of JO146 were smaller than those formed in the control (DMSO-treated cells) in all clinical isolates as well as for the *C. trachomatis* D ($p < 0.0001$) (Fig. 1).

3.2. *JO146 treatment during mid-replicative phase of chlamydial development in McCoy B cells resulted in a loss of infectious progeny for clinical isolates*

The effect of 16 h PI JO146 treatment on the development of infectious progeny was tested. No infectious progeny (44 h PI) were observed for all clinical isolates treated with 50 and 100 µM JO146 except for isolate 1-017(13) (Fig. 2). For isolate 1-017(13) treatment with 50 µM JO146 resulted in approximately 1 log less infectious yield compared to DMSO and media controls ($p<0.001$) and 100 µM resulted in a complete loss of infectious progeny as

consistent with the other clinical isolates. Treatment of the type strain *C. trachomatis* D with 50 and 100 µM JO146 both resulted in ~1.5 log less infectious progeny compared to that observed in the DMSO control (p < 0.0001) (Fig. 2).

The reduction in infectious progeny and reduced inclusion sizes (Fig. 1) should correlate with a decrease in the detection of chlamydial protein. A western blot to detect levels of MOMP 8 h after JO146 treatment, showed a reduction of MOMP levels compared to matched DMSO controls for most of the isolates (Supplementary Fig.S2).

3.3. JO146 requires long treatment times to be effective against C. trachomatis in McCoy B cultures

In order to determine if JO146 activity against chlamydial clinical isolates is effective with a short duration of treatment, we removed JO146 from the cultures 8 h after addition. This resulted in recovery of infectious progeny at 44 and 68 h PI in all clinical isolates (Fig. 3) in contrast to complete loss of progeny when the compound was left in the cultures (Fig. 2). In *C. trachomatis* clinical isolates 1-028(1) and 1-019(1), there were fewer infectious EBs in cells treated with 100 µM JO146 compared to the cells treated with 0 µM JO146 (media only) and DMSO controls either at 44 or 68 h PI (Fig. 3). A non-significant increase was observed in the number of infectious progeny at 68 h PI compared to 44 h PI with 100 µM JO146 for isolates 1-017(13), 1-079(1), 1-048(1) and 1-020(1) (Fig. 3). Overall, an 8 h JO146 treatment of the cultures resulted in a minor loss of infectious progeny compared to when the compound was left in the cultures until the end of the developmental cycle (Fig. 2), suggesting that in McCoy B cultures there is a need for longer treatment duration for efficacy.

4.   Discussion

Here, we set out to determine if these previous observations of a critical function for HtrA during chlamydial replicative phase are relevant for recent clinical isolates of *C. trachomatis*. We demonstrate that addition of JO146 during mid-replicative phase resulted in a complete loss of infectious progeny if the compound is left in the cultures until the completion of the developmental cycle, providing preliminary support that the previous data in type strain cultures can be extrapolated to clinical isolates.

These experiments were conducted in McCoy B cells, as consistent with other previously reported studies on *C. trachomatis* clinical isolates that all mainly use McCoy B cells (mouse fibroblasts) as host cells [19-21]. As we have not extensively studied the effect of JO146 on *Chlamydia* grown on McCoy B cells, we included *C. trachomatis* D as a control in all experiments. We previously demonstrated that treatment of HEp-2 cultures with the CtHtrA inhibitor JO146 resulted in diminishing chlamydial inclusion size, eventual loss of the inclusions, and loss of infectious progeny without being toxic to the host cells [8]. CtHtrA was found to be essential for the reversion and recovery to viability from penicillin persistence and during heat stress [12]. The critical role that CtHtrA plays during the replicative phase of the chlamydial developmental cycle was also demonstrated to be conserved among other *C. trachomatis* strains and other *Chlamydia* species such as *C. pecorum, C. suis*, and *C. caviae* [22]. The exact role of CtHtrA for the replicative phase remains unknown, and it is potentially and indirect effect of CtHtrA, and that one or more specific extra-cytoplasmic protein substrates that rely on CtHtrA for assembly is the required factor for replicative phase.

The lack of lethality of 100 μM JO146 on type strain *C. trachomatis* D grown in McCoy B cells contrasts our previous result in HEp-2 cells, in which we observed complete loss of progeny for this same strain. These results suggest that there could be differences in

the pharmacokinetics of JO146 between mouse fibroblast (McCoy B) and human epithelial (HEp-2) cell lines, or in the chlamydial susceptibility during growth in these different cell lines. This difference also impacted on inclusion sizes actually sightly increasing over time during the cultures, again this may be due to differences in stability or degradation of JO146 in the fibroblast cells. Previous studies have demonstrated different pharmacokinetics and bioactivity of drugs such as erythromycin in different mammalian cell lines [23, 24].

In the present study the chlamydial inclusions were not completely lost from the host cells after JO146 treatment (in contrast to complete inclusion loss previously observed in HEp-2 cells), however these inclusions did not contain infectious progeny (Fig 1. and Fig 2.) [18]. Infectious progeny (with comparatively minor loss compared to controls) were observed for all strains tested when the cultures were treated with JO146 for 8 h at the replicative phase whereas there was lethality when the inhibitor was left in the cultures until the end of the developmental cycle. This result indicates that the inhibitory effect of JO146 is reversible by removal of the compound from the cultures and may be bacteriostatic, or may require longer than 8 h treatment to be effective in McCoy B cells. Here in McCoy B cells the inclusions fail to increase in size and do not make infectious progeny. This difference in the underlying process leading to loss of infectious progeny could be explained by several reasons; in these fibroblast-like cells perhaps the dosing is reduced due to cellular processes or once the *Chlamydia* in the inclusion have been inhibited by the JO146 treatment in HEp-2 a distinct host process than that of these fibroblasts is able to target the vacuoles. Nonetheless it is clear that CtHtrA is a valid possible target should future therapeutics need to be developed against *Chlamydia*, based on the effectiveness against these clinical isolates.

The subtle differences in JO146 efficacy observed between the isolates and the type strain *C. trachomatis* D is not able to be explained by differences in CtHtrA amino acid sequence. The CtHtrA sequence is highly conserved with at most 4 amino acids different in

the 647 amino acid sequence across the published *C. trachomatis* genomes to date. The *C. trachomatis* genomes cluster into three predominant clades (LGV, T1, T2) [25], the CtHtrA sequences that we have determined so far from our clinical isolates are consistent with the T2 clade (1-017(13), 1-017(1), 1-028(1), 1-048(1) (sequences will be published elsewhere). The amino acid variation is not near the residues that form active site where JO146 binds to the protein and is not likely to explain the variation. However, other differences that might impact on growth kinetics of pharmacokinetics of the drug on the chlamydial strains, as well as differences on the host cell factors do require further investigation in the future.

5.   Conclusions

This preliminary study supports that JO146 is effective against recent clinical isolates of *C. trachomatis.* The data indicates that *in vitro* application of an inhibitor compound that targets CtHtrA during the replicative phase of recent clinical isolates of *C. trachomatis* prevents development of infectious progeny. CtHtrA therefore, could be a potential target for future drug development for *C. trachomatis* and we suggest that CtHtrA inhibition and JO146 should be further investigated for improved drug development.

Author's contributions

VAO and AL performed laboratory experiments, and analysed data. PT, LAV, SNT, JSH, WMH were involved in participant collection study, and data analysis. KWB and JAA analysed data. All authors contributed to the manuscript preparation.

Competing interests

The authors declare they have no competing interests.

Acknowledgments

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Fig. 1. Inclusion sizes at 20, 24 and 40 h PI for DMSO and JO146 treated isolates of *C. trachomatis* grown in McCoy B cells are shown in the graphs. Each isolate is shown in a separate graph: A. 1-017(13), B. 1-079(1), C. 1-028(1), D. 1-048(1), E. 1-020(1), F. 1-019(1), G. *C. trachomatis* D (UW-3/Cx). Statistical analysis was conducted using Two-Way ANOVA with Bonferroni's multiple comparison tests. The bar colours represent treatment conditions; black: DMSO, and grey 100 μM JO146. Data are presented as mean ± S.E.M (n=30), ** indicates p < 0.01, ****p < 0.0001.

Fig. 2. Inclusion forming units after treatment with JO146 at 16 h PI or each of the strains is shown on the graph. The bars are shaded by grey scale (as indicated on the right) to represent the different concentrations of JO146. The identity of each of the isolates is indicated on the x-axis and the IFU ml^{-1} are indicated on the y-axis in log scale. The cultures were harvested at 44 h PI and the infectious progeny determined by measuring inclusion forming units that formed infections in a new monolayer of host cells. Statistical analysis was conducted using Two-Way ANOVA with a post hoc Bonferroni multiple comparison test relative to the DMSO control. Error bars represent the standard error of the mean (n=27) (non-logarithmic data), *** indicates p < 0.001, ****p < 0.0001. # indicates no detectable inclusion forming units.

Fig. 3. Inclusion forming units ml^{-1} at 44 and 68 h PI after JO146 addition at 16 h PI and removal after 8 h (at 24 h PI). Conditions are represented by the coloured bars on the right (black: media only (0 μM JO146), grey: DMSO, white: 100 μM JO146). Each isolate is shown in a separate graph: A. 1-017(13), B. 1-079(1), C. 1-028(1), D. 1-048(1), E. 1-020(1), F. 1-019(1), G. *C. trachomatis* D (UW-E/Cx). The IFU ml^{-1} are indicated on the y-axis and the two time points (44 and 68 h PI) are indicated on the x-axis. The cultures were harvested at 44 h PI and the infectious progeny determined by measuring inclusion forming units that

formed infections in a new monolayer of host cells. Statistical analysis was conducted using Two-Way ANOVA with a post hoc Bonferroni multiple comparison test relative to the DMSO control. The error bars represent the standard error of the mean (n=27) (error bars are for the non-logarithmic scale), ****$p < 0.0001$.

Supplementary material

Supplementary Fig. S1. Confocal microscopy images of *C. trachomatis* clinical isolates treated with JO146 or DMSO at 16 h PI and examined at 20, 24, and 40 h PI. Representative images of control (DMSO treated) cultures are shown on the left panel while representative images of cultures treated with JO146 are shown on the right for each time point (time points indicated above figure) (strain identity indicated to left of the figure). The image colours are as follows, green; MOMP (major outer membrane protein); blue: host cell nucleus (stained by DAPI); red: β actin (stained by phalloidin 594). Scale bar (bottom left) indicates 10 μm.

Supplementary Fig S2. Western blot for MOMP in cell lysates of JO146-treated and DMSO-treated *C. trachomatis* clinical isolates in McCoy B cells. JO146 or DMSO were added at 16 h PI and cells were harvested at 24 h PI (i.e. 8 hours after treatment). Treatments are indicated above each lane at the top of each isolate name. "+" denotes treatment with JO146 and "-" denotes treatment with DMSO control. Laboratory strain *C. trachomatis* D (UW-3/Cx) (CtD) was included as a control strain. The size of relevant molecular weight markers are indicated to the right of the figure and the western blot identity (i.e. MOMP or β-actin) are indicated to the left.
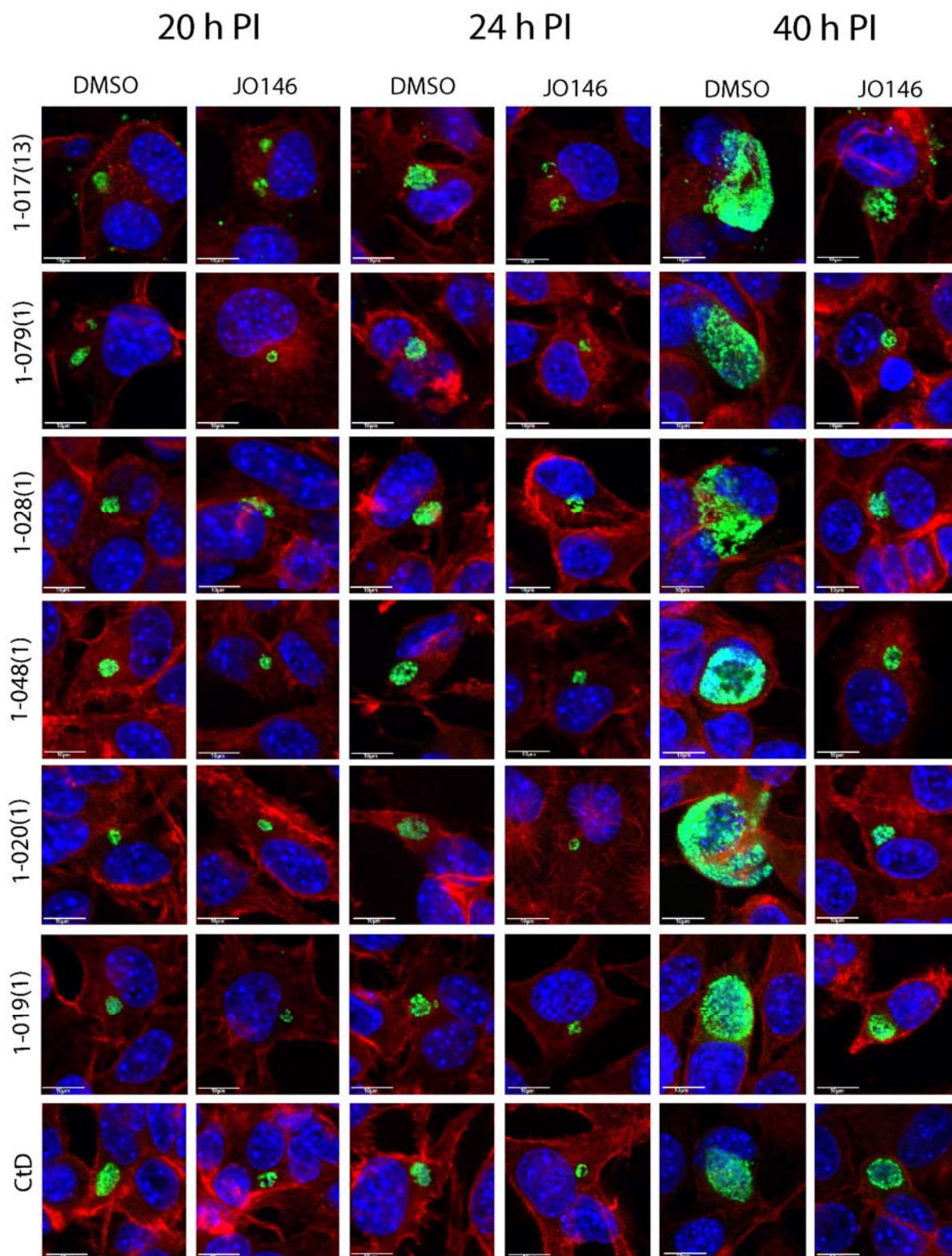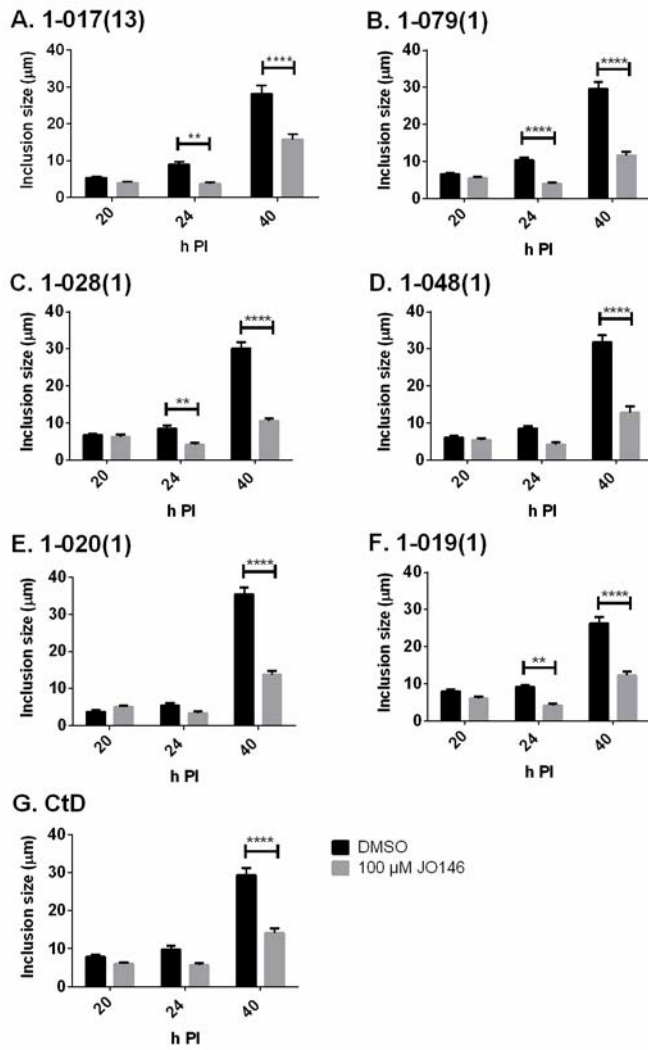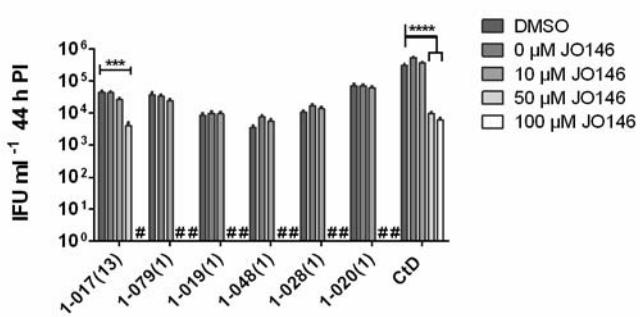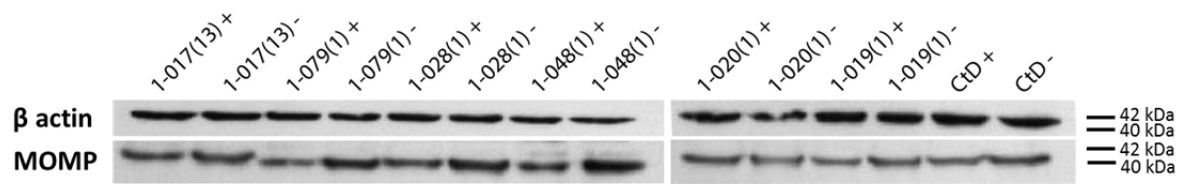
20 h PI   24 h PI   40 h PI

| | DMSO | JO146 | DMSO | JO146 | DMSO | JO146 |

Fig S1

Fig 1

Fig2

Fig S2

Fig 3