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Original Article

# Anti-chlamydial activities of cell-permeable hydrophobic dipeptide-containing derivatives☆

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ABSTRACT

The obligate intracellular bacteria chlamydia is major human pathogen that causes millions of trachoma, sexually transmitted infections and pneumonia worldwide. We serendipitously found that both calpain inhibitors z-Val-Phe-CHO and z-Leu-Nle-CHO showed marked inhibitory activity against chlamydial growth in human epithelial HeLa cells, whereas other calpain inhibitors not. These peptidomimetic inhibitors consist of *N*-benzyloxycarbonyl group and hydrophobic dipeptide derivatives. Both compounds strongly restrict the chlamydial growth even addition at the 12 h post infection. Notably, inhibitors-mediated growth inhibition of chlamydia was independent on host calpain activity. Electron microscopic analysis revealed that z-Val-Phe-CHO inhibited chlamydial growth by arresting bacterial cell division and RB-EB re-transition, but not by changing into persistent state. We searched and found that z-Leu-Leu-CHO and z-Phe-Ala-FMK also inhibited chlamydial growth. Neither biotin-hydrophobic dipeptide nor morpholinoureidyl-hydrophobic dipeptide shows inhibitory effects on chlamydial intracellular growth. Our results suggested the possibility of some chemical derivatives based on z-hydrophobic dipeptide group for future therapeutic usage to the chlamydial infectious disease.



## 1. Introduction

A family Chlamydiaceae is major intracellular pathogens that cause a wide variety of disease, such as sexually transmitted infections (STIs), trachoma, pneumonia or atherosclerosis [1]. Chlamydia has the biphasic developmental cycle: a small, infectious but metabolically inactive elementary bodies (EBs) and a larger metabolically active reticulate body (RB). Chlamydial RBs replicate only in parasitophorous vacuole termed “inclusion” inside the host cells [2]. At the late phase of infection cycle, RBs stop replication and re-differentiate to infective EBs, which are subsequently released from the inclusion to outside of the host cells. Under some circumstances such as low nutrient, IFN-γ mediated tryptophan starvation or presence of antibiotic, intracellular chlamydia turns into “persistent state” with morphologically enlarged, non-replicative, aberrant RBs. When the conditions are suited for growth, the atypical RBs return to the developmental cycle and yielding infectious EBs [3,4].

Chlamydia infection evokes a variety of immunological response of host cells, including IL-1α secretion [5–7]. The activation of calcium-dependent protease calpain is required for IL-1α maturation and secretion [8]. Calpains have been reported to regulate numerous cellular processes including cell cycle, motility, proliferation, signal transduction, differentiation, membrane fusion or cellular death by processing a wide variety of substrate proteins [9]. It has been reported that calpains are important for intracellular pathogen, such as *Plasmodium falciparum*, *Toxoplasma gondii* [10], *Listeria monocytogens* [11], *Cryptosporidium parvum* [12], Group B coxsackievirus [13], *Helicobacter pylori* [14] and *Shigella flexneri* [15]. These pathogens utilize host calpains for the escape from host immune response, or their growth. Likewise, inhibition of calpain causes the blockage of Golgi ministack formation in the *C. trachomatis* infected cells [16], hence it is plausible that chlamydia may require host calpain function for intracellular maturation.

In this study, we examined the necessity of calpains for the chlamydial growth using calpain inhibitor and shRNA strategy. Although the genetic silencing of host calpain did not affect the chlamydial growth, a certain calpain inhibitors dramatically inhibit the

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chlamydial growth. Therefore we report our serendipitous finding that *N*-benzyloxycarbonyl hydrophobic dipeptide-containing aldehyde or fluoroketones are very effective for the inhibition of intracellular growth of chlamydia.

## 2. Materials and methods

### 2.1. Reagents

Calpain inhibitors z-Val-Phe-CHO (common name MDL-28170), z-Leu-Nle-CHO (Calpeptin), ac-Ala-Leu-Leu-Nle-CHO (ALLN), calpastatin peptides, L-trans-Epoxysuccinyl-Leu-3-methylbutylamide-ethyl ester (E-64d), 3-(4-Iodophenyl)-2-mercapto-(Z)-2-propenoic Acid (PD-150606) and Mu-Val-HPh-FMK (Calpain inhibitor V) were purchased from Merck. Cathepsin B inhibitor L-trans-Epoxysuccinyl-Ile-Pro-OMe propylamide (CA-074Me), cathepsin L inhibitor z-Phe-Tyr-CHO and PPARγ antagonist H-Trp-Glu-OH (G3335) were also from Merck. Gamma-secretase inhibitor z-Leu-Leu-CHO was from Peptide institute. Irreversible cysteine protease inhibitor z-Phe-Ala-FMK was from Cayman Chemicals. Cysteine protease inhibitor Biotin-Phe-Ala-FMK was from KAMIYA BIOMEDICAL COMPANY. z-hydroxylamine was from Sigma Aldrich. z-N-ε-Boc-L-lysine was from Santa Cruz. z-S-phenyl-L-cysteine was from Toronto Research Chemicals Inc. These compounds described above were separately dissolved in dimethyl sulfoxide (DMSO) at 10 mM concentration, and then diluted with Dulbecco's Modified Eagle's Medium (DMEM). Three dipeptides H-Leu-Ile-OH, H-Phe-Leu-OH and H-Val-Phe-OH were obtained from AnaSpec. Penicillin G potassium (200,000 unit for injection) was from Meiji Seika. FITC-conjugated anti-chlamydia LPS mAb (clone ACI) was from PROGEN.

### 2.2. Bacterial culture, infection and chemical treatment

*C. trachomatis* serovar L2 (strain:434, ATCC VR-902B), *C. trachomatis* serovar D (strain:UW-3/Cx, ATCC VR-885), *C. muridarum* (mouse pneumonitis strain Nigg II, ATCC VR-123) and *C. pneumoniae* (strain AR-39, ATCC 53592) were propagated in HeLa or HEp-2 cells as described previously [17]. For experiment, host cells were plated in 24-well tissue culture dishes and infected with chlamydia at indicated multiplicity of infection (MOI). After inoculation, plate was centrifuged for an hour at 900×*g* at room temperature. Each chemical compound was added to DMEM supplemented with 10% fetal bovine serum at indicated concentrations. One μg/ml of cycloheximide was added in all studies here. For induction of chlamydial persistent infection, infected cells were treated 5 units/ml of Penicillin G at 6 h post infection (hpi) for 42 h.

### 2.3. Cytotoxicity assay

Cell cytotoxicity was measured using CytoTOX 96® Non-Radioactive Cytotoxicity Assay kit (Promega) according to the

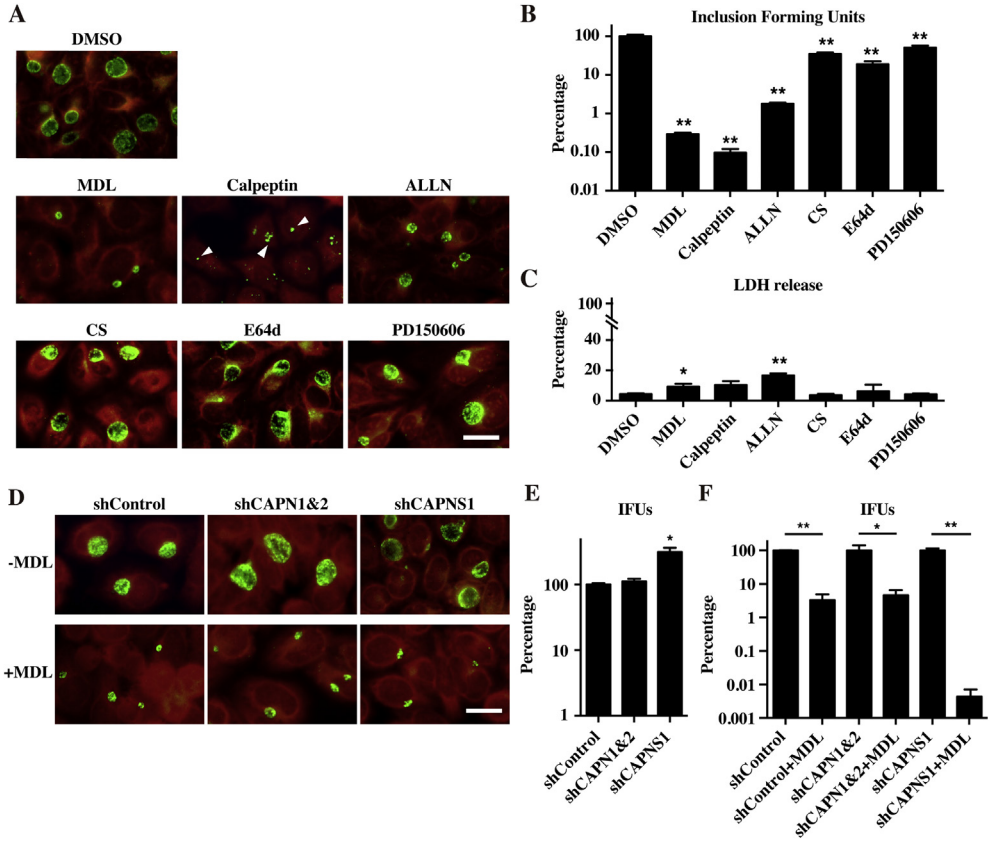


**Fig. 1. z-Val-Phe-CHO and z-Leu-Nle-CHO severely inhibit the growth of *C. trachomatis* independently of host calpain activity.** (A) HeLa cells were infected with *C. trachomatis* L2 and cultured with z-Val-Phe-CHO (MDL), z-Leu-Nle-CHO (Calpeptin), ac-Ala-Leu-Leu-Nle-CHO (ALLN), calpastatin peptide (CS), E-64d and PD-150606 (50 μM each) for 24 h. Cell were fixed with ice-cold methanol and stained using FITC-conjugated anti-chlamydia LPS antibody. Red, Evans blue dye as counter staining. Arrowheads indicated growth-arrested chlamydial inclusions. 1% DMSO used as a solvent control. Scale bar: 10 μm. (B) Infected cells were harvested at 48 hpi and re-infected fresh HeLa cells. The numbers of inclusion were counted and calculated the inclusion-forming units (IFUs) scores. (C) HeLa cells were separately treated with indicated chemicals for 24 h (50 μM each). Each culture supernatant was collected and released LDH activity was measured. Total cell lysate was used as 100% cytotoxicity control. (D) *C. trachomatis* infected cells were treated with or without 50 μM z-Val-Phe-CHO and then stained at 24 hpi. Scale bar: 10 μm. (E) IFU scores at 48 hpi. shControl sample was defined as 100% control. (F) Comparisons of IFU score between z-Val-Phe-CHO-untreated and treated samples. Each z-Val-Phe-CHO-untreated sample is defined as 100% control. Data are presented as mean ± S.D. of three independent wells. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with each control sample by Welch's *t*-tests.

manufacturer's protocol. Culture supernatant with 0.1% Triton X-100 was used as 100% cytotoxic control.

### 2.4. Electron microscopy

Chlamydia-infected HeLa cells were harvested at 48 hpi and fixed via 2% glutaraldehyde for an hour and followed by 1% osmium tetroxide ($OsO_4$) at 4 °C for overnight. Fixed samples were dehydrated by graded ethanol extraction and propylene oxide, and then embed with EPON 812 epoxy resin (TAAB Laboratories Equipment) for 96 h. Thin sections were formerly stained with 5% uranyl acetate, and then post-stained using lead citrate solution before imaging on H-7100 transmission electron microscope (Hitachi).

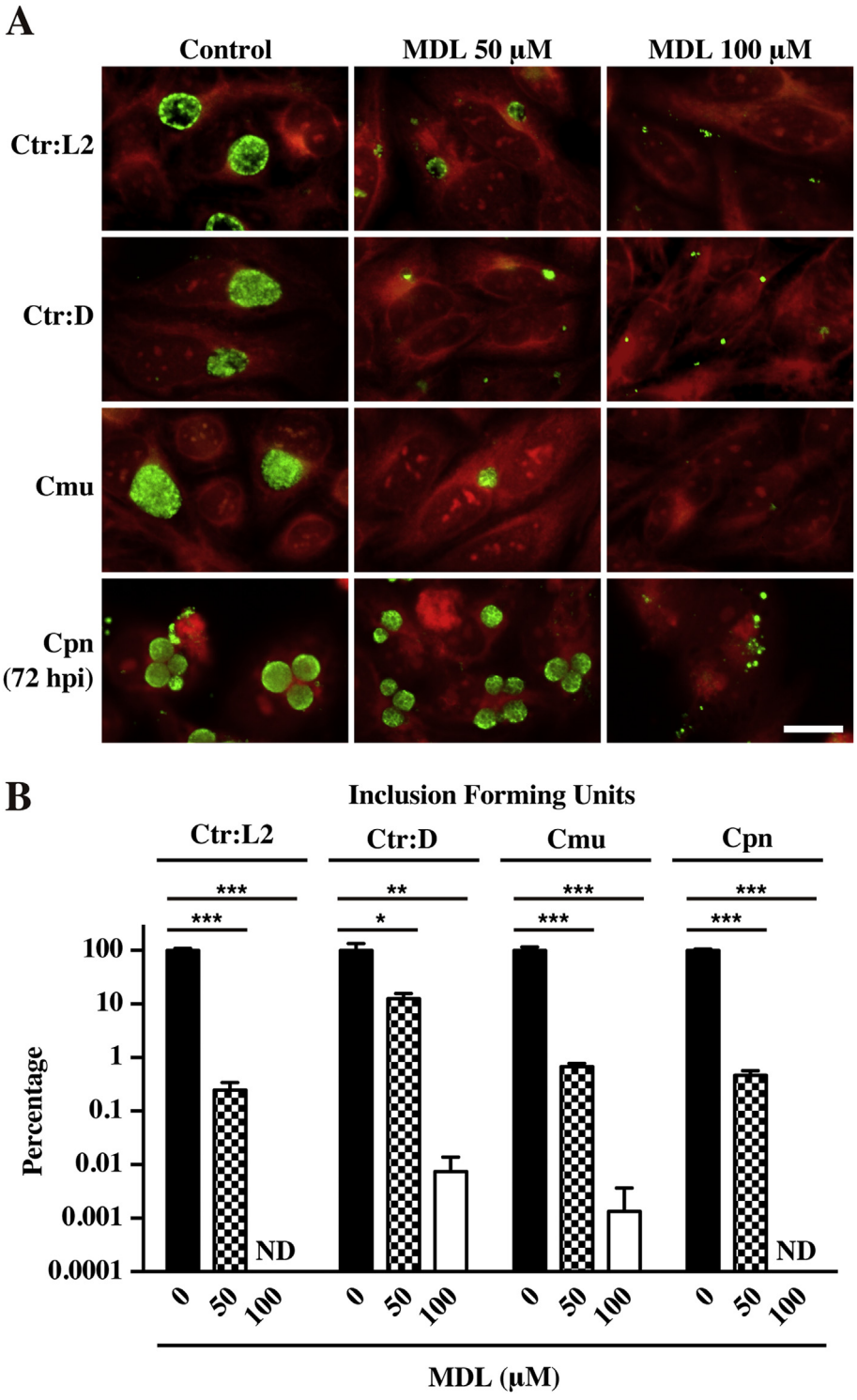


**Fig. 2. z-Val-Phe-CHO inhibits the growth of all *Chlamydia* species.** (A) HeLa cells were infected with *C. trachomatis* serovar L2 (Ctr: L2), *C. trachomatis* serovar D (Ctr: D), *C. muridarum* (Cmu) and *C. pneumoniae* AR39 (Cpn) respectively and cultured for 24 h (all *C. trachomatis*) or 72 h (Cpn) with or without z-Val-Phe-CHO (MDL) at indicated concentrations. Cell were fixed and stained as in Fig. 1A. Scale bar: 5 μm. (B) Infected cells were cultured with z-Val-Phe-CHO (MDL) and harvested at 48 hpi (all *C. trachomatis*) or 72 hpi (Cpn) and calculated the IFU scores. ND, not detected (detectable IFU threshold = 500). Data are presented as mean ± S.D. of three independent wells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by multiple *t*-tests.

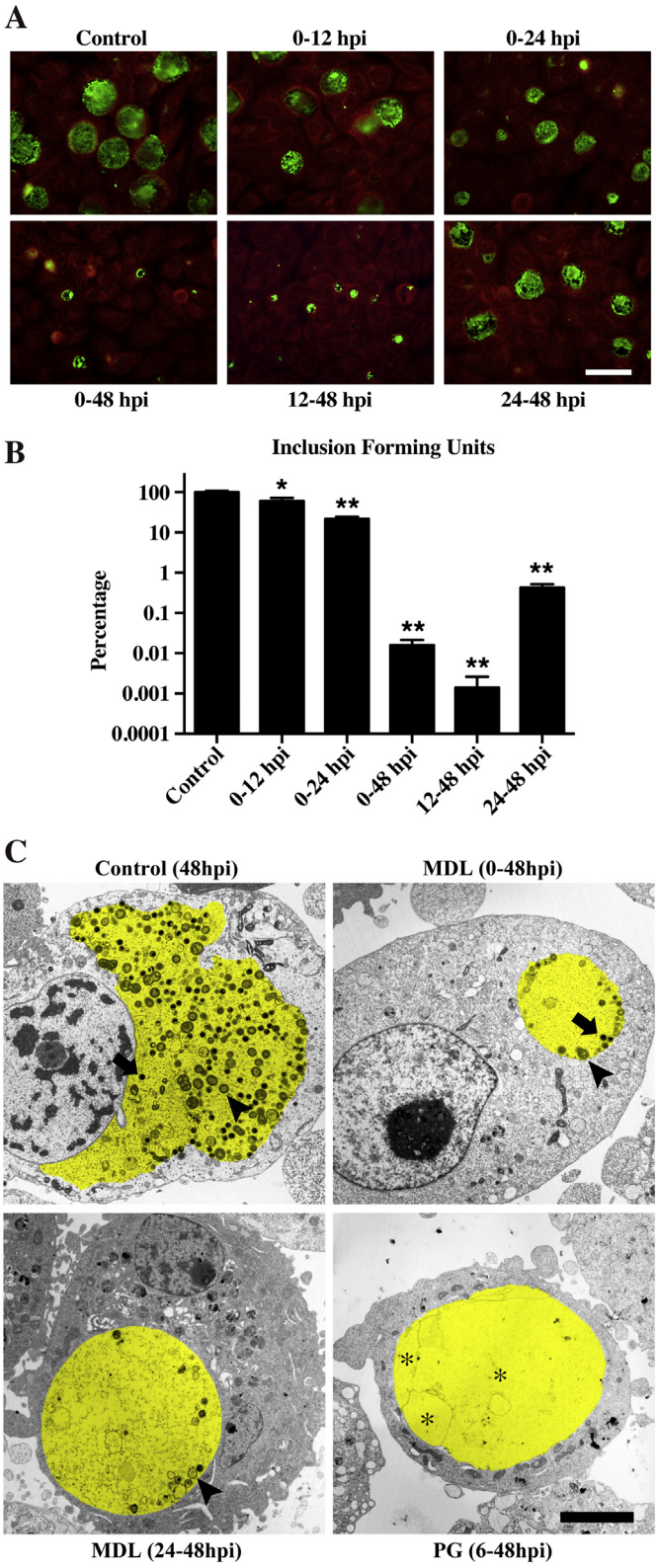


**Fig. 3. z-Val-Phe-CHO is critically arresting the middle to late stage of the chlamydial developmental stages.** *C. trachomatis* L2-infected HeLa cells were incubated with or without 50 μM z-Val-Phe-CHO (MDL) or 5 units/ml of Penicillin G (PG) as indicated time period. (A) After 48 h post infection, cell were stained as in Fig. 1A. Scale bar: 10 μm. (B) IFU scores in harvested cells at 48 hpi were calculated as in Fig. 1B. (C) All cell samples were harvested at 48 hpi and analyzed by electron microscopy (Magnification ×3000). Yellow area, chlamydial inclusion. Arrows, chlamydial EB. Arrowheads, chlamydial RB. Asterisks, aberrant RB. Bar, 4 μm. Data are presented as mean ± S.D. of three independent wells. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control sample by Welch's *t*-tests.

### 2.5. Calpain 1/2/S1 knock down method

HeLa cells were separately infected with Calpain 1 shRNA (h) lentiviral particles (sc-29885-v, Santa Cruz), Calpain 2 shRNA (h) lentiviral particles (sc-41459-v, Santa Cruz), Calpain reg shRNA (h) lentiviral particles (sc-29887-v, Santa Cruz), or control shRNA lentiviral particles-A (sc-108080, Santa Cruz) according to the manufacturer's protocol. Each stable transformant was isolated using 5 μg/ml Puromycin.

## 3. Results

### 3.1. A certain type of calpain inhibitors markedly inhibit the chlamydial growth independent of host calpain function

We first examined the effect of treatment with various calpain inhibitors (50 μM) on chlamydial growth in HeLa cells. Among six calpain inhibitors examined, both z-Val-Phe-CHO (MDL in figure) and z-Leu-Nle-CHO (Calpeptin) caused the striking decrease in the size of chlamydial inclusion at 24 hpi (Fig. 1A) and the chlamydial EB production at 48 hpi (Fig. 1B). Treatment with ac-Ala-Leu-Leu-Nle-CHO (ALLN) demonstrated mild inhibition of chlamydial growth. The degree of chlamydial growth inhibition mediated by other three inhibitors CS peptide, E-64d and PD-150606, were very weak. Both z-Val-Phe-CHO and ac-Ala-Leu-Leu-Nle-CHO showed very slight toxic effect against host cell at 50 μM concentration (Fig. 1C). To clarify whether inactivation of host calpain affect the chlamydial growth, we used HeLa cells stably expressing shRNAs against both *CAPN1*/*CAPN2* genes or *CAPNS1* gene, which ubiquitously-expressing calpain-1 (μ-calpain), calpain-2 (m-calpain) and small subunit-1, respectively. *C. trachomatis* L2 grew normally in these calpain gene-silenced cells (Fig. 1D, *upper*) and the production of infectious progeny was not diminished (Fig. 1E). More importantly, z-Val-Phe-CHO treatment showed a similar level of inhibition of chlamydial growth compared to shRNA control cells (Fig. 1D *lower* and F).

### 3.2. z-Val-Phe-CHO inhibits the generation of infectious progeny of different species of Chlamydia

Next we examined whether z-Val-Phe-CHO also inhibit the intracellular growth of other species of *Chlamydiae*. HeLa cells were infected with four *Chlamydiae*, *C. trachomatis* serovar L2, *C. trachomatis* serovar D, *C. muridarum* and *C. pneumoniae*, respectively, and cultured in the presence of increasing dose of z-Val-Phe-CHO. We found that z-Val-Phe-CHO significantly decreased the size of chlamydial inclusion of all four tested *Chlamydiae* in a dose dependent manner (Fig. 2A). In line with this, z-Val-Phe-CHO (50 μM) treatment caused more than 99% decrease of the infectious progeny numbers in *C. trachomatis* L2 (0.25 ± 0.09%), *C. muridarum* (0.68 ± 0.10%) and *C. pneumoniae* (0.47 ± 0.10%) (Fig. 2B). The treatment of z-Val-Phe-CHO at 100 μM caused almost complete inhibition of the generation of infectious progeny of all four tested *Chlamydiae*. We also found that z-Leu-Nle-CHO (50 μM) greatly inhibit all chlamydial growth (data not shown).

### 3.3. z-Val-Phe-CHO arrests chlamydial replication during mid-cycle development and later RBs-EBs differentiation, but does not induce the persistent form

To determine which stage is important for the inhibitory effect of z-Val-Phe-CHO during chlamydial growth, *C. trachomatis* L2-

infected cells were treated with z-Val-Phe-CHO at various time periods. Cells treated with z-Val-Phe-CHO for 48 h (0–48 hpi) and for latter 36 h (12–48 hpi) revealed the great inhibition of the chlamydial inclusion size (Fig. 3A), which corresponded well with IFU data (0–48: 0.02 ± 0.005%, 12–48: 0.001 ± 0.001%) (Fig. 3B). The cells which treated z-Val-Phe-CHO for former 12 h (0–12 hpi) or 24 h (0–24 hpi) showed the moderate sizes of chlamydial inclusions (Fig. 3A) and the slight decrease of chlamydial progeny

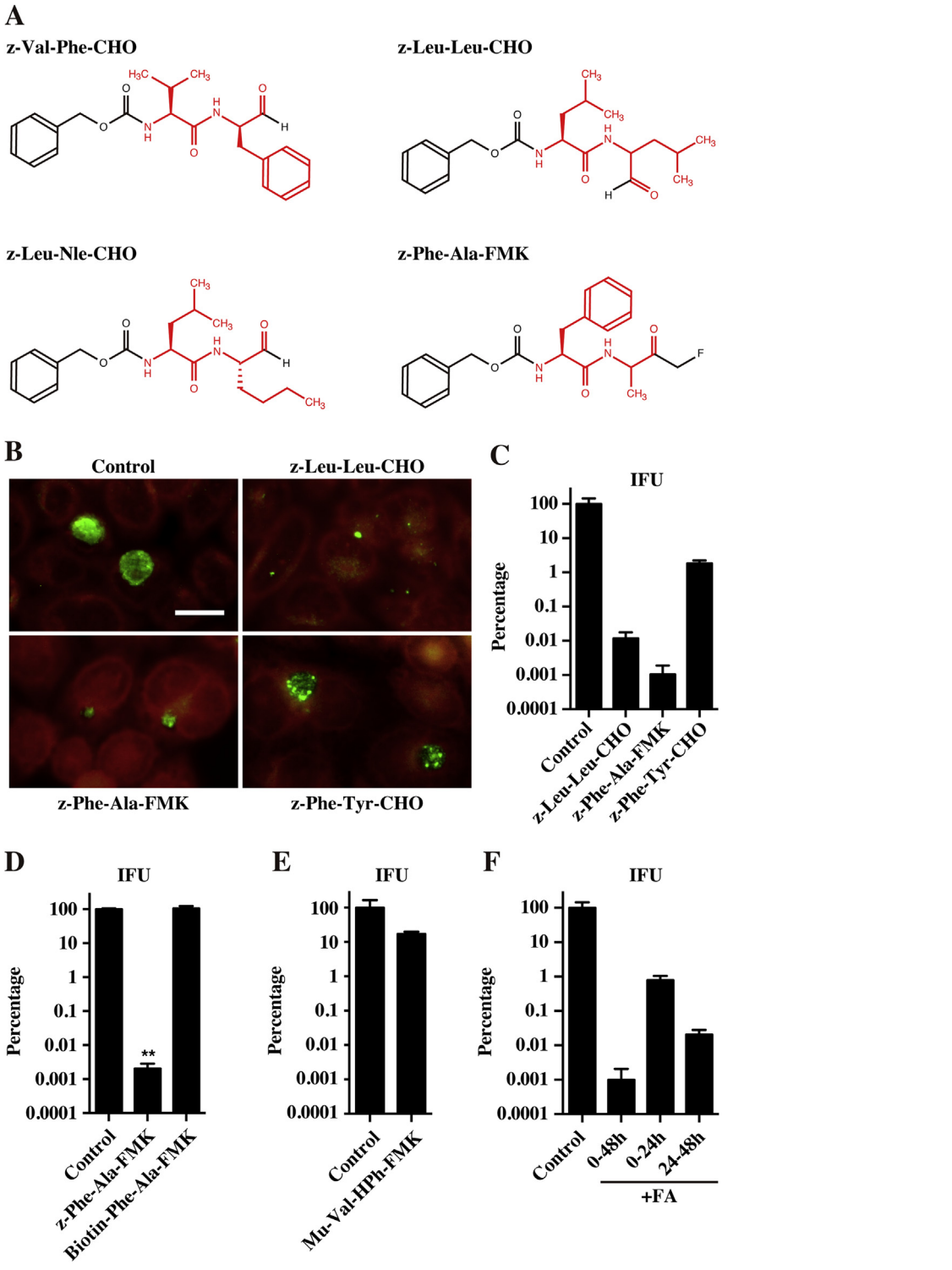


**Fig. 4. z-hydrophobic dipeptide inhibitors severely inhibit the chlamydial growth.** (A) Chemical structures of z-Val-Phe-CHO, z-Leu-Nle-CHO, z-Leu-Leu-CHO and z-Phe-Ala-FMK. Hydrophobic polypeptide skeletons are represented red color. (B) Infected cell were treated with z-Leu-Leu-CHO, z-Phe-Ala-FMK or z-Phe-Tyr-CHO respectively and then stained as in Fig. 1A. Scale bar: 10 μm. (C) IFU scores at 48 hpi were evaluated as in Fig. 1B. (D, E) Effects of z-Phe-Ala-FMK, Biotin-Phe-Ala-FMK or Mu-Val-HPh-FMK were examined by IFU assay. (F) Infected cells were incubated with or without 50 μM z-Phe-Ala-FMK (+FA) as indicated time period. Then cells were harvested at 48hpi and IFU scores were analyzed. Data are presented as mean ± S.D. of three independent wells. $^{**}p < 0.01$ by Welch's *t*-tests.

numbers (0–12: 60.6 ± 11.5%, 0–24: 21.7 ± 2.8%) (Fig. 3B). It is notable that z-Val-Phe-CHO treatment at the late 24 h (24–48 hpi) significantly decreased the generation of infectious progeny (0.45 ± 0.09%), whereas the inhibitory effect on the size of chlamydial inclusions was very minor. Electron microscopic analyses of infected control cells at 48 hpi revealed that chlamydial inclusions contained many chlamydial EBs, indicating late-stage RB-EB retransition (Fig. 3C, control 48 hpi.). In contrast, infected cells treated with z-Val-Phe-CHO for 48 h had a small inclusion contained very few chlamydial RBs lining the rim of inclusion body (MDL (0-48hpi)). Infected cells treated with z-Val-Phe-CHO during 24–48 hpi also revealed a chlamydial inclusion body containing a scarce number of chlamydial RB (MDL (24-48hpi)). It is noteworthy that there was not any aberrant RBs observed in z-Val-Phe-CHO treated infected cells, whereas penicillin G-treated infected cells showed bizarre-looking inclusion bodies, which were compacted with aberrant RBs, typical characteristic of experimentally-induced persistent infection (PG (6-48hpi)).

### 3.4. *N*-benzyloxycarbonyl group containing hydrophobic dipeptide inhibit chlamydial growth

Both z-Val-Phe-CHO and z-Leu-Nle-CHO contain N-terminal benzyloxycarbonyl (z) group and hydrophobic dipeptide (Fig. 4A). Thus we examined whether these unique molecular structures common to these two particular calpain inhibitors are required for the growth inhibition of chlamydia. We investigated three chemical compounds containing *N*-benzyloxycarbonyl group but not having hydrophobic dipeptide skeleton: z-HNOH, z-Lys(Boc)-OH and z-Cys(Ph)-OH (Fig. 5A). These chemicals did not show any inhibition of chlamydial growth (Fig. 5B).

Next, we examined whether the hydrophobic dipeptide skeleton is important for the anti-chlamydial activity. Both γ-secretase inhibitor III (z-Leu-Leu-CHO) and a cysteine protease inhibitor z-Phe-Ala-FMK contain *N*-benzyloxycarbonyl group and hydrophobic dipeptide skeleton (Fig. 4A). We found that both chemicals have a very strong anti-chlamydial activity and cause remarkable blockade

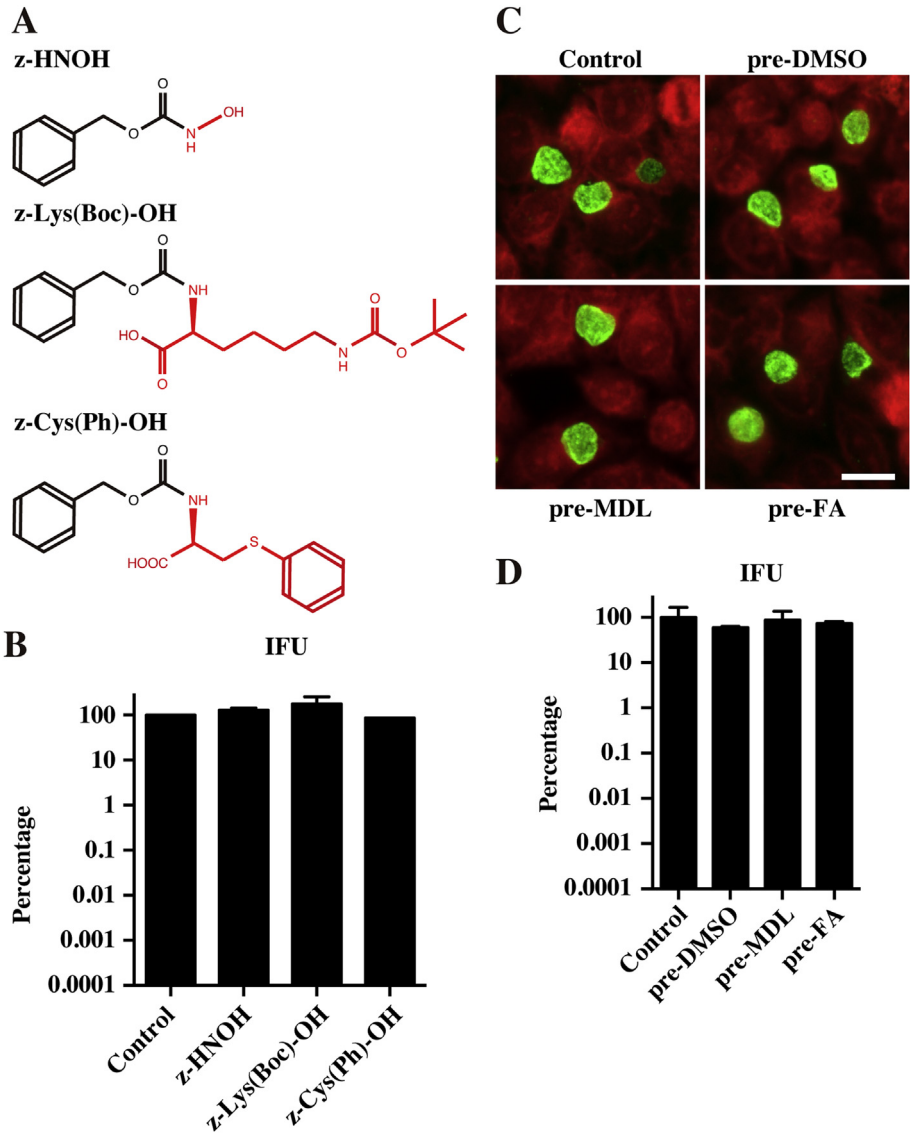


**Fig. 5. *N*-benzyloxycarbonyl group or pre-treatment does not affect chlamydial growth.** (A) Chemical structures of z-HNOH, z-Lys(Boc)-OH, and z-Cys(Ph)-OH. *N*-benzyloxycarbonyl groups are represented black. (B) Infected cells were separately treated with or without chemicals as in (A), harvested at 48hpi, and then IFU scores were analyzed. (C) Elementary bodies of *C. trachomatis* L2 were separately treated with 50 μM of z-Val-Phe-CHO (pre-MDL), z-Phe-Ala-FMK (pre-FA) and 0.5% of DMSO (pre-DMSO) in SPG for 30 min, and then infected onto HeLa cell monolayer. (D) Cells were harvested at 48 hpi and IFU scores were evaluated. Data are presented as mean ± S.D. of three independent wells.

of chlamydial inclusion formation and the EB production (z-Leu-Leu-CHO: 0.10 ± 0.02%, z-Phe-Ala-FMK: 0.001 ± 0.001%) (Fig. 4B–C). It is noteworthy that a cathepsin L inhibitor z-Phe-Tyr-CHO, which is z-dipeptide-aldehyde that has one hydrophilic residue tyrosine, showed a weak inhibition of chlamydial growth (1.81 ± 0.42%), which was 1800-times lesser than z-Phe-Ala-FMK activity (Fig. 4B, C). H-Val-Phe-OH, H-Leu-Ile-OH, or H-Phe-Leu-OH did not show any inhibitory effect on chlamydial growth in HeLa cells (Table 1). Furthermore, Biotin-Phe-Ala-FMK or Mu-Val-HPh-FMK did not significantly inhibit the intracellular growth of chlamydia in HeLa cells (Fig. 4D, E). We also confirmed that z-Phe-Ala-FMK, similarly to z-Val-Phe-CHO, is critically inhibiting or arresting the middle/late of the chlamydial developmental stages and may inhibit both replication of RBs and RB-EB re-transition (Fig. 4F). In line with these observations, we further confirmed that pre-treatment of chlamydial EBs with z-Val-Phe-CHO or z-Phe-Ala-FMK did not affect chlamydial infectivity and the growth in HeLa cells (Fig. 5C, D).

## 4. Discussion

Our data support the conclusion that *N*-benzyloxycarbonyl (z) group containing hydrophobic dipeptide inhibited the intracellular growth of chlamydia. We demonstrate here that the requirement of z-group and the necessity of hydrophobicity of dipeptide compound for successful inhibition of chlamydial replication. Moreover, we observed the chlamydial growth inhibition is observed in the stage of RBs division and RBs into EBs re-differentiation stage.

The above four z-hydrophobic dipeptide compounds belong to cysteine protease inhibitor (calpain or cathepsin). It has been reported that some pathogenic organisms utilize host calpains for invasion step, phagosome escape, vesicle trafficking and RNA replication [10–15,18]. Inversely, calpain inhibition strongly increased the number of viable intracellular Group B *Streptococcus* by cytoskeleton alterations and reduced macrophage phagocytosis [19]. The role of calpain system on chlamydial infection has been largely unknown except the role of golgi fragmentation [16]. We reason that the participation of host calpains in chlamydial growth inhibition by z-hydrophobic dipeptide is unlikely based on the following reasons. Firstly, our study demonstrated that three calpain inhibitors CS peptides, E-64d and PD-150606 had very weak, if any, inhibitory effect on chlamydial growth (Fig. 1). If calpain activity is indispensable for chlamydial growth, all calpain inhibitors are, more or less, supposed to show inhibitory effects on the chlamydial replication. Secondly, knock down of calpain-1, calpain-2 and calpain-S1 method did not reveal the inhibition of chlamydial growth (Fig. 1D, E). Furthermore, chlamydial intracellular growth inhibition of z-Val-Phe-CHO was still observed in calpain-gene silenced HeLa cells (Fig. 1D, F), suggesting that the calpain function is dispensable for chlamydial growth. Thus we believe that chlamydial growth inhibitory effects of z-hydrophobic dipeptide compounds are not mediated by host calpain inhibition.

It has been reported that cathepsin inhibitors inhibit the growth of chlamydia which is co-opted by chlamydia to acquire essential amino acids [20]. In fact mild (weak) inhibitory effect of CA-074Me (Table 1) and z-Phe-Tyr-CHO (Fig. 4) can be considered as chlamydial growth inhibition mediated by cathepsin inhibition. However, the degree of growth inhibition brought about by z-Val-Phe-CHO, z-Leu-Nle-CHO, z-Leu-Leu-CHO and z-Phe-Ala-FMK were far more advanced. These results strongly suggest that these growth inhibitory effects are not mediated by cathepsin inhibition.

Several reports revealed that some peptidomimetic protease inhibitors act as antagonist of replications of bacteria, virus, parasites and fungi. For example, z-Val-Phe-CHO has been shown to inhibit the intracellular *T. cruzi* growth *in vitro* [21] and treatment with some FMK-derivatized pseudopeptides rescued mice from the protozoan parasite *T. cruzi* lethal infection, by inhibiting the major *T. cruzi* cysteine protease cruizan [22]. The dipeptide cysteine protease inhibitor z-Phe-Ala-FMK inhibited reovirus replication in the host cells *in vitro* [23]. Therefore, the possibility of some peptidomimetic protease inhibitors as a novel chemotherapy for infectious disease has been discussed [24–27]. Our study is the first report showing the effects of z-hydrophobic dipeptide on chlamydial infection. The requirement of *N*-benzyloxycarbonyl (z) group may suggest that these inhibitors are needed to incorporation into chlamydial inclusions and probably into chlamydial RBs via strong hydrophobicity. If this is the case, z-hydrophobic dipeptide aldehyde or fluoromethyl ketone may be targeting chlamydial protein enzyme, which is essential for both RB division and RB-EB re-transition. It is noteworthy that both z-Val-Phe-CHO and z-Phe-Ala-FMK suppressed in chlamydial growth during treatment, however, when the compound removed, chlamydial proliferation immediately recovered within 24 h and IFU scores were increased (Fig. 3A–B and Fig. 4F, sample “0–24 h”). These results strongly suggest that anti-chlamydial activities of four chemical compounds are not bactericidal effect but bacteriostatic effect. Therefore, it could be possible

**Table 1**
Summary of chlamydial growth-inhibition abilities of chemicals.

| Chemicals | Common Name | % of IFU (50 μM) | Inclusion size |
|---|---|---|---|
| z-Phe-Ala-FMK | Cysteine protease inhibitor | 0.001 ± 0.001 | Small |
| z-Leu-Leu-CHO | γ-secretase inhibitor III | 0.012 ± 0.006 | Small |
| z-Leu-Nle-CHO | Calpeptin | 0.097 ± 0.02 | Small |
| z-Val-Phe-CHO | MDL-28170 | 0.3 ± 0.03 | Small |
| Ac-Leu-Leu-Nle-CHO | ALLN | 1.8 ± 0.1 | Medium |
| z-Phe-Tyr-CHO | Cathepsin L inhibitor II | 1.8 ± 0.4 | Medium |
| L-trans-Epoxysuccinyl-Ile-Pro-OMe propylamide | CA-074 Me | 1.9 ± 0.2 | Medium |
| Mu-Val-HPh-FMK | Calpain Inhibitor V | 17.1 ± 2.3 | Large |
| L-trans-Epoxysuccinyl-Leu-3-methylbutylamide-ethyl ester | E-64d | 18.9 ± 3.6 | Large |
| a 27-amino acid petide from human calpastatin | CS peptide | 34.8 ± 3.2 | Large |
| 3-(4-Iodophenyl)-2-mercapto-(Z)-2-propenoic Acid | PD-150606 | 51.0 ± 6.7 | Large |
| z-S-phenyl-L-cysteine | z-Cys(Ph)-OH | 86.2 ± 0.01 | Large |
| H-Leu-Ile-OH | | 86.6 ± 12.6 | Large |
| H-Phe-Leu-OH | | 97.9 ± 20.7 | Large |
| Biotin-Phe-Ala-FMK | Cysteine protease inhibitor | 105.1 ± 17.6 | Large |
| H-Val-Phe-OH | | 112.8 ± 2.9 | Large |
| z-HNOH | z-Hydroxylamine | 127.4 ± 16.1 | Large |
| H-Trp-Glu-OH | G3335 | 149.1 ± 57.0 | Large |
| z-N-ε-(t-butoxycarbonyl)-L-lysin | z-Lys(Boc)-OH | 177.4 ± 75.8 | Large |

that some hydrophobic dipeptide skeleton derivatives bactericidal effect against the chlamydial growth by selecting more appropriate functional groups, and it has the possibility for therapeutic usage against the chlamydial infectious disease.

Further studies will be needed to identify the target proteins of these z-group hydrophobic dipeptide compounds in chlamydia infected cells. And now, we have searching for a series of novel inhibitors that has anti-chlamydial activity comparable to existing antimicrobials according to the rules based on our finding.

## Conflicts of interest

None.

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