THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 24, pp. 16691–16699, June 16, 2006

Metalloprotease Inhibitors GM6001 and TAPI-0 Inhibit the Obligate Intracellular Human Pathogen *Chlamydia trachomatis* by Targeting Peptide Deformylase of the Bacterium*

Received for publication, December 22, 2005, and in revised form, March 21, 2006 Published, JBC Papers in Press, March 24, 2006, DOI 10.1074/jbc.M513648200

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Chlamydia trachomatis **is an obligate intracellular bacterium responsible for a number of human diseases. The mechanism underlying the intracellular parasitology of Chlamydiae remains poorly understood. In searching for host factors required for chlamydial infection, we discovered that *C. trachomatis* growth was effectively inhibited with GM6001 and TAPI-0, two compounds known as specific inhibitors of matrix metalloproteases. The inhibition was independent of chlamydial entry of the cell, suggesting that the loss of extracellular metalloprotease activities of the host cell is unlikely to be the mechanism for the growth suppression. Nucleotide sequences of candidate metalloprotease genes remained unchanged in a chlamydial variant designated GR10, which had been selected for resistance to the inhibitors. Nevertheless, GR10 displayed a single base mutation in the presumable promoter region of the gene for peptide deformylase (PDF), a metal-dependent enzyme that removes the *N*-formyl group from newly synthesized bacterial proteins. The mutation correlated with an increased PDF expression level and resistance to actinonin, a known PDF inhibitor with antibacterial activity, as compared with the parental strain. Recombinant chlamydial PDF was covalently labeled with a hydroxamate-based molecular probe designated AspR1, which was developed for the detection of metalloproteases. The AspR1 labeling of the chlamydial PDF became significantly less efficient in the presence of excessive amounts of GM6001 and TAPI-0. Finally, the PDF enzyme activity was efficiently inhibited with GM6001 and TAPI-0. Taken together, our results suggest that the metalloprotease inhibitors suppress chlamydial growth by targeting the bacterial PDF. These findings have important biochemical and medical implications.**

Chlamydiae are Gram-negative eubacteria that are divided into four species: *Chlamydia trachomatis*, *C. pneumoniae*, *C. psittaci*, and *C. pecorum* (1). *C. trachomatis* and *C. pneumoniae* are widespread human pathogens (1, 2). *C. pneumoniae* is a common cause of pneumonia and bronchitis and may also be a cofactor for cardiovascular disease (3). *C. trachomatis* consists of three biovars. Biovar trachoma is responsible for preventable blindness and is also a major cause of sexually transmitted infections characterized by cervicitis, endometritis, and salpingitis in women and urethritis in men, whereas biovar lymphogranuloma venereum causes a more invasive sexually transmitted syndrome that attacks subepithelial and lymphatic tissues. Biovar mouse is not a human pathogen; however, it is a useful organism modeling human chlamydial infection in mice (2).

Chlamydiae are strictly dependent upon host eukaryotic cells for their growth (4). They have a distinct developmental cycle, which begins with attachment of an infectious elementary body (EB)[2] to the host cell that internalizes the bacterium into a vacuole termed inclusion. In the inclusion, the EB differentiates into the non-infectious, metabolically active reticulate body (RB), which replicates by binary fission. Around the midpoint of their developmental cycle, the majority of RBs start to reorganize back to EBs, which are then released to infect additional cells (4).

Due to their strict requirement for intracellular growth and the lack of methodology for genetic transformation, the molecular mechanisms underlying the intracellular development of Chlamydiae remain largely undefined. We are particularly interested in identifying host factors that are important for supporting chlamydial infection. In a screen of chemical compounds that target a variety of cellular activities, we unexpectedly discovered that reagents developed for suppressing matrix metalloproteases acted as highly effective inhibitors of *C. trachomatis* infection. However, detailed analyses pointed to peptide deformylase (PDF), a metal-dependent enzyme key to protein maturation in bacteria, as the inhibitors' target. Thus, these studies identify PDF as an essential enzyme for chlamydial replication and may also be a therapeutic target for chlamydial diseases, provided inhibitory specificity can be achieved.

EXPERIMENTAL PROCEDURES

Reagents—GM6001, GM6001 Negative Control (GMNC), and TAPI-0 were purchased from Calbiochem. The structures of these three compounds are given in Fig. 1. Cycloheximide, sodium formate, $NiSO_4$, imidazole, β-nicotinomide adenosine dinucleotide (NAD), and *Candida boidinii* formate dehydrogenase were purchased from Sigma (St. Louis, MO). The structure of the AspR1 probe is also shown in Fig. 1.

* This work was supported in part by grants from the University of Medicine and Dentistry of New Jersey Robert Wood Johnson Medical School, the National Center of the American Heart Association (Grant 0330335N), and the National Institutes of Health Grant 1R21AI064441 (to H. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: EB, elementary body; RB, reticulate body; L2, *C. trachomatis* serovar L2; GR10, an L2 mutant resistant to GM6001 and TAPI-0; MoPn, *C. trachomatis* mouse pneumonitis; PDF, peptide deformylase; IFU, inclusion-forming unit; MOMP, major outer membrane protein; GMNC, GM6001 negative control; fMAS, *N*-formyl-methionine-alanine-serine; IPTG, isopropyl β-D-1-thiogalactopyranoside; IC_{50}, concentration that causes 50% inhibition.

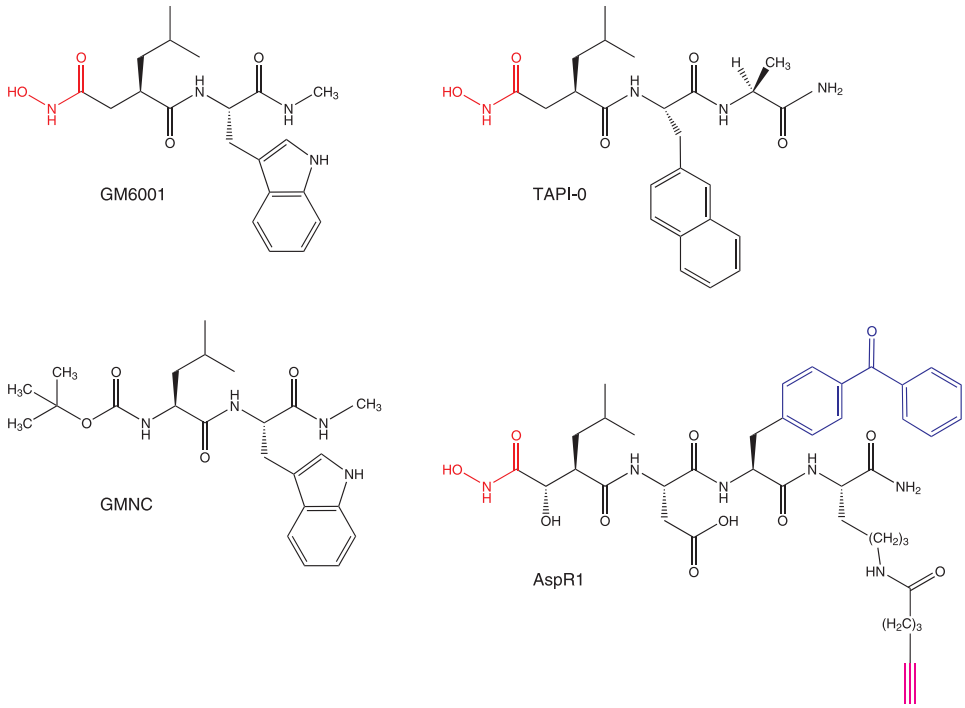


FIGURE 1. **Structures of metalloprotease inhibitors, GM6001 and TAPI-0, their control compound GMNC, and the AspR1 probe.** GM6001 and TAPI-0 are peptide-based compounds in which the hydroxamic group shown in *red* with the catalytic zinc of metalloproteases and consequently inhibits their activities. Modification of the hydroxamic group in GMNC results in a loss of inhibitory activity (19). The AspR1 probe is a hydroxamate that carries a benzophenone group shown in *blue*, which allows for cross-linking with target enzymes upon photoactivation, and an alkyne tag shown in *magenta*, which allows for conjugation with the fluorochrome Rhodamine by copper(I)-catalyzed azide-alkyne cycloaddition reaction after cross-linking (5, 18, 37).

The protocol for synthesizing an earlier version of the AspR1 probe has been described (5). The procedures for synthesizing the AspR1 probe have also been reported.[3] [6-^{3}H]Uridine (specific activity, 20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). *N*-Formyl-methionine-alanine-serine (fMAS) was purchased from Bachem Biosciences Inc. (King of Prussia, PA). Cobalt-conjugated Sepharose was purchased from Clontech (Mountain View, CA). Glutathione-agarose and the ECL kit were purchased from Amersham Biosciences (Piscataway, NJ). The Precision Plus Protein All Blue molecular weight standards (for covalent labeling experiments) and the broad range prestained protein standards (for all other experiments) were purchased from Bio-Rad (Hercules, CA).

Wild-type Chlamydial Strains—Strain 434/bu of *C. trachomatis* serovar L2 (L2), strain UW-3/Cx of *C. trachomatis* serovar D, and strain Nigg II of *C. trachomatis* mouse pneumonitis (MoPn) represent biovars lymphogranuloma venereum, trachoma, and mouse, respectively. They were purchased from American Type Culture Collection (Manassas, VA). Strain stocks were amplified using HeLa cells with the aid of 1 μg/ml cycloheximide added to the culture medium to block host protein synthesis (6).

Selection and Cloning of Resistant Mutant—To select *Chlamydia* resistant to GM6001, L2 was cultured in HeLa cells in the presence of 6, 7, and 10 μM GM6001 for 3, 7, and 4 passages, respectively. The last passage was harvested, expanded in the absence of GM6001, and designated GR10. To obtain homogeneous clonal populations, we 1:10 serially diluted a GR10 stock before it was added onto HeLa cell monolayers grown on 35-mm culture dishes. Six replicate dishes were inoculated with each dilution of GR10 and were incubated at 37 °C. Two hours later free EBs were removed by three washes with medium. The infected cells were cultured for an additional 36 h. The monolayers were viewed carefully under an Olympus IX-41 inverted phase-contrast microscope with a 40× objective to identify inclusions. There was less than one inclusion per field in wells infected with a 1:10^6-diluted stock. An inclusion-containing cell (and the surrounding uninfected ones) was picked from each of the six dishes infected with that dilution. The cells were transferred into a tube containing 500 μl of culture medium, briefly sonicated to release EBs, and inoculated onto new HeLa monolayers to expand the resistant clones.

Immunostaining of Chlamydial Inclusions—HeLa and A549 cells grown on coverslips were exposed to an EB stock for 2 h. Unless specified, infected cells were washed with medium to remove free EBs and were cultured in medium with or without the supplementation of 1 μg/ml cycloheximide and a metalloprotease inhibitor. Infected cells were fixed with cold methanol 40 h after infection and reacted to a monoclonal antibody against the major outer membrane protein (MOMP) of L2 (7, 8). MoPn- and serovar D-infected cells were fixed at 30 and 48 h after infection, respectively, and reacted to a monoclonal antibody against chlamydial lipopolysaccharide (7, 8). Free primary antibodies were removed by three washes with phosphate-buffered saline. Coverslips were then reacted with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (7, 8). After three additional washes, coverslips were mounted onto glass slides and viewed with an Olympus IX-51 fluorescence microscope (9). For experiments comparing wild-type L2 and GR10, the multiplicities of infection for both were 5 inclusion-forming units (IFUs)/cell (see below).

Quantitation of EB Production—For experiments comparing sensitivities of L2 and GR10 to GM6001 and TAPI-0, the infectivities of their EB stocks were titrated in parallel by measuring their abilities to form inclusions in HeLa cell monolayers grown on coverslips in 24-well plates. Six replicate wells with HeLa monolayers were infected with each dilution of a stock. Infected cells were fixed with cold methanol 40 h after infection and reacted to a monoclonal antibody. Inclusions were scored following immunostaining as described above (9). Essentially the same IFU values were obtained in three repeated titration experiments for each of the stocks. Titration of EB production for other purposes was carried out in essentially the same manner, except only duplicate wells were infected with each dilution.

Determination of DNA Synthesis Activity of MoPn—HeLa cells in 24-well plates were infected with MoPn and cultured in the presence of

[3] Sieber, S. A., Niessen, S., Hoover, H. S., and Cravatt, B. F. (2006) *Nat. Chem. Biol.* **2,** 274–281.

TABLE 1
Sequences of primers used to amplify genes encoding proteins containing the HEXXH motif

Locus tag	Protein	Accession no.	5′ primer	3′ primer
CT072	Metalloprotease	AAC67663	5′TTTCGATAGTGGTGCGG3′	5′AGCATACAACAATCTCCTA3′
CT422	Metalloprotease	AAC68019	5′GCTGTTCAGCATCCAC3′	5′GGCTCTGCGGTGAGAAG3′
CT824	Metalloprotease (insulinase)	AAC68421	5′GTAGGCTGTCTTTCTTA3′	5′GAATGCTTATCCCCAGA3′
CT859[a]	LytB	NP 220381, AAC68457	5′TGGTTTGTCAGCTGATC3′	5′AAGAGCTTAAAGATCATTGG3′
CT353	Peptide deformylase	AAC67948	5′GACCAGACCCGAGACC3′	5′GCACTCCTAAACACGCC3′

[a] Note that CT859 was annotated "metalloprotease" under the accession number AAC68457 at the time the sequence analysis was performed. However, under the accession number NP220381 this annotation has been revised to the 4-hydroxy-3-methylbut-2-enyl diphosphate reductase or LytB.

indicated concentrations of GM6001 as well as cycloheximide (10, 11). Twenty-four hours later, the incorporation of [6-^{3}H]uridine into chlamydial DNA was determined as previously reported (10, 11).

Growth Determination of Other Bacteria—LB medium was used to culture *Escherichia coli* and *Salmonella enterica*, while MRS medium (ATCC) was used to grow *Lactobacillus delbrueckii*. An *E. coli* MG1655 colony was inoculated into 10 ml of LB broth, which was divided into 2-ml aliquots. TAPI-0 was added to the aliquots to the final concentrations indicated. After overnight culture, A_{600} values were taken. Overnight *L. delbrueckii* and *S. enterica* cultures were diluted 100- and 30-fold, respectively, then cultured in the presence or absence of 20 μM GM6001. A_{600} values were measured at the indicated time points. Determination of *S. enterica* growth in HeLa cells was carried out as previously described (12). HeLa cells were infected with a fresh culture of *S. enterica* grown to stationary phase. After washes to remove free bacteria, cells were cultured with medium containing 50 μg/ml gentamycin (to kill any residual free bacteria) plus or minus 20 μM GM6001 for indicated time periods. Gentamycin was then removed by washes. Intracellular bacteria were released by 0.1% Triton X-100, serially diluted, and plated onto LB agar plates. After overnight incubation at 37 °C, colony-forming units were scored (12).

Immunoblotting—HeLa or A549 cells were seeded onto 24-well plates without coverslips; infection and inhibitor treatment were carried out as described for immunostaining. For Western blotting, medium was aspirated 24 h after infection, and cells were lysed in SDS-PAGE gel loading buffer. Sample processing, electrophoresis, blotting, and antibody detection have been detailed previously (9, 13). For dot blotting, cells were lysed in 200 μl of H_2O; a 50-μl sample of the resulting EB/RB extract was blotted onto Zeta Probe membrane (Bio-Rad). The procedure for antibody detection of MOMP was the same as that for Western blotting.

Sequence Determination—EBs of wild-type L2 and GR10 obtained from infected HeLa cells were digested overnight with 100 μg/ml Proteinase K. The chlamydial genomic DNA released was purified by phenol-chloroform extraction and ethanol precipitation. Genes of interest were amplified using 50 ng of genomic DNA (per reaction) as the template. The primer sequences used for amplification are listed in Table 1. The resulting PCR fragments were gel-purified and subjected to automated sequencing analyses using primers that were 500 bases apart on both strands. The actual reading capacities of sequencing reactions were 600–750 bases.

Expression and Purification of Glutathione S-Transferase-PDF Fusion Protein—The PDF open reading frame of *C. trachomatis* (CT353) (14) was amplified with PCR and inserted into the pGEX-6p-2 *E. coli* expression vector using the BamH1 and NotI digestion sites. The glutathione *S*-transferase-PDF fusion protein was produced in the BL21 strain of *E. coli*, purified with glutathione-agarose as previously described (15).

Production of Antibody against Chlamydial PDF—Two Balb/c mice were immunized once every 2 weeks for a total of four times with the purified glutathione *S*-transferase-PDF fusion protein. Sera containing polyclonal antibodies against the chlamydial PDF were collected the 10th day after the final immunization.

Production of His-tagged Chlamydial PDF—The PDF open reading frame was cloned into the pET21 T7-expression vector in-frame to the carboxyl-terminal $(His)_6$-tag using the NdeI and SalI restriction sites. The resulting pET21-PDF plasmid was transformed into the BL21(ΔDE3)pLysS strain of *E. coli*. Transformed pLysS cells were cultured at 37 °C till A_{600} reached 0.6 and then were cultured at 30 °C for 3 h in the presence or absence of 0.1 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). The bacteria were collected by centrifugation, lysed by three cycles of freezing and thawing, and sonicated to shear genomic DNA. After centrifugation at 25,000 × *g* for 30 min, the supernatant was collected and used for enzyme assays or for labeling with the AspR1 probe or for further purification. Cobalt-conjugated agarose was used to absorb the $(His)_6$-PDF. Elution of the recombinant protein was carried out with 150 mM imidazole solution containing 5 mM $NiSO_4$.

PDF Activity Assay—An assay previously developed for *E. coli* PDF (16) was modified to measure the enzyme activity of chlamydial PDF. The assay mix, in a total reaction volume of 200 μl, contained 50 mM Hepes, 10 mM NaCl, 50 or 250 ng of purified His-tagged PDF or 5 μg of crude bacterial extract, and 4 mM fMAS. For testing the effects of chemical compounds on the deformylase, fMAS was added after 50 ng of His-tagged PDF had been incubated in the presence of desired concentrations of compounds for 10 min (17). The deformylation reaction was allowed to proceed for 30 min at 37 °C, terminated by heating at 95 °C for 3 min, and then placed on ice. The amount of formate generated from fMAS through the action of chlamydial PDF was reported by formate dehydrogenase that uses NAD as hydrogen recipient, which has a lower A_{340} value compared with its reduced form NADH. Thus, the heat-inactivated PDF reaction mix was mixed with 800 μl of 50 mM Hepes containing 0.5 unit of formate dehydrogenase, 10 mM NaCl, and 1 mM NAD. The A_{340} value was recorded immediately at the end of setting up the reporting reaction mix and again after a 3-h incubation at 37 °C. To estimate the amounts of formate generated by chlamydial PDF, a series of standard assays with various sodium formate inputs (ranging from 1 μM to 1 mM) was performed. These reactions generated a straight line (data not shown). The amounts of formate generated in all reactions with the chlamydial PDF are within the range of formate standards used.

Labeling of PDF with the AspR1 Probe—Binding of the AspR1 probe to the chlamydial PDF was performed with procedures previously developed for labeling matrix metalloproteases with hydroxamate-based molecular probe (5). Extracts prepared from untransformed pLysS cells or from the bacteria transformed with the pET21-PDF vector were mixed with 1 μM probe in the presence or absence of excessive GM6001 or TAPI-0 or GMNC. The mixtures were incubated on ice for 15 min and irradiated at 365 nm for 1 h. Rhodamine was conjugated to the probe bound to the protein by copper(I)-catalyzed azide-alkyne cycloaddition reaction (18). Rhodamine-azide, Tris(2-carboxyethyl)phosphine hydrochloride, and $CuSO_4$ (final concentrations: 12.5 μM, 100 μM, and 1 mM,

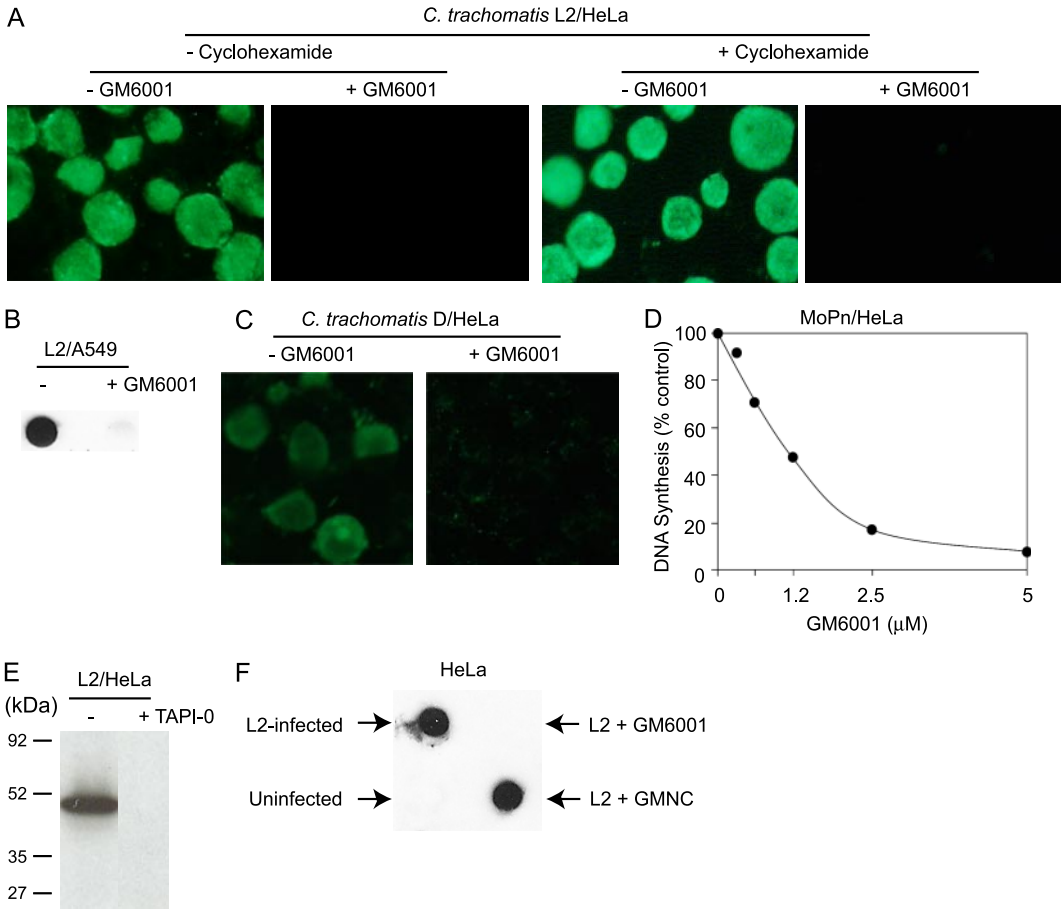


FIGURE 2. **Inhibition of chlamydial growth by hydroxamates.** *A*, MOMP immunofluorescence microscopy showing a lack of inclusion formation in L2-infected HeLa cells treated with GM6001 in the presence and absence of cycloheximide. *B*, dot blot analysis of MOMP revealing inhibition of L2 growth in A549 cells by GM6001. *C*, immunostaining of chlamydial lipopolysaccharide demonstrating inhibition of *C. trachomatis* serovar D by GM6001. *D*, dose-dependent inhibition of MoPn DNA synthesis by GM6001. *E*, Western blotting of MOMP showing inhibition of L2 growth in HeLa cells by TAPI-0. *F*, dot blot analysis showing a lack of an anti-*Chlamydia* effect in GMNC. The final concentration of GM6001 was either 25 μM (*A*, *B*, and *F*) or 20 μM (*C*) except in *D*. The final concentrations of TAPI-0 (*E*) and GMNC (*F*) were 50 μM and 25 μM, respectively. Cycloheximide was used in *C* and *D* but not in *B*, *E*, and *F*.

respectively) were added to the complex. The conjugation reaction was allowed to take place for 1 h. Labeled samples were resolved by SDS-PAGE and visualized in-gel with a Hitachi FMBio II flatbed scanner (MiraiBio, Alameda, CA).

RESULTS

Inhibition of C. trachomatis by GM6001 and TAPI-0—GM6001 is a hydroxamate-based compound that was developed as a specific inhibitor of matrix metalloproteases. Acting as a pseudosubstrate, the compound binds to the active center of the enzymes where the hydroxamic group bonds with the catalytic zinc element of the enzymes (19). Interestingly, GM6001 exhibited strong inhibitory effects on *C. trachomatis* infection. The biovar lymphogranuloma venereum organism L2 failed to form inclusion in the human cervical carcinoma HeLa cells cultured in the presence of 25 μM GM6001 (Fig. 2*A*). The strong inhibitory effect remained unchanged even when cycloheximide was used to inhibit the host protein synthesis, and consequently to inhibit host RNA and DNA syntheses, and thereby maximize chlamydial replication (Fig. 2*A*). GM6001 showed no visible cytotoxic effects on L2 and mock infected cells (data not shown). In fact, when subconfluent HeLa cells grown in 35-mm plates (2×10^6 cells per plate) were infected with L2 and cultured in the absence of cycloheximide, there were $(3.7 \pm 0.58) \times 10^6$ cells per plate in the five replicate plates treated with 10 μM GM6001 at the end of the 40-h culture period, whereas there were only $(2.3 \pm 0.29) \times 10^6$ cells per plate in the five untreated control plates at the same time. These corresponded to average increases of 1.9- and 1.2-fold in the cell number for treated and control experiments, respectively. This difference was found to be statistically significant ($p < 0.001$). Thus, GM6001 appeared to be able to reverse the inhibition of host cell proliferation caused by chlamydial infection.

In addition to HeLa cells, the inhibition of L2 growth by GM6001 was also observed in human lung carcinoma A549 cells as demonstrated by dot blot analysis (Fig. 2*B*) as well as immunostaining of inclusions (data not shown). Moreover, GM6001 effectively suppressed inclusion formation by serovar D of biovar trachoma (Fig. 2*C*). Finally, by measuring chlamydial DNA synthesis as an indicator of bacterial growth (10, 11), a dose-response curve of GM6001 inhibiting MoPn of biovar mouse was obtained (Fig. 2*D*). Taken together, these results suggest that GM6001 inhibits the growth of all three biovars of *C. trachomatis* in multiple cell types.

TAPI-0 is another hydroxamic inhibitor of metalloproteases (13, 20). Like GM6001, TAPI-0 showed a striking inhibitory effect on L2 growth (Fig. 2*E*). Therefore, inhibition of chlamydial infection appears to be a common property of hydroxamate-based inhibitors of metalloproteases. In contrast, GMNC, a chemical derivate of GM6001 containing very minor modifications (as shown in Fig. 1) which cause a loss of binding to the catalytic zinc of metalloproteases (19), had no detectable effect on chlamydial growth (Fig. 2*F*). These results suggest that

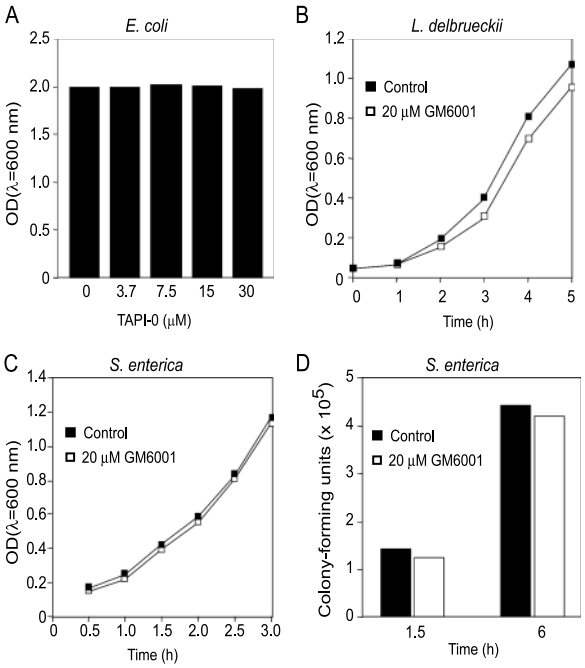


FIGURE 3. **Lack of inhibition of *E. coli*, *L. delbrueckii*, and *S. enterica* growth by TAPI-0 and GM6001.** *A*, *E. coli* was cultured in LB broth containing indicated concentrations of TAPI-0. A_{600} values were measured after overnight incubation. *B*, *L. delbrueckii* was cultured in MRS broth containing 20 μM GM6001. Samples were taken for A_{600} measurement at the indicated time points. *C*, *S. enterica* was cultured in LB broth containing 20 μM GM6001 for the indicated periods of time. A_{600} values of the culture were measured at the indicated time points. *D*, HeLa cells were infected with *S. enterica*, washed and cultured with medium containing gentamycin plus or minus 20 μM GM6001 for the indicated periods of time. After washes with antibiotic-free medium, intracellular bacteria were released by Triton X-100 and plated onto LB plates. The resulting colonies were scored.

GM6001 and TAPI-0 inhibit chlamydial infection by targeting one or more metalloproteases.

Lack of Growth Inhibition by GM6001 and TAPI-0 against Common Bacterial Species—The observation of strong inhibitory effects of GM6001 and TAPI-0 on chlamydial replication prompted us to address whether the growth of other bacterial species is also sensitive to these compounds. Neither GM6001 nor TAPI-0 showed any inhibitory effects on the growth of *E. coli*, *L. delbrueckii*, and *S. enterica* in liquid cultures (Fig. 3, *A–C*). Because *S. enterica* is a facultative intracellular bacterium, we also assessed whether its intracellular growth is affected by inhibition of metalloproteases. Clearly, GM6001 did not inhibit the growth of *Salmonella* in HeLa cells (Fig. 3*D*). These results indicate that GM6001 and TAPI-0 specifically inhibit Chlamydiae without affecting the growth of facultative intracellular or free-living bacteria.

Entry-independent Inhibition—Mammalian cells express a wide range of metalloproteases (21); many of them are inhibited by hydroxamates (19, 21). The chlamydial genome also encodes several candidate zinc metalloproteases (14, 22–24). Because many metalloproteases in mammalian cells in general are secreted or membrane-anchored ectoenzymes (21), a host metalloprotease sensitive to GM6001 and TAPI-0 might mediate chlamydial entry. However, this appears unlikely, because inhibition of chlamydial growth was obtained when GM6001 and TAPI-0 were added to cultures at the end of the 2-h attachment/entry period (Figs. 2), a point where free unbound EBs had been removed by extensive washes. The entry-independent inhibition was also seen by titrating infectious EBs produced by infected HeLa cells (Table 2). Accordingly, 20 μM GM6001, added after the attachment/entry period, exhibited a better than 99.99% inhibition of EB production. A comparable inhibitory activity was also noted when GM6001 was added 8 h after infection. At this point, EBs have already completed the entry phase and have differentiated into RBs in inclusions (25). Even when GM6001 treatment was started 24 h after infection, a point where the number of RBs peaks during the chlamydial developmental cycle (25), a 99% reduction in EB production was observed (Table 2). These results suggest that GM6001 (and TAPI-0) inhibit Chlamydiae by targeting a step subsequent to cell entry.

TABLE 2
Cell entry-independent targeting of *Chlamydia* by GM6001
An L2 stock was added to HeLa cell monolayers. Cells were incubated for 2 h at 37 °C, washed three times, and cultured with medium. GM6001 (final concentration: 20 μM) was added at the indicated time points. Cells were lysed at 40 h after infection; numbers of EBs in the cell lysates were titrated on HeLa cells using MOMP immunofluorescence microscopy and are expressed as IFUs.

	No GM6001	GM6001 added at times after infection		
		2 h	8 h	24 h
IFUs	5.2×10^7	1.4×10^2	5.5×10^2	5.2×10^5
% inhibition	0	>99.99	>99.99	99

Selection of GR10, an L2 Mutant Resistant to GM6001 and TAPI-0—To further address whether GM6001 and TAPI-0 suppress chlamydial growth through inhibition of a bacterial component, we set out to isolate chlamydial mutants that are resistant to these compounds. As described under "Experimental Procedures," we obtained an L2 mutant designated GR10. GM6001 resistance in GR10 was demonstrated by its markedly increased abilities to form inclusions (Fig. 4*A*) and to generate infectious EBs (Fig. 4*B*) at high GM6001 concentrations. The resistance to GM6001 in GR10 is a very stable phenotype, as judged by its ability to overcome GM6001 inhibition after five passages in the absence of the inhibitor. All clones isolated from GR10 and expanded in the absence of GR10 were resistant to GM6001 when they were grown in the presence of GM6001 again (data not shown). Significantly, GR10 is also cross-resistant to TAPI-0 (Fig. 4*C*), suggesting that GM6001 and TAPI-0 inhibit chlamydial growth by the same mechanism.

Lack of Mutations in Candidate Metalloproteases in GR10—Because GM6001 and TAPI-0 are believed to be specific inhibitors of metalloproteases, we sequenced three candidate metalloprotease genes, CT072, CT422, and CT824, in a GR10 clone and parental L2 (even though none of the proteins encoded by these genes had been proven as a true protease). We also sequenced CT859, which was annotated a metalloprotease as well under the accession number AAC68457 at the time our sequencing analysis was performed (however, this annotation has been recently revised to LytB that encodes the putative 4-hydroxy-3-methylbut-2-enyl diphosphate reductase under the accession number NP220381). No mutations were found in any of the four genes analyzed in GR10 (data not shown). Therefore, the three candidate metalloproteases analyzed and CT859 are unlikely to be the targets of the inhibitors.

Mutation and Overexpression of PDF in GR10—We reasoned that another yet-to-be identified metalloprotease might be targeted by GM6001 and TAPI-0. We searched for additional chlamydial proteins with an HE*XX*H motif that is conserved in metalloproteases. Interestingly, we found that PDF (whose gene is designated CT353 in the *C. trachomatis* genome project) contains HETDH. PDF is a metal-dependent hydrolase that catalyzes the removal of the *N*-formyl group from newly synthesized proteins in bacteria. This modification step is required for bacterial neopeptides to become biologically active (26, 27). We found a single point (C → A) mutation at −245 relative to the ATG initiation codon of PDF in GR10 (Fig. 5*A*). All three GR10 clones that were sequenced also showed the same mutation (data not shown).

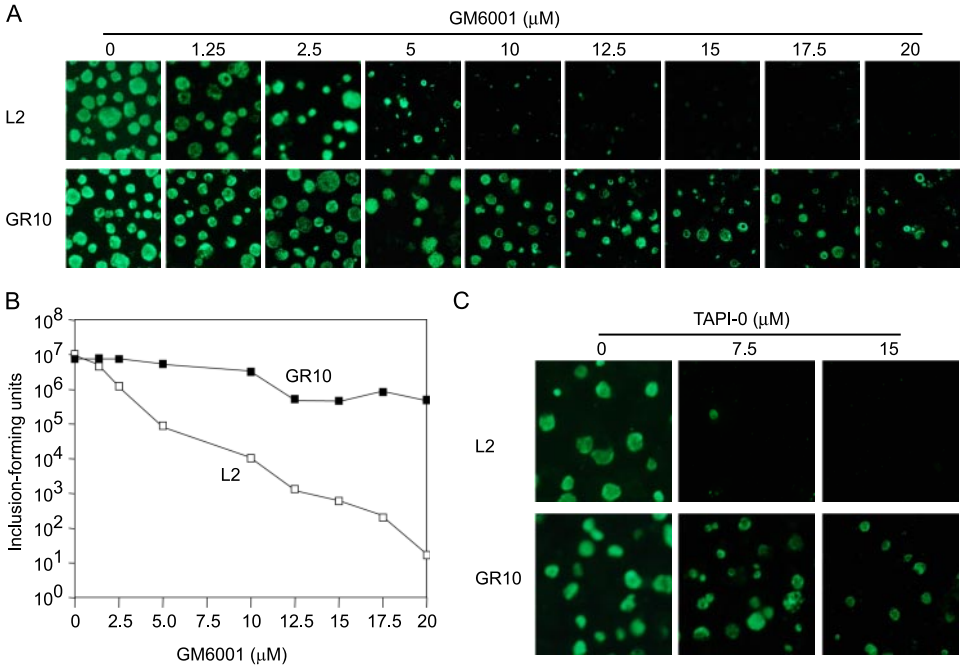


FIGURE 4. **Characterization of GM6001/TAPI-0-resistant L2 mutant GR10.** *A*, increased capacity to form inclusions in the presence of a wide range of GM6001 concentrations as shown by MOMP immunostaining. *B*, increased EB production in the presence of GM6001 as determined by quantifying inclusions formed in secondary cultures inoculated with the lysates of GM6001-treated *Chlamydia*-infected cells. *C*, cross-resistance of GR10 to TAPI-0 as demonstrated by its increased capability of inclusion formation in the presence of the compound.

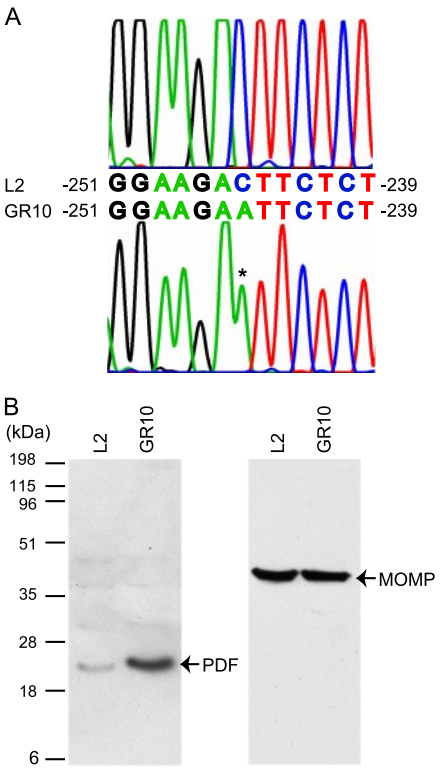


FIGURE 5. **Point mutation in the PDF gene and overexpression of the PDF protein in GR10.** *A*, genomic DNA was extracted from GR10 and parental L2. The PDF gene (as well as parts of the neighboring CT352 and CT354) was amplified from the genomic DNA using the primers listed in Table 1. The PCR fragment was subjected to automated sequencing after purification. The substituting adenosine at base −245 (relative to the translation initiation codon of PDF) found in GR10 is underlined, and the corresponding fluorescence signal on the wave form is marked with an *asterisk*. *B*, extracts were prepared from HeLa cells infected with GR10 or L2 and were subjected to Western blotting. The polyvinylidene difluoride membrane was initially probed with a polyclonal antibody for PDF (*left*), stripped with SDS and 2-mercaptoethanol, and then reprobed with the MOMP antibody (*right*).

These findings suggest that PDF instead of a metalloprotease might be the target of GM6001 and TAPI-0.

Because the mutated base is located in a non-coding region of the PDF gene in GR10, we predicted that the mutation would increase the expression of PDF and as a result would confer resistance to GM6001 and TAPI-0. Indeed, Western blotting detected an elevated expression level of the 20-kDa PDF protein in GR10 as compared with L2 (Fig. 5*B left*), whereas the same levels of MOMP were detected in GR10 and L2 after the polyvinylidene difluoride membrane was treated with SDS and 2-mercaptoethanol to strip off the anti-PDF antibody and then reprobed with the anti-MOMP antibody (Fig. 5*B*, *right*). Densitometry analysis showed there was a 5-fold increase of PDF protein in GR10 as compared with L2.

Binding of the AspR1 Probe to the Recombinant Chlamydial PDF and Inhibition of Binding by GM6001 and TAPI-0 but Not GMNC—Hydroxamate-based molecular probes carrying a benzophenone photocross-linker that allows for covalent labeling of metalloproteases upon photoactivation have been developed recently (5). We here demonstrate covalent labeling of recombinant chlamydial PDF with the AspR1 probe (see Fig. 1 for structural information) by photoactivated cross-linking and subsequent attachment of the fluorescent dye Rhodamine to the probe. Accordingly, a prominent fluorescent protein band corresponding to the 21-kDa recombinant protein carrying a polyhistidine tag was readily detected in the extracts prepared from *E. coli* BL21(ΔDE)pLysS cells transformed with a pET21 expression vector containing the PDF gene. As expected, a more strongly labeled PDF signal was seen in the extract prepared from bacteria cultured with IPTG to induce PDF expression as compared with the extract prepared from the uninduced culture (Fig. 6*A*). The fluorescently labeled PDF protein band was completely absent from the control extract prepared from untransformed pLysS cells (Fig. 6*A*). Evidently, binding of the AspR1 probe to the recombinant PDF was significantly weakened in the presence of excessive amounts of GM6001 and TAPI-0 but not GMNC (Fig. 6*B*). Thus, the abilities of these compounds to compete with the AspR1 probe in binding the recombinant chlamydial PDF correlates with their capacities to inhibit chlamydial growth in cells.

Inhibition of PDF Activity by GM6001 and TAPI-0 but Not GMNC—To prove chlamydial PDF as the target of GM6001 and TAPI-0, we performed PDF enzyme assays in the presence and absence of the compounds. With fMAS as a substrate, only a very low basal peptide

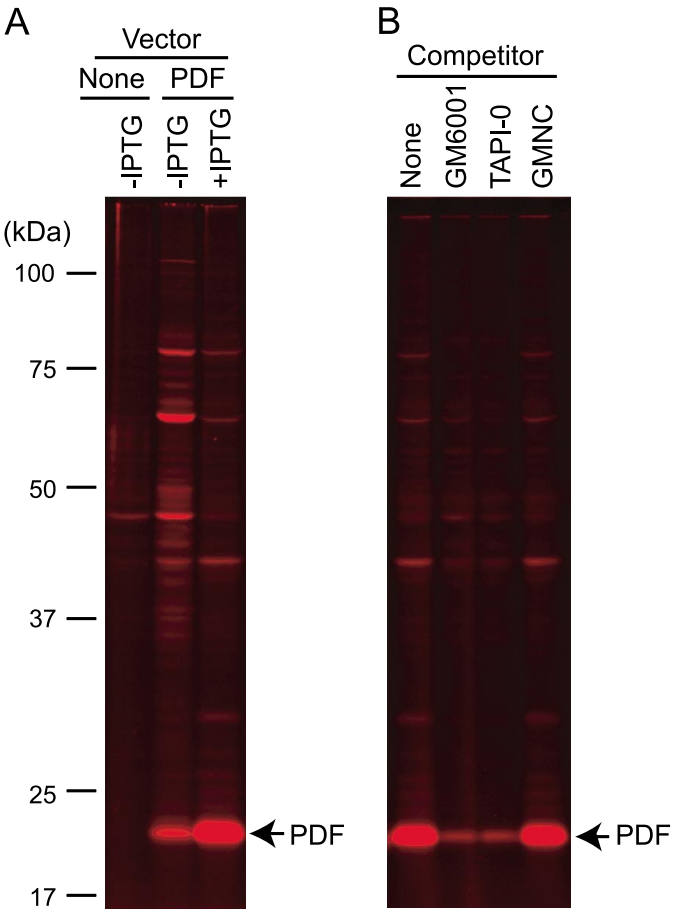


FIGURE 6. **Binding of the AspR1 probe to recombinant chlamydial PDF.** *A*, extracts were prepared from untransformed BL21(ΔDE3)pLysS *E. coli* or bacteria, which were transformed with the pET21-PDF expression vector and cultured in the absence or presence of IPTG, incubated with 1 μM AspR1 probe, and exposed to UV. A copper(I)-catalyzed azide-alkyne cycloaddition reaction was performed to attach Rhodamine to the probe (18, 37). Labeled proteins were resolved by SDS-PAGE and detected by a Typhoon fluorescence scanner. *B*, extract of pLysS cells induced with IPTG was subjected to AspR1 labeling in the presence or absence of the indicated competitors. The concentrations of the probe and the competitors were 1 and 100 μM, respectively.

deformylation activity, representing the activity of the endogenous *E. coli* PDF, was detected in the extract prepared from untransformed pLysS bacteria (Table 3). Transformation with the pET21-PDF expression vector led to an ~5-fold elevation of enzyme activity, which increased additionally nearly 3-fold following the induction of recombinant expression with IPTG (Table 3). As expected, the purified His-tagged PDF protein yielded the highest specific enzyme activity (Table 3). Importantly, our enzyme assays showed that GM6001 and TAPI-0 but not GMNC are highly effective inhibitors of chlamydial PDF. Accordingly, the IC_{50} of GM6001 and TAPI, assayed against purified recombinant PDF, were determined to be 38 and 18 nM, respectively (Fig. 7, *A* and *B*). In contrast, even 10 μM GMNC showed a lack of inhibition of chlamydial PDF activity (Fig. 7*C*).

Inhibition of Chlamydial Growth and PDF Enzyme Activity with Actinonin, a Known PDF Inhibitor—Actinonin is a hydroxamate produced by an actinomycete with antibiotic activity. It has been shown that actinonin is an efficient inhibitor of bacterial PDF (17, 28). Evidently, actinonin is also an extremely efficient inhibitor of chlamydial PDF with an IC_{50} of 1.2 nM (Fig. 8*A*). The PDF inhibitor also strongly inhibited chlamydial growth. Accordingly, no inclusions were detected in L2-infected cells cultured in the presence of 0.5 μM actinonin (Fig. 8*B*). In a strong contrast, the growth of GR10 tolerated significantly higher actinonin concentrations (Fig. 8*B*). Thus, similar to other bacteria, actinonin inhibits the growth of *Chlamydia* by blocking peptide deformylation.

TABLE 3

Enzyme activity of recombinant chlamydial PDF expressed using the BL21 (ΔDE3)pLysS strain of *E. coli*

fMAS was used as the substrate for PDF. The amounts of formate generated were detected by formate dehydrogenase using NAD as hydrogen recipient.

	Specific activity
	nmoles of formate generated/mg protein/min
Extract of untransformed pLysS	58
Extract of pET21-$(His)_6$-PDF-transformed pLysS, uninduced	307
Extract of pET21$(His)_6$-PDF-transformed pLysS, IPTG-induced	886
Purified $(His)_6$-PDF	12,400

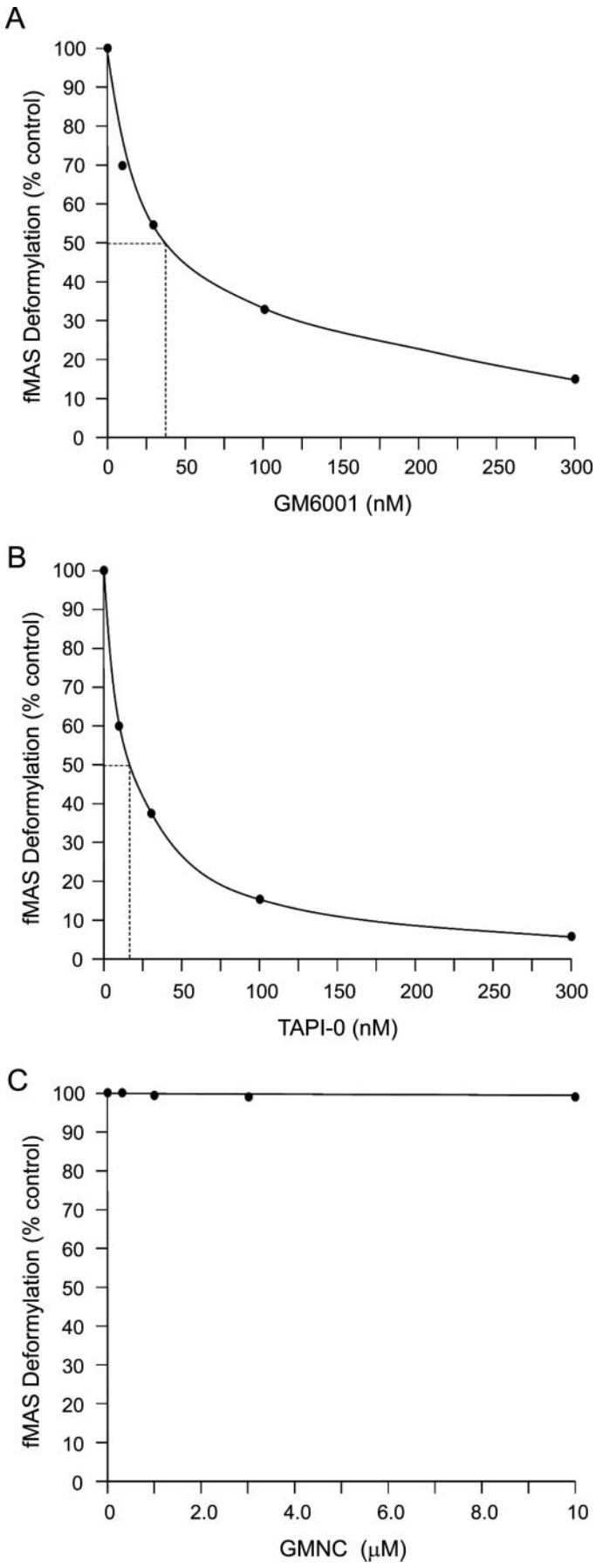
DISCUSSION

Newly synthesized proteins in eubacteria are initiated with *N*-formylmethionine. In most cases, the neopeptides are converted to mature proteins through the sequential removals of the *N*-formyl group and methionine by PDF and methionine aminopeptidase, respectively. In rare cases, the formyl group is removed, but the initiator methionine is retained (26). Deletion of the PDF gene in *E. coli* results in a lethal phenotype (27), indicating this enzyme as a potential target for antibacterials. Indeed there are tremendous efforts in the pharmaceutical industry to design novel PDF inhibitors for bacterial pathogens, particularly for those that are resistant to multiple current antibiotics (29–32).

In this study, we have presented evidence that GM6001 and TAPI-0 are inhibitors of chlamydial PDF, and they inhibit *C. trachomatis* growth most likely by targeting this important enzyme. Accordingly, a mutation found in the PDF gene leading to overexpression of the PDF protein correlated with resistance to GM6001 and TAPI-0 as well as to actinonin, a known antibacterial PDF inhibitor. In addition, the hydroxamate-based AspR1 probe, which shares structural features with GM6001 and TAPI-0 covalently labeled the recombinant chlamydial PDF, and furthermore, GM6001 and TAPI-0 competed off the probe in the labeling of the parasitic enzyme. Finally, *in vitro* assays directly demonstrated that both GM6001 and TAPI-0 are strong inhibitors of chlamydial PDF. The IC_{50} values of GM6001 and TAPI-0 against the PDF are in the nanomolar range, which are quite comparable to their IC_{50} values against metalloproteases (5, 33).

GM6001 and TAPI-0 were developed as specific inhibitors of matrix metalloproteases (19, 20). Recent studies have shown that they are also active against other types of metalloproteases (5). The findings of GM6001 and TAPI-0 as inhibitors of chlamydial PDF raise the question of whether these compounds may also target other metallohydrolases (besides metalloproteases and PDF), which modify polypeptides. Similar to GM6001 and TAPI-0, actinonin also inhibits certain metalloproteases (34). Thus, the possibility that GM6001 and TAPI-0 inhibit *Chlamydia* by simultaneously targeting an unrecognized metalloprotease(s), or another hypothetical protein(s) that is also required for chlamydial growth cannot be excluded even though no mutations have been found in the candidate metalloprotease genes in GR10 that are resistant to GM6001, TAPI-0, and actinonin. A definitive answer to this may require sequencing of the entire genome of the mutant, because *Chlamydia* is yet genetically untransformable.

The mutation that renders overexpression of the PDF protein in GR10 is located 245 bp upstream of the ATG translation initiation



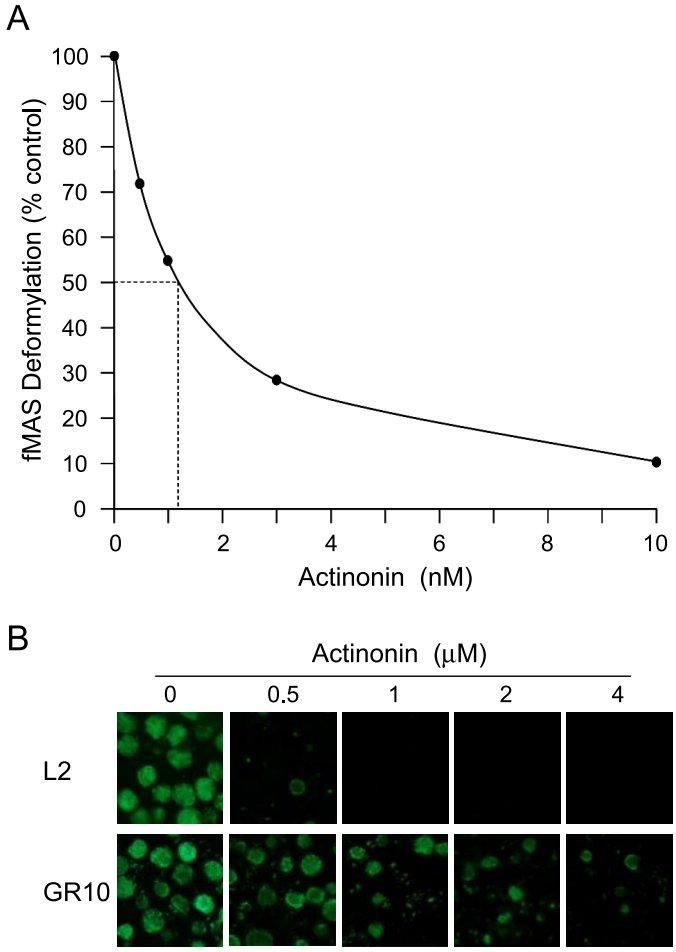


FIGURE 8. **Inhibition of chlamydial PDF activity (*A*) and growth (*B*) by actinonin.** *A*, PDF enzyme activity was determined as descried in Fig. 7 legend. *B*, growth inhibition by actinonin was performed using HeLa cells. Refer to Fig. 1 for assay conditions.

codon of PDF. This base is likely part of the PDF (*i.e.* CT353) gene instead of the neighboring CT354 gene, because it is 7 bases outside the transcription termination signal of CT354. Furthermore, because the mutated base is relatively far away from the hypothetical ribosomal binding signal (which is usually immediately before the initiation codon in bacteria), the region containing the mutation is likely to control the transcription rather than the translation of PDF. Like most other chlamydial genes, a −35 promoter sequence in the PDF gene was not readily identifiable (35, 36). Therefore, identifying a definitive role of this region in the transcription of PDF will require further experimental analyses.

It is thought that peptide deformylation is ubiquitously required by all prokaryotes. Indeed PDF is found in all prokaryotic genomes that have been sequenced. While GM6001 and TAPI-0 are strong inhibitors of Chlamydiae, they exhibited no effects on the growth of three other bacteria tested. Because the amino acid sequence of chlamydial PDF is only 33–40% identical to those of *E. coli*, *Salmonella*, and *Lactobacillus* PDFs (data not shown), it is tempting to hypothesize that the structure of the catalytic center of the chlamy-

FIGURE 7. **Inhibition of chlamydial PDF activity *in vitro* by GM6001 (*A*) and TAPI-0 (*B*) but not GMNC (*C*).** Purified *E. coli*-expressed His-tagged chlamydial PDF was preincubated with the indicated concentrations of compounds before the addition of fMAS. The amounts of formate generated were determined by formate dehydrogenase using NAD as hydrogen-recipient. Note the *x*-axes in *A* and *B* are on the nanomolar scale, whereas the *x*-axis in *C* represents the micromolar scale.

dial enzyme differs significantly from those of other PDFs and therefore that the chlamydial PDF has unique affinity to GM6001 and TAPI-0. Nevertheless, the existence of other potential resistant mechanisms such as bacterial wall and/or membrane impermeability to the compounds, and efflux as well as higher expression levels of the enzyme (as compared with *C. trachomatis*) in the resistant organisms should also be considered. Directly testing whether these compounds inhibit the PDFs from the resistant bacteria would be a starting point to distinguish among these possibilities.

Infection with *C. trachomatis* is widespread and often asymptomatic. When left untreated, it frequently leads to infertility and other serious conditions. The discovery that PDF is critical for chlamydial growth has implications for the treatment and prevention of chlamydial infections. The ability of GM6001 and TAPI-0 to inhibit Chlamydia without affecting the growth of several other bacteria tested suggests that it may be possible to develop PDF inhibitors that specifically target chlamydial infection with minimal or no adverse effects on normal microbial flora.

Acknowledgments—We thank Drs. Ann Stock (Center for Advanced Biotechnology and Medicine) and Thomas Montville (Rutgers University) for supplying S. enterica and L. delbrueckii, respectively. We also thank Dr. Yadong Yang (Harvard School of Public Health) for performing statistical analysis on the cell proliferation data, Dr. Soumik Biswas for drawing the compound structures in Fig. 1, Dr. Yuh-hwa Wang for help with densitometry, and colleagues at the Robert Wood Johnson Medical School as well as the Fan laboratory for valuable discussions. A. B. thanks Dr. Xiaojin Li for technical advice during the early phase of this study.

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