Chloramphenicol Causes Mitochondrial Stress, Decreases ATP Biosynthesis, Induces Matrix Metalloproteinase-13 Expression, and Solid-Tumor Cell Invasion

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Overuse and abuse of antibiotics can increase the risk of cancer. Chloramphenicol can inhibit both bacterial and mitochondrial protein synthesis, causing mitochondrial stress and decreased ATP biosynthesis. Chloramphenicol can accelerate cancer progression; however, the underlying mechanisms of chloramphenicol in carcinogenesis and cancer progression are still unclear. We found that chloramphenicol can induce matrix metalloproteinase (MMP)-13 expression and increase MMP-13 protein in conditioned medium, resulting in an increase in cancer cell invasion. Chloramphenicol also activated c-Jun N-terminal kinases (JNK) and phosphatidylinositol 3-kinase (PI-3K)/Akt signaling, leading to c-Jun protein phosphorylation. The activated c-Jun protein has been proven to activate binding to the MMP-13 promoter and also upregulate the amount of MMP-13. Both the SP 600125 (JNK inhibitor) and LY 294002 (PI-3K/Akt inhibitor) can inhibit chloramphenicol-induced c-Jun phosphorylation, MMP-13 expression, and cell invasion. Overexpression of the dominant-negative JNK and PI-3K p85 subunit also negate chloramphenicol-induced responses. Other antibiotics that cause mitochondrial stress and a decrease in ATP biosynthesis also induce MMP-13 expression. These findings suggest that chloramphenicol-induced PI-3K/Akt, JNK phosphorylation, and activator protein 1 activation might function as a novel mitochondrial stress signal that result in an increase of MMP-13 expression and MMP-13-associated cancer cell invasion. The findings of this study confirms that chloramphenicol, and other 70S ribosomal inhibitors, should be administered with caution, especially during cancer therapy.

Key Words: chloramphenicol; matrix metalloproteinase; cell invasion; antibiotics; JNK; PI-3K, mitochondria stress.

Chloramphenicol, extracted from *Streptomyces venezuelae*, is an antibiotic with a small molecular size and highly lipid soluble. Chloramphenicol is indicated for the treatment of wound healing and ocular infections. Chloramphenicol reaches therapeutic concentrations in the cerebrospinal fluid and is used for the treatment of meningitis in the third-world countries.

Also, it is used as the last-line defense for multiple drug-resistant organisms, such as vancomycin-resistant *Enterococcus* (Hammett-Stabler and Johns, 1998). Aside from its pharmaceutical application, chloramphenicol has become a major contaminant in farmed seafood (especially shrimps, crab, and fish). Due to the fact that some farmed seafood contain excessive chloramphenicol residue, new concerns about its toxicity have been raised. Adverse effects caused by overdose and overuse of chloramphenicol include aplastic anemia, gray baby syndrome, and leukemogenesis (Hammett-Stabler and Johns, 1998; Holt *et al.*, 1993; Robin *et al.*, 1981). In a mouse T-cell model, chloramphenicol induced abnormal cell differentiation and inhibited apoptosis, leading to the development of leukemia-like syndrome (Yuan and Shi, 2008). Based on epidemiologic studies, chloramphenicol did not contribute to carcinogenesis when applied topically. However, its causal association with leukemia, especially through systemic use, was probably not adequately tested in these studies (Smith *et al.*, 2000). From a thorough review by the International Agency for Research on Cancer, chloramphenicol is listed as a probable carcinogen to humans (Group 2A). The underlying mechanism of chloramphenicol in human solid-tumor progression is still unclear.

Chloramphenicol reversibly binds to the 50S subunit of the 70S ribosome, inhibiting peptidyl transferase in both prokaryotic organisms and mitochondria (Balbi, 2004). In histopathological studies, chloramphenicol-stressed mitochondria showed morphological and functional deterioration (Duewelhenke *et al.*, 2007). Mitochondrial damage is an important contributor to human aging and aging-related diseases. Thus, we assumed that chloramphenicol-treated somatic cells may show senescence-related phenotypes. In 2005, we found that chloramphenicol caused senescence-associated morphological changes and senescence biomarker SA-βGal activation in somatic cancer cells (Li *et al.*, 2005). Besides morphological alteration, senescent cells often exhibit cell cycle blockage, decreased sensitivity to growth factors and apoptosis stimulators, and changes in their

gene expression pattern. A study using complementary DNA (cDNA) array showed that a variety of genes involved in cell cycle regulation, cytoskeleton, stress response, and metabolism are altered during senescence (Kang *et al.*, 2003).

Upregulation of matrix metalloproteinases (MMPs) during senescence may contribute to matrix degradation and senescent phenotype appearance (Kang *et al.*, 2003). MMPs are a group of zinc-dependent endopeptidases. It has been demonstrated that MMPs participate in several physiological (including embryonic development and wound healing) and pathological events (rheumatoid arthritis and tumor metastasis) (Kuzuya and Iguchi, 2003; Leeman *et al.*, 2002). At least 23 different MMPs have been identified in humans. MMPs have been classified into gelatinases, collagenases, stromelysins, and membrane-type MMPs. The MMP-13 (also called collagenase-3) can hydrolyze collagen types I, II, III, IV, X, and XIV; gelatin; aggrecan; tenascin C; and fibronectin (Knäuper *et al.*, 1997a; Leeman *et al.*, 2002). Because of its proteolytic potency and wide substrate specificity, the expression of MMP-13 is restricted to specific tissues, including human fibroblasts, keratinocytes, and solid-tumor cells (Knäuper *et al.*, 1997a). High levels of MMP-13 often correlate with malignant tumor invasion, suggesting an important role for MMP-13 in tumor progression (Knäuper *et al.*, 1997a; Leeman *et al.*, 2002).

The rationale of the study is to verify chloramphenicol-induced senescence-like responses, especially the mitochondria-to-nucleus stress signals and MMP expression in chloramphenicol-treated H1299 cells. We found that chloramphenicol treatment activates PI-3 kinase/Akt/c-Jun N-terminal kinases (JNK) signaling and increases nuclear c-Jun activity, which contribute to MMP-13 expression and H1299 cell invasion.

MATERIALS AND METHODS

Reagents, cell culture, and treatments. All chemicals used were of analytical grade and, unless otherwise indicated, were purchased from Sigma (St Louis, MO). Chloramphenicol was dissolved in ethanol, prepared freshly, and protected from light. The solvent content was < 5 μl/ml in all experiments. The H1299 (non–small-cell lung cancer), HepG2 (hepatocarcinoma), A549 (lung adenocarcinoma), and HaCaT cell (immortalized keratinocyte) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, and cultured in a humidified atmosphere of 5% CO_2 at 37°C.

Transient transfection of dominant-negative-JNK and DN p85. H1299 cells were seeded in complete medium in six-well plates for 24 h before transfection. Cells were transfected with 2-μg plasmid DNA containing dominant-negative (DN) JNK (a gift from Dr H.-F. Yang-Yen, Institute of Molecular Biology, Academia Sinica, Taiwan) or DN p85 (δ-p85; a gift from Dr W. M. Fu, Institute of Pharmacology, National Taiwan University, Taiwan) using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in serum-free medium, according to the manufacturer's protocol. The medium was replaced with complete medium 6 h later. Cells were then incubated for another 24 h and were then exposed to chloramphenicol.

Cellular ATP content measurement. Total cellular ATP levels were determined by ATP-bioluminescent assay. Cell lysate was prepared as describe in the manufacturer's protocol (BioVision, Mountain View, CA). Each reaction

was performed by mixing 5–10 μl lysates in 90 μl reaction buffer in a 96-well plate. Finally, luciferase and its substrates were added and luminescence intensity was counted immediately, using a luminometer (TopCount; Packard, Ramsey, MN) calibrated with appropriate ATP standards.

RNA isolation, reverse transcription–PCR, and real-time quantitative PCR. Total RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction as described previously (Li *et al.*, 2005). To analyze MMP messenger RNA (mRNA) expression, reverse transcription (RT)-PCR were performed by evaluating MMP mRNA content and that of a housekeeping gene, β-actin, as an internal control. In brief, the first strand cDNA was synthesized from 3 μg total RNA at 42°C for 60 min. Specific MMP cDNAs were amplified as follows: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s and a final 10 min extension at 72°C. The PCR products were separated by 2% agarose gel electrophoresis, with ethidium bromide staining. The PCR primer sets for MMPs and β-actin are listed in Supplementary table 1.

The real-time quantitative PCR (qPCR) and quantitative measurements were performed with SYBR-Green PCR Mix essentially as described by the manufacturer (Bio-Rad, Hercules, CA). For MMP-13, the RT-PCR primer set was purchased from SABiosciences (Frederick, MD). Normalized expression levels were then calculated using the starting levels of β-actin in each sample to normalize for differences in total RNA content in individual samples.

Nuclear protein extraction. Nuclear extracts were prepared using nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL). According to the manufacturer's protocol, cell monolayer on 100-mm dishes were scraped and resuspended in cytoplasmic extraction reagent by repetitive pipetting. The cytosolic fraction was separated by centrifugation (15,000 × g, 5 min). The nuclear protein was extracted with nuclear extraction reagent by intermittently vortexing over 40 min.

Western blots. To evaluate the MMP-13 content in the culture medium, serum-free DMEM-conditioned medium was collected and precipitated with 10% trichloroacetic acid at 4°C for 30 min (Ahmed *et al.*, 2003). The precipitated proteins were separated by centrifugation (14,000 × g) and redissolved in 2× sample buffer (125mM Tris/HCl, 4% SDS, 20% glycerin, β-mercaptoethanol, and 0.25% bromophenol blue, pH 6.8) and boiled for 10 min. To obtain total cellular protein extracts, a cell monolayer was washed with PBS and lysed in RIPA lysis buffer (50mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 150mM NaCl, 1mM ethylene glycol tetraacetic acid, 1mM NaF, 1mM Na_3VO_4, and proteinase inhibitor cocktail). The protein concentration was determined by the Bradford method (Bio-Rad). Proteins were separated by 10 or 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene fluoride (PVDF) membranes. For immunodetection, the PVDF membrane was blocked in nonfat milk and incubated in Tris-buffered saline with Tween-20 (TBST) with antibodies specific to Erk, phospho-Erk, p38-MAPK, phospho-p38-MAPK, Akt, phospho-Akt, JNK, phospho-JNK, and phospho-cJun (Santa Cruz, Santa Cruz, CA), MMP-13 (BioVision), and β-actin (Sigma). For chemiluminescent detection, blots were incubated with horserad-ish peroxidase-conjugated secondary antibodies (1:5000 in TBST) for 2 h at room temperature, followed by enhanced chemiluminescence detection, according to the manufacturer's protocol (PerkinElmer).

In vitro invasion assay. The *in vitro* invasion assay was carried out in transwell plates with 8-μm pores. Each transwell was precoated with 25–30 μl diluted Matrigel (BD Biosciences, Belford, MA) (1:3 dilution with serum-free DMEM) as described previously (Yagel *et al.*, 1989). H1299 (2.5×10^4 cells per well) or A549 (5×10^4 cells per well) was seeded on top of the Matrigel layer and incubated for 12–48 h. Noninvading cells and the Matrigel layer were removed using a cotton bud and the migrated cells, attached to the lower membrane, were fixed by immersion in 4% formaldehyde for 3 min. Finally, the invading cells were counterstained with 0.05% crystal violet and counted.

Lung colonization assay. Experimental *in vivo* metastatic potential of the H1299 (with or without chloramphenicol treatment) was measured by the lung

colonization assay (Kim *et al.*, 2009). Cells used for injection were grown to subconfluence, treated with chloramphenicol for 24 h, trypsinized, washed, and single-cell suspensions were prepared in serum-free DMEM. The cell suspensions (5×10^4 single cells/0.2 ml DMEM) were injected into the tail vein of 4- to 6-week-old male mice. C57BL/6J mice were obtained from National Taiwan University Animal Center and housed aseptically in the animal facilities. All experimental procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*. Twenty days later, the mice were anesthetized with isoflurane and sacrificed. The lungs were fixed with Bouin's solution, and metastatic colonies on the lung surface were counted macroscopically.

Gelatin zymography. The enzymatic activity of MMP-13 was assayed by gelatin zymography (Yoon *et al.*, 2002). Conditioned medium was mixed with sample buffer without reducing agent. The sample was loaded into a gelatin (1 mg/ml)-containing SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was washed twice in washing buffer (50mM Tris-HCl, pH 7.4; 150mM NaCl; and 2.5% Triton X-100) for 30 min and then incubated in incubation buffer (50mM Tris-HCl, pH 7.4; 150mM NaCl, 0.02% NaN$_3$, 10mM CaCl$_2$, and 1μM ZnCl$_2$) at 37°C. After 12 h of incubation, the gel was stained with 0.1% Coomassie brilliant R-250 in 50% methanol and 10% acetic acid for 30 min and destained with staining solution without Coomassie brilliant R-250. The clear zone of gelatin digestion indicated the presence of MMP protein.

Chromatin immunoprecipitation. The assay was performed using the chromatin immunoprecipitation (ChIP) kit (Upstate Biotech, Lake Placid, NY). Living cells were treated with formaldehyde to generate protein-DNA cross-links. Then, sheer chromatin (with an average DNA fragment size to ~500 base pairs) was prepared by repeated sonication. The extract was divided to INPUT and ChIP portions. Anti-phospho-cJun IgG (Catalog sc-822; Santa Cruz) were added to the ChIP portions at 1:100 dilution for immunoprecipitation. The nonspecific IgG was used as a negative control. The immunocomplexes were pulled down by protein A/Sepharose and washed out of the nonspecific binding. The cross-links in the immunoprecipitated chromatin were reversed by heating overnight with proteinase K at 65°C. The DNA was purified by phenol/chloroform extraction and ethanol precipitation. The purified ChIP-captured DNA of samples and of input DNA samples were amplified by the PCR method as described before. The following oligonucleotides: 5′-CACCCACAT-CAGGAACCCCCCCATC-3′, 5′-GAGGAAGACCTCCAGTTTGCA GAG-3′ (MMP-13) and 5′-GATATCGCCGCGCTCGTCGTCGAC-3′, 5′-ACCATCAC GCCCTGGTGCCTGGGG-3′ (β-actin) were used for PCR detection.

Statistical analysis. All data were expressed as mean ± SD from at least three independent experiments. Statistical analysis was carried out using Student's *t*-test or one-way ANOVA, and values of $p < 0.05$ were considered statistically significant.

RESULTS

Chloramphenicol Caused Mitochondrial Stress, ATP Restriction, and Increased Cancer Cell Invasion

Chloramphenicol testing dosages (10–100 μg/ml) used in these studies were without cytotoxicity in MTT assay (Supplementary fig. 1). The clinical dosage of chloramphenicol normally used against infectious diseases is 10–30 μg/ml in serum, which corresponds to a dose of 50–100 mg/kg/day (Balbi, 2004). Chloramphenicol inhibited mitochondrial protein translation (Fig. 1A), caused mitochondrial stress, and decreased ATP biosynthesis (Fig. 1B) in H1299 cells in a dose-dependent manner. A transwell assay showed that the ATP-restricted cell condition could promote cancer cell invasion (Fig. 1C).
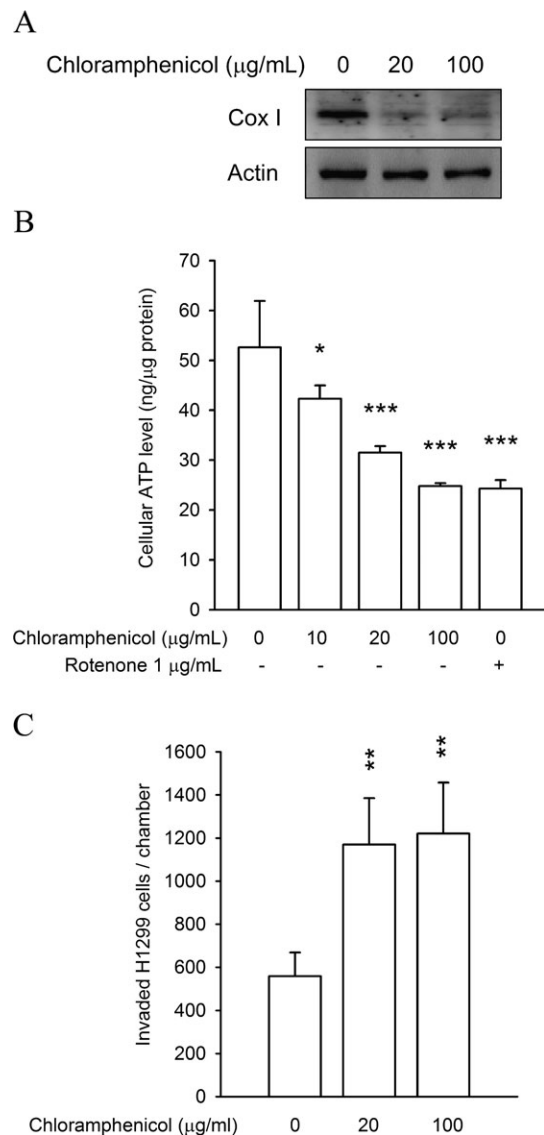
FIG. 1. Chloramphenicol treatment caused mitochondria stress, ATP restriction, and increased cancer cell invasion. (A) Chloramphenicol treatment suppressed mitochondria translation of mtDNA-encoded cytochrome c oxidase subunit I (Cox I) in H1299 cell. (B) Total ATP was determined by using ATP bioluminescent assay kit as described in the ''Materials and Methods'' section. Mitochondria inhibitor rotenone was used as positive control. Chloramphenicol treatment decreased ATP biosynthesis in H1299 cell. The ATP restriction condition resembled the hypoxia. (C) To evaluate the invasion capacity of chloramphenicol-treated H1299 cell, the Matrigel-coated 8-μm transwells were used as described in the ''Materials and Methods'' section. The data showed that chloramphenicol-induced ATP-restricted H1299 cell exhibited stronger invasion activity than control. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a statistical difference with the control.

Chloramphenicol Induced MMP-13 mRNA Expression in Cancer Cells and Facilitated Cancer Cell Invasion
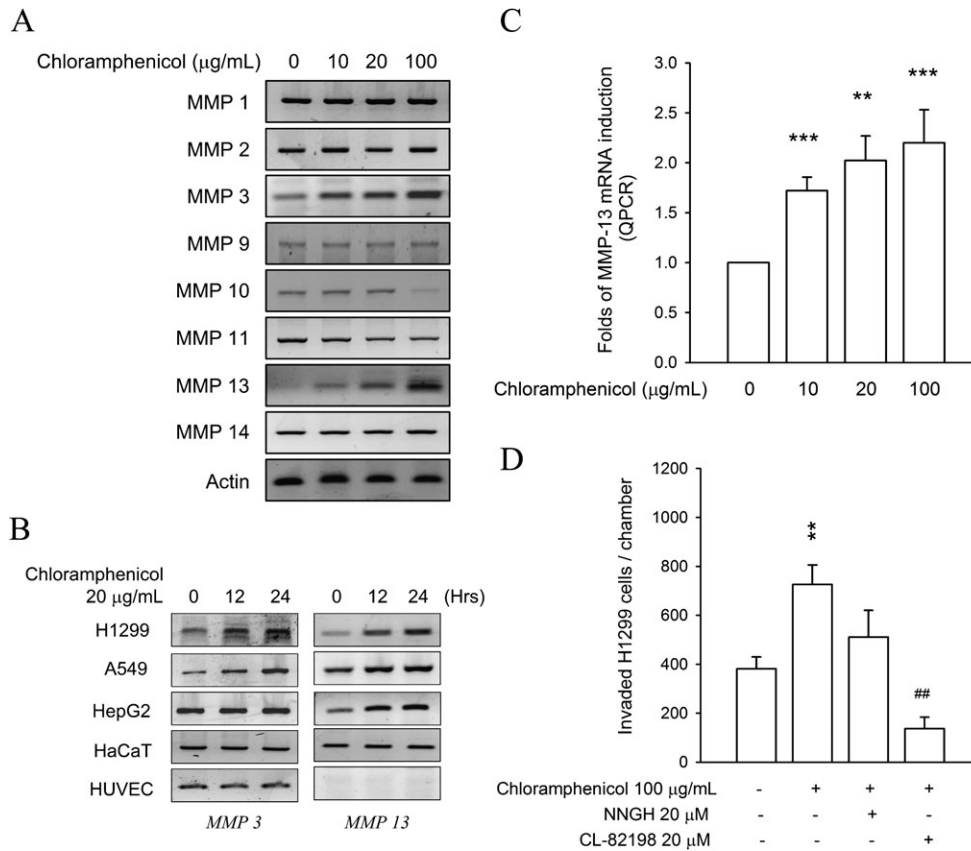
The mRNA levels of MMPs were analyzed by RT-PCR in chloramphenicol-treated H1299 cells. Cellular MMP-3 (stromelysin-1) and MMP-13 (collagenase-3) mRNA levels

were increased in both dose- and time-dependent manner (Figs. 2A and 2B). In a qPCR assay, the MMP-13 mRNA was also increased in chloramphenicol-treated H1299 cells (Fig. 2C). Under the testing conditions, the collagenases (MMP-1), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-10 and MMP-11), and MMP-14 remained either unaffected or down-regulated (Fig. 2A). Chloramphenicol-induced MMP-13 mRNA expression was observed in H1299, A549, and HepG2, whereas the MMP-3 mRNA expression was observed only in H1299 and A549. Chloramphenicol-induced MMP-13 expression was observed in nine solid-tumor cell lines (including H1299, HepG2, A549, CL-10, NCI-N87, Huh7, SAS, GBM8401, and Hep1) but not in leukemia suspensions (including THP-1, HL-60, and Jurkat) (Supplementary fig. 2). Leukemia suspensions usually have less mitochondria copy number than somatic cells (Mendoza et al., 2004; Shen et al., 2008; Shmookler Reis and Goldstein, 1983). The less mitochondria copy number in leukemia suspensions may diminish the mitochondria-to-

nucleus stress signaling, resulting in less response of cells to chloramphenicol.

Both MMP-3 and MMP-13 mRNA expression levels remained unaffected in immortalized normal cells (including HaCaT keratinocytes, CCD-966SK fibroblast, IMR-90, retinal pigmented epithelial cell [ARPE]) and primary cultures (human umbilical vein endothelial cell, human kidney epithelial cell, mouse lung cell, rat cardiocyte, and rat vascular smooth muscle cell) (Supplementary fig. 2). Our findings suggest that the chloramphenicol-induced MMP-13 expression only existed in solid-tumor cells but not in leukemia suspensions and normal cells.

Recently, both MMP-3 and MMP-13 have been demonstrated to enhance extracellular matrix degradation and cancer cell invasion. However, the chloramphenicol-induced invasive activities were inhibited by coincubation with the specific MMP-13 inhibitor CL-82198 but not the MMP-3 inhibitor NNGH (N-isobutyl-N-[4-methoxyphenylsulphonyl] glycyl hydroxamic acid) (Fig. 2D).

FIG. 2. The MMP-13 mRNA was induced in chloramphenicol-treated cancer cell and then facilitated cancer cell invasive activity. The mRNA levels of MMPs in chloramphenicol-treated cells were analyzed by RT-PCR as described in the ''Materials and Methods'' section. (A) Both MMP-3 and MMP-13 mRNA expression were induced in chloramphenicol (10–100 µg/ml)-treated H1299 cell in a dose-dependent manner. (B) Chloramphenicol (20 µg/ml)-induced MMP-13 mRNA expression was observed in cancer cell lines H1299, A549, and HepG2 in a time-dependent manner. However, chloramphenicol-induced MMP-3 mRNA expression was observed only in cancer cell lines H1299 and A549. Both MMP-3 and MMP-13 mRNA expression levels remained unaffected in immortalized HaCaT and primary human umbilical vein endothelial cell . (C) The qPCR data showed that chloramphenicol treatment increased MMP-13 mRNA expression. (D) Chloramphenicol-induced cancer cell invasion could be inhibited by MMP-13 inhibitor, CL-82198 (20µM), but not by MMP-3 inhibitor, NNGH (20µM). $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicate a statistical difference with the control. $^{#}p < 0.05$, $^{##}p < 0.01$, and $^{###} p < 0.001$ indicate a statistical difference with chloramphenicol-treated control.

A

B

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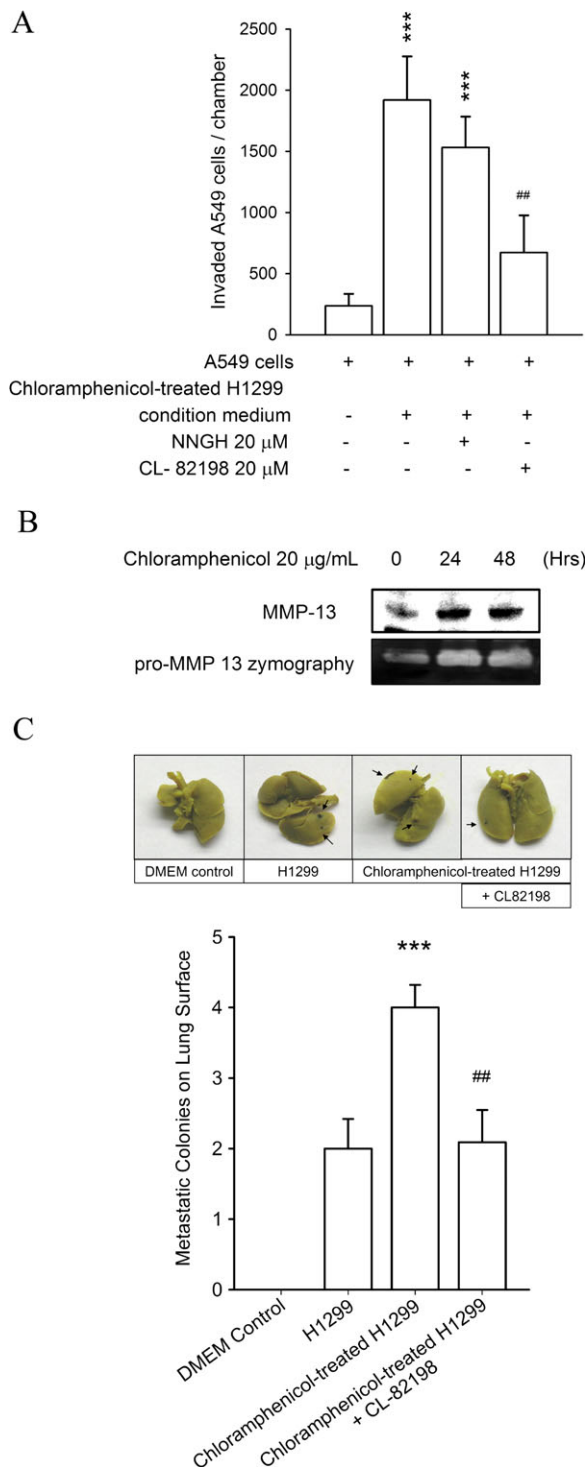
FIG. 3. The MMP-13 protein in chloramphenicol-treated H1299 condition medium facilitated A549 cell invasion. (A) About 5×10^4 A549 cells were seeded into transwells and incubated with condition medium of chloramphenicol-treated H1299 cell for 24 h. The data showed that secreted MMP-13 in condition medium could enhance A549 cells invasion. The increase in invasive A549 cell counts could be prevented by MMP-13 inhibitor, CL-82198 (20μM), but not by MMP-3 inhibitor, NNGH (20μM). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a statistical difference with the control. [#]$p < 0.05$, [##]$p < 0.01$, and

We also found that the conditioned medium of chloramphenicol-treated H1299 cells could enhance the invasive ability of A549 cells, which have previously been demonstrated to have weak invasive ability (Mooradian *et al.*, 1992). This increase could also be inhibited by CL-82198 (Fig. 3A). During the entire process, the Western blots and gelatin zymography also showed strong proteolytic activity of the MMP-13 protein in conditioned medium with chloramphenicol-treated H1299 cells (Fig. 3B). Next, we investigated the effect of chloramphenicol-induced MMP-13 expression on cancer cell metastasis *in vivo*. In comparison with H1299, treatment with 100 μg/ml chloramphenicol for 24 h led to a remarkable increase in metastatic colony formation on lung surface. The MMP-13 inhibitor, CL-82198, inhibited metastatic activity of chloramphenicol-treated cells (Fig. 3C). The *in vivo* data supported that chloramphenicol-induced MMP-13 expression is strongly associated with metastatic capacity of solid-tumor cells.

Chloramphenicol Activated Mitogen-Activated Protein Kinases and PI-3 Kinase/Akt Signaling

Mitogen-activated protein kinases (MAPKs), including p42/p44 ERK, JNK and p38-MAPK, and Akt, were activated in chloramphenicol-treated H1299 cells in both a dose-dependent (Fig. 4A) and a time-dependent manner (Supplementary fig. 3).

The Effects of MAPKs and PI-3 Kinase Inhibitors on Chloramphenicol-Induced MMP-13 Expression

To assess the involvement of MAPK and phosphatidylinositol 3-kinase (PI-3K)/Akt signaling in chloramphenicol-induced MMP-13 mRNA expression, cells pretreated with MAPKs and PI-3K inhibitors were cotreated with chloramphenicol and assayed by RT-PCR. Results showed that both SP 600125 and LY 294002 suppressed chloramphenicol-mediated MMP-13 mRNA expression. However, the induction of MMP-13 by chloramphenicol treatment remained unaffected by PD 98059 or SB 203580 (Fig. 4B). The SP 600125 and LY 294002

[###]$p < 0.001$ indicate a statistical difference with chloramphenicol-treated condition medium control. (B) Chloramphenicol-treated H1299 condition medium was collected, and the protein was precipitated by 10% trichloroacetic acid. MMP-13 protein in condition medium was detected by Western blotting. For gelatin zymography, the condition medium was concentrated. The gelatin enzymatic digestion was performed as described in the ''Materials and Methods'' section. Both MMP-13 protein level and activity were increased in chloramphenicol-treated H1299 condition medium in a time-dependent manner. (C) The *in vivo* metastatic activity was evaluated by lung colonization assay. The H1299 or chloramphenicol-treated H1299 cells were iv injected into living C57BL/6J mice. Twenty days later, the colonies formation on lung surface was counted. No colony was found in mice received DMEM injection. Treatment with 100 μg/ml chloramphenicol for 24 h led to a remarkable increase of metastatic colonies formation on lung surface compared to H1299 cell control. MMP-13 inhibitor, CL-82198, treatment inhibited metastatic activity to a resting state. ***$p < 0.001$ indicate a statistical difference with the H1299 control. [##]$p < 0.01$ indicate a statistical difference with chloramphenicol-treated H1299 group.
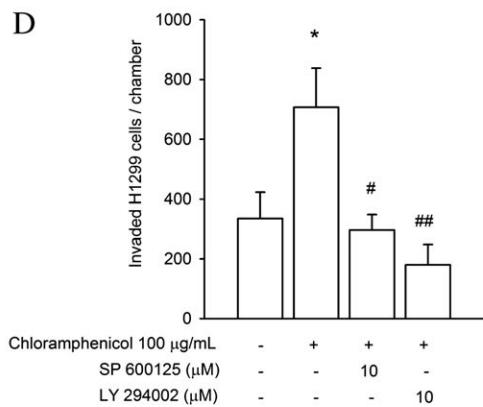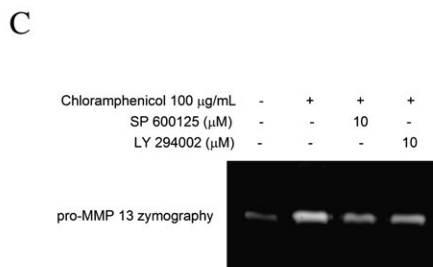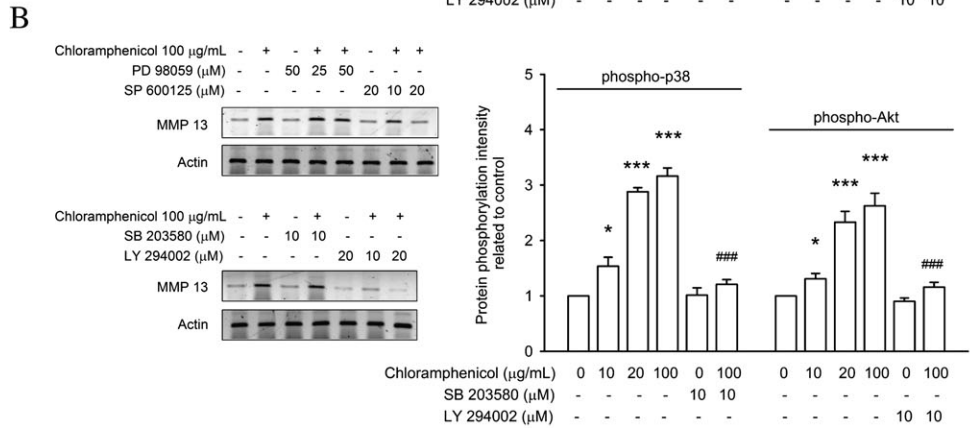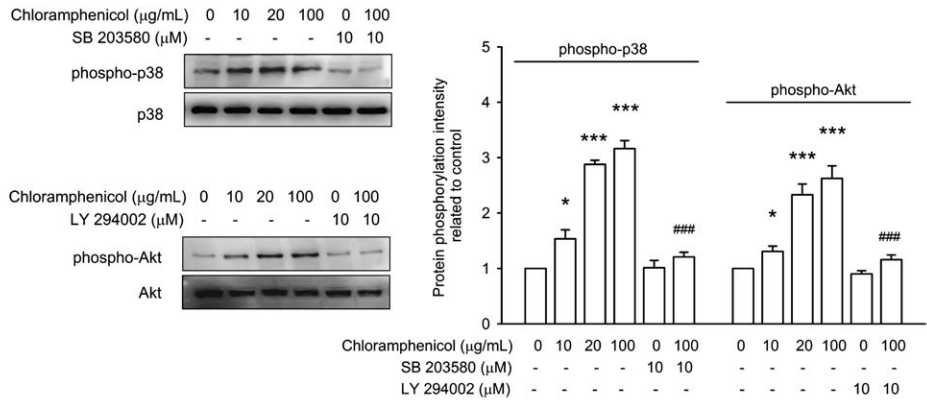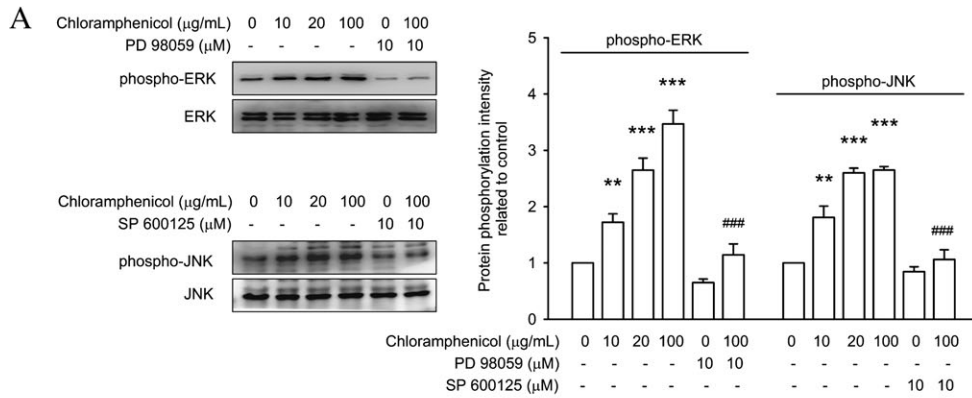
also inhibited MMP-13 activity by gelatin zymography (Fig. 4C) and chloramphenicol-induced cell invasion (Fig. 4D). Chloramphenicol-induced JNK phosphorylation could also be inhibited by LY 294002, whereas SP 600125 did not abolish Akt activation (Fig. 5A). The data suggest that PI-3K/Akt/JNK signaling is essential for chloramphenicol-associated MMP-13 induction and cancer cell invasion.

PI-3 Kinase/Akt/JNK Signaling Required for Chloramphenicol-Induced c-Jun Phosphorylation and MMP-13 Expression

Coincubation with SP 600125 or LY 294002 inhibited chloramphenicol-induced c-Jun phosphorylation in the nucleus. The proliferating cell nuclear antigen (PCNA) protein was used as an internal control in the nuclear extracts (Fig. 5A).

Plasmids encoding DN JNK or the p85 subunit of PI-3K (DN p85) were transfected into H1299 cells. The HA-tag protein confirmed the successful expression of the DN JNK and p85 proteins. In these successfully transfected cells, chloramphenicol-induced JNK activation and c-Jun phosphorylation were reduced, and there was no MMP-13 activity in gelatin digestion (Fig. 5B).

Chloramphenicol Increased Activator Protein 1 DNA–Binding Activity and Induced MMP-13 Expression

The transcription factor activator protein 1 (AP-1) component protein, phospho-cJun, increased in nuclear extracts from chloramphenicol-induced H1299 cells in both a dose-dependent (Fig. 6A) a and time-dependent manner (Supplementary fig. 6). MMP-13 expression could be repressed by the AP-1 inhibitor curcumin (Fig. 6B), but not by the nuclear factor-κB inhibitor pyrrolidine dithiocarbamate or Bay–17–7085 (Supplementary fig. 4). The direct DNA binding between phospho-cJun protein and AP-1 consensus sequence (TGACTCA) were demonstrated by EMSA studies in vitro (data not shown).

ChIP was used for assaying the phospho-cJun protein–binding activity to the MMP-13 promoter. Validation of the method is shown in Supplementary figure 5. The phospho-cJun protein was coimmunoprecipitated together with the MMP-13 promoter in both a dose-dependent (Fig. 6C) and a time-dependent manner (Supplementary fig. 6). Coincubation with SP 600125 or LY 294002 inhibited the interaction between phospho-cJun and MMP-13 promoter (Fig. 6D). This finding suggests that the transcription factor AP-1 is required for
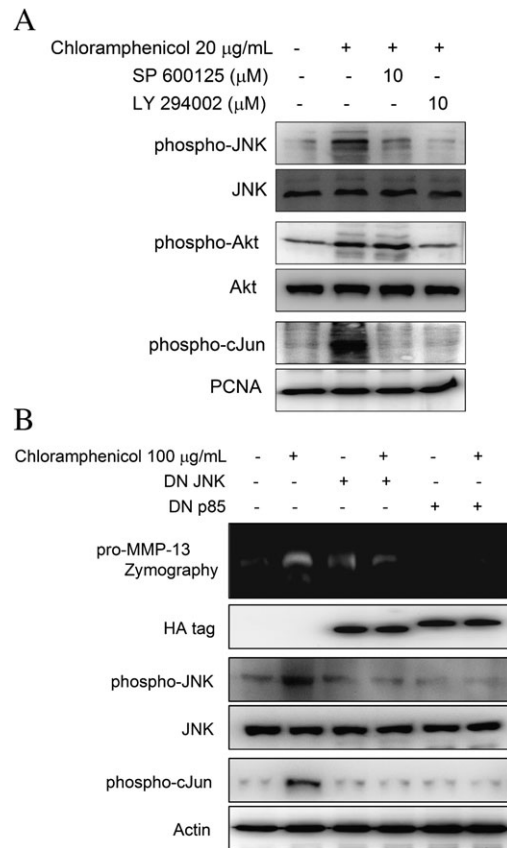

FIG. 5. JNK and PI-3 kinase/Akt signaling were required for chloramphenicol-induced c-Jun phosphorylation and MMP-13 expression. (A) The blot showed that LY 294002 inhibited both JNK and Akt phosphorylation. SP 600125 inhibited JNK phosphorylation, whereas the Akt phosphorylation was unaffected. The data suggested that the involvement of PI-3K/Akt signaling in chloramphenicol-induced JNK activation. (B) Plasmids encoded DN JNK and p85 subunit of PI-3K were transfected into H1299 cell as described in the ''Materials and Methods'' section. The HA-tag indicated the successful transfection of DN protein. The condition medium was collected and analyzed by gelatin zymography. The DN JNK- or DN p85-overexpressing H1299 cell were reduced in pro-MMP-13 activity in relation to chloramphenicol-treated control.

chloramphenicol-induced MMP-13 expression and further MMP-13–mediated cell invasion.

Antibiotics Causing Mitochondrial Stress and ATP Restriction Induced MMP-13 Expression

In addition to chloramphenicol, other antibiotics, including minocycline, doxycycline, and clindamycin, may also inhibit

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FIG. 4. Effects of MAPKs and PI-3 kinase inhibitors on chloramphenicol-induced MMP-13 mRNA expression. (A) H1299 cells were treated with 0–100 μg/ml chloramphenicol for 24 h, and the phosphorylation of MAPKs and Akt were detected by Western blotting. The blots showed that ERK, JNK, p38-MAPK, and Akt were activated in respond to chloramphenicol treatments. Chloramphenicol-induced activation of MAPKs and Akt can be inhibited by specific inhibitors, PD 98059 (ERK inhibitor), SP 600125 (JNK inhibitor), SB 203580 (p38-MAPK inhibitor), and LY 294002 (PI-3K inhibitor). The fold changes of protein phosphorylation were analyzed by densitometry. (B) Chloramphenicol-mediated MMP-13 expression was inhibited by SP 600125 and LY 294002 but not by PD 98059 and SB 203580. The fold changes of MMP-13 mRNA were analyzed by densitometry. (C) The gelatin zymography showed SP 600125 and LY 294002 could inhibit chloramphenicol-mediated increase in pro-MMP-13 activities. (D) Chloramphenicol-induced increase in invasion activity was inhibited by SP 600125 and LY 294002. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a statistical difference with the control. #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ indicate a statistical difference with chloramphenicol-treated control.
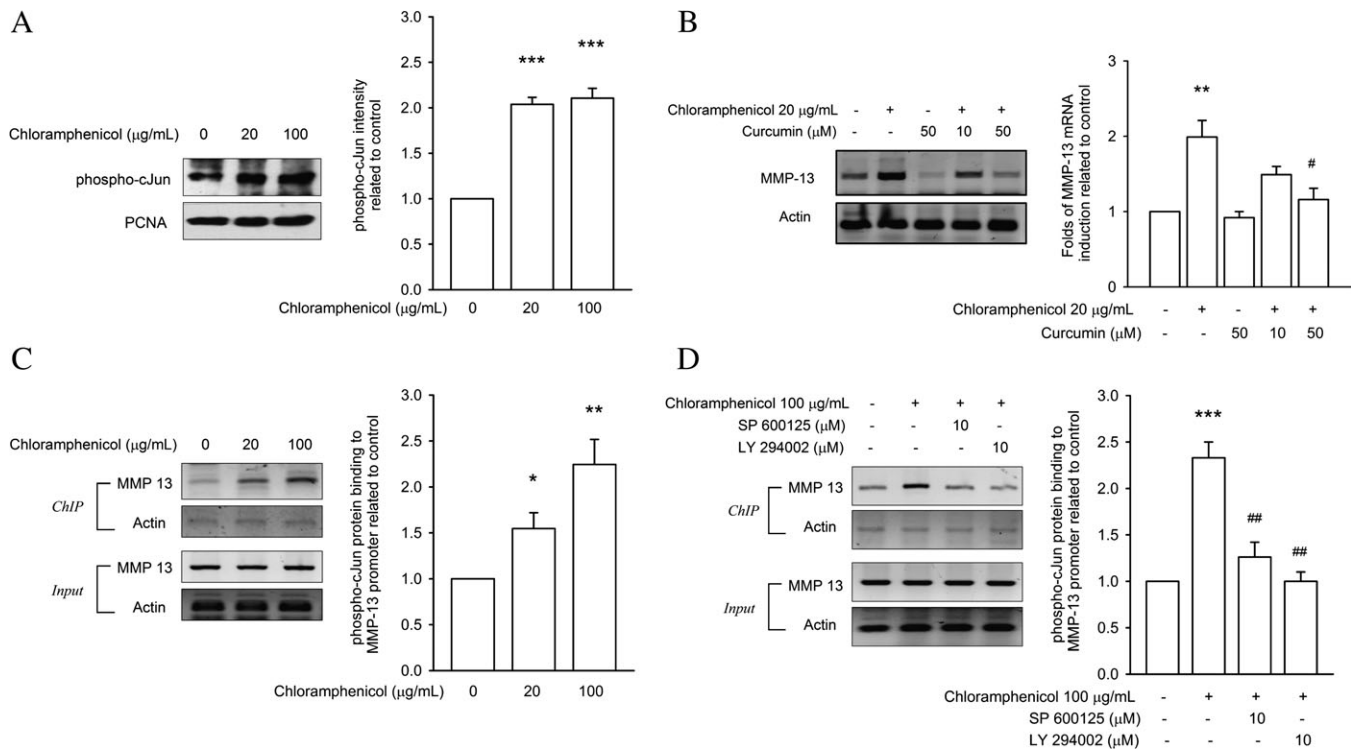
FIG. 6. Chloramphenicol treatment increased AP-1 DNA–binding activity to MMP-13 promoter. (A) The blot showed that the phospho-cJun was accumulated in nuclear extract of chloramphenicol-treated H1299 cell. The PCNA was used as internal control of nuclear extract. (B) Chloramphenicol-induced MMP-13 expression was inhibited by the transcription factor AP-1 inhibitor, curcumin (10–50μM). The fold changes of MMP-13 mRNA were analyzed by densitometry. (C) The ChIP was used for assay the interaction between phospho-cJun and MMP-13 promoter. In chloramphenicol-treated cell, the ChIP-captured MMP-13 promoter was increased in dose-dependent manner. (D) Coincubation with SP 600125 and LY 294002 decreased the DNA-binding activity of phospho-cJun protein to MMP-13 promoter. These data suggested that JNK, PI-3K/Akt, and AP-1 activities were required for chloramphenicol-induced MMP-13 mRNA induction. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicate a statistical difference with the control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ indicate a statistical difference with chloramphenicol-treated control.
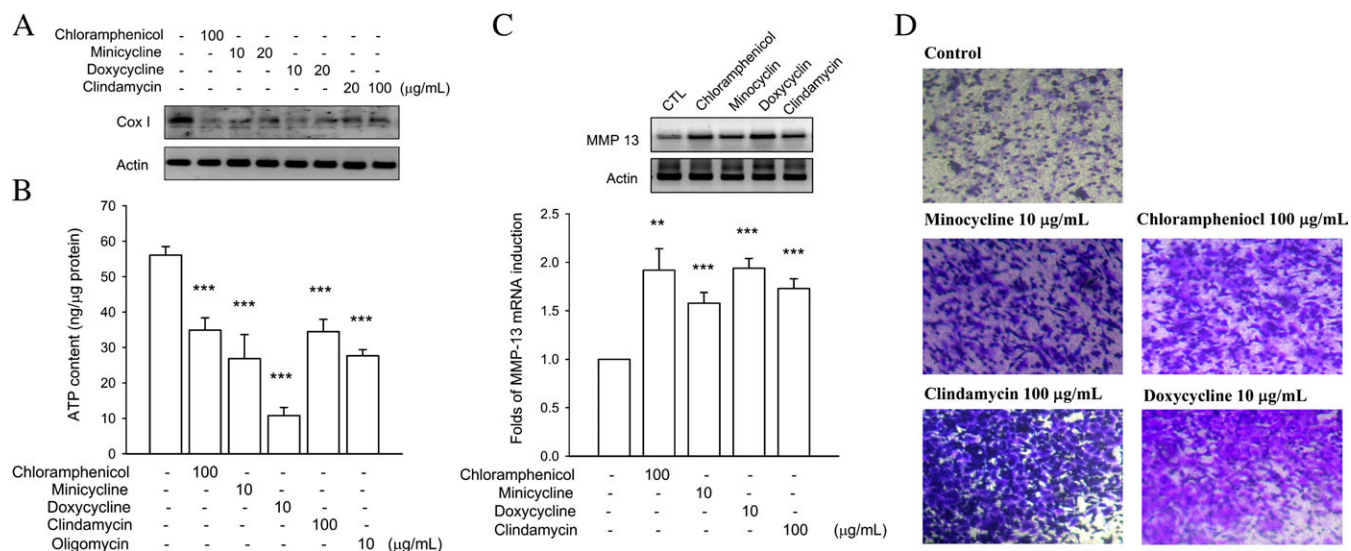
mitochondrial translation (Fig. 7A), cause ATP restriction (Fig. 7B), induce MMP-13 mRNA expression (Fig. 7C), and facilitate cell invasion (Fig. 7D). It has been reported previously that doxycycline inhibits MMPs. For MMP-13, doxycycline did not function as an inhibitor below 200μM (48 μg/ml). The inhibition concentration may be difficult to achieve *in vivo* (Smith *et al.*, 1998). However, the 10 μg/ml doxycycline induced MMP-13 expression in H1299 cell.

DISCUSSION

Tumor cells usually change their mitochondrial function, triggers a mitochondria-to-nucleus signaling, and changes gene expression that make the tumor cells more likely to invade (Fosslien, 2008; Uchiyama *et al.*, 2004). The MMPs are well-known metastatic factors (Kuzuya and Iguchi, 2003). Tumor metastatic potential correlated with enzymatic degradation of type IV collagen. MMP-13 shows the highest proteolytic activity among the collagenases and could degrade type IV collagen at both α2(IV) and α1(IV) chains. Thus, MMP-13–expressing cells often have strong invasive capacities (Knäuper

et al., 1997a). Clinical evidence support the fact that MMP-13 appearances is highly relevant to the invasive of squamous cell carcinomas, non–small cell lung cancer, malignant peripheral nerve sheath tumors, and breast tumors (Holtkamp *et al.*, 2007; Hsu *et al.*, 2006; Nil *et al.*, 2004; Zhang *et al.*, 2008). Our previous studies showed that chloramphenicol treatment caused mitochondrial stress and senescence-associated apoptosis resistance in cancer cells (Li *et al.*, 2005). In this study, we further proved that chloramphenicol treatment caused ATP restriction and enhanced cancer cell invasion both *in vitro* and *in vivo*. The involvement of MMP-13 was supported by the fact that both MMP-13 protein and mRNA were induced by chloramphenicol treatment. This enhanced invasion was blocked by MMP-13 inhibitors. The activation of proMMP-13 can be regulated by membrane-type MMPs (MMP-14) and stromelysin (MMP-10) (Itoh and Seiki, 2006; Nakamura *et al.*, 1998). In this study, the expression of MMP-14 did not change significantly. However, MMP-10 and MMP-11 were down-regulated, suggesting that the invasive effects mentioned above resulted directly from MMP-13 induction. Furthermore, the activated MMP-13 can activate gelatinases and enhance invasion indirectly (Knäuper *et al.*, 1997b).

FIG. 7. Antibiotics causing mitochondria stress and ATP restriction also induced MMP-13 expression. (A) In addition to chloramphenicol, antibiotics including minocycline, doxycycline, and clindamycin, also inhibited mitochondrial translation. (B) Minocycline, doxycycline, and clindamycin caused ATP restriction in H1299 cell. The oilgomycin was used as positive control. (C) Chloramphenicol, as well as minocycline, doxycycline, and clindamycin, induced MMP-13 mRNA expression. The induction was evaluated by densitometer. (D) Antibiotics treatment facilitated H1299 cancer cell invasion in transwell assay. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ indicate a statistical difference with the control.

MMP-13 can be induced by parathyroid hormone, platelet-derived growth factor, basic fibroblast growth factor, TGF-β, and interleukins (Rydziel et al., 2000; Selvamurugan et al., 1998; Solís-Herruzo et al., 1999; Varghese et al., 2000). The JNK/AP-1, p38-MAPK/NF-κB, p38-MAPK/Smad, PI-3K/Akt, and NO/cGMP/ERK have been associated with MMP-13 expression as previously reported (Ahmed et al., 2003; Barchowsky et al., 2000; Lechuga et al., 2004; Leivonen et al., 2002; Zaragoza et al., 2002). Our data showed that PI-3K/Akt/JNK was activated in chloramphenicol-treated cells. Inhibition of JNK and PI-3K/Akt (by chemical inhibitor or by DN kinases) could negate chloramphenicol-induced c-Jun phosphorylation, MMP-13 expression, and cell invasion. The c-Jun phosphorylation proved that transcription factor AP-1 is essential. The AP-1–responsive genes, including proteases (MMPs and cathepsins) and actin cytoskeleton regulators, are strongly associated with cell invasion (Ozanne et al., 2000). An AP-1 consensus sequence was found in the proximal region of MMP-13 promoter (Tardif et al., 1997), and the binding of phospho-cJun protein with MMP-13 promoter was demonstrated by ChIP assay. These findings suggest that chloramphenicol-induced MMP-13 upregulation is dependent on PI-3K/JNK/AP-1 activity (Supplementary fig. 7).

Mitochondrial stress, by mitochondrial DNA depletion, hypoxia, or aging, resulted in altered energy metabolism and intracellular calcium homeostasis. Mitochondrial stress also induced mitochondria-to-nucleus stress signaling, such as MAPKs and PI-3 kinase/Akt signaling (Uchiyama et al., 2004). The specific stress signaling confirmed apoptosis resistance and enhanced cancer cell invasion (Fosslien,

2008). Recently, 2,3,7,8-tetrachlorodibenzo-p-dioxin has been demonstrated to interfere with mitochondrial transcription, inhibit apoptosis, and lead to invasive phenotype formation (Biswas et al., 2008). In mitochondria-damaged cells, with the increase of cytosolic calcium, calcineurin-sensitive NF-κB/Rel factor is activated. This signaling strengthens the expression of cathepsin L and cathepsin B and leads to an increase in invasive ability (Amuthan et al., 2001; Biswas et al., 2008; Hamer et al., 2009).

In our study, we found that chloramphenicol-induced PI-3K/JNK/AP-1 activation might function as a novel mitochondria-to-nucleus stress signal. The signal increased MMP-13 expression and MMP-13–associated cancer cell invasion. Moreover, antibiotics with the ability to inhibit mitochondrial translation would also cause senescence-associated apoptosis resistance (Li et al., 2005) and MMP-13 expression. These antibiotics have been used for many years in the treatment of skin and other infections. Hence, the clinical significance of our study is that chloramphenicol application, as well as other 70S ribosomal inhibitors, should be used with caution, especially during cancer therapy. As a result, the chloramphenicol-treated solid-tumor cell also provides a model for further investigation of mitochondrial damage and cancer progression.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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