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Curcumin reduces the antimicrobial activity of ciprofloxacin against *Salmonella* Typhimurium and *Salmonella* Typhi

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Objectives: Typhoidal and non-typhoidal infection by *Salmonella* is a serious threat to human health. Ciprofloxacin is the last drug of choice to clear the infection. Ciprofloxacin, a gyrase inhibitor, kills bacteria by inducing chromosome fragmentation, SOS response and reactive oxygen species (ROS) in the bacterial cell. Curcumin, an active ingredient from turmeric, is a major dietary molecule among Asians and possesses medicinal properties. Our research aimed at investigating whether curcumin modulates the action of ciprofloxacin.

Method: We investigated the role of curcumin in interfering with the antibacterial action of ciprofloxacin *in vitro* and *in vivo*. RT–PCR, DNA fragmentation and confocal microscopy were used to investigate the modulation of ciprofloxacin-induced SOS response, DNA damage and subsequent filamentation by curcumin. Chemiluminescence and nitroblue tetrazolium reduction assays were performed to assess the interference of curcumin with ciprofloxacin-induced ROS. DNA binding and cleavage assays were done to understand the rescue of ciprofloxacin-mediated gyrase inhibition by curcumin.

Results: Curcumin interferes with the action of ciprofloxacin thereby increasing the proliferation of *Salmonella* Typhi and *Salmonella* Typhimurium in macrophages. In a murine model of typhoid fever, mice fed with curcumin had an increased bacterial burden in the reticuloendothelial system and succumbed to death faster. This was brought about by the inhibition of ciprofloxacin-mediated downstream signalling by curcumin.

Conclusions: The antioxidant property of curcumin is crucial in protecting *Salmonella* against the oxidative burst induced by ciprofloxacin or interferon γ (IFNγ), a pro-inflammatory cytokine. However, curcumin is unable to rescue ciprofloxacin-induced gyrase inhibition. Curcumin's ability to hinder the bactericidal action of ciprofloxacin and IFNγ might significantly augment *Salmonella* pathogenesis.

Keywords: antioxidant, gyrase, reactive oxygen species, interferon γ

Introduction

Salmonella is a causative agent of salmonellosis, one of the major reasons for morbidity and mortality throughout the world. The successful treatment of typhoid patients is a challenge, especially in immunocompromised individuals. The emergence of multidrug-resistant strains further calls for alternative drugs against typhoid.[1] Ciprofloxacin is a promising alternative antibiotic because of its ability to penetrate into the macrophages[2–4] and its ability to kill multidrug-resistant strains.[4–6] However, in the past few years the emergence of ciprofloxacin-resistant *Salmonella* strains has been reported.[7–9] The efficacy of ciprofloxacin is attributed to its ability to affect various activities of bacterial DNA gyrase[10,11] without affecting mammalian topoisomerases at a concentration <150 mg/L.[12] In Gram-negative bacteria ciprofloxacin and other fluoroquinolones mainly target gyrase. They bind to the gyrase–DNA complex trapping the gyrase on the DNA. This induces formation of a cleaved complex.[13–15] The gyrase–drug–DNA complex impedes the function of DNA and RNA polymerases, thus blocking replication and transcription.[16,17] The formation of cleaved DNA complexes leads to the induction of the SOS response[18–20] via the RecBC-mediated system. Induction of the SOS response blocks cell division,[21] making the bacteria filamentous.

Ciprofloxacin and other quinolones, at lethal concentration, induce the formation of reactive oxygen species (ROS) that contributes to bacterial cell death.[22–25] Bactericidal drugs acting via other mechanisms also induce the generation of ROS in both Gram-positive and Gram-negative bacteria.[25,26] These antibiotics disturb the bacterial metabolism and respiration, inducing ROS in

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the bacterial cells.[26] The superoxide thus produced subsequently damages iron–sulphur clusters, leading to the formation of cytotoxic hydroxyl radicals via the Fenton reaction. ROS, produced by the host cells in response to microbial infection, may also contribute to bacterial death. Cell-penetrating iron chelators and hydroxyl radical quencher have been found to reduce the damage caused by bactericidal antibiotics.[25] It has also been demonstrated that antioxidants, like glutathione and ascorbic acid, protected the bacteria against ciprofloxacin action.[23] This argues against the consumption of antioxidants during antibiotic treatment.

In our study, we assessed the effect of curcumin, a dietary antioxidant, on *Salmonella* pathogenesis and its interference with the antimicrobial activity of ciprofloxacin in the murine model of typhoid fever. Curcumin possesses antioxidant[27] and anti-inflammatory properties.[28] The UV-induced SOS response was shown to be inhibited by curcumin.[29] The role of all these properties in rescuing *Salmonella* from the detrimental effects of ciprofloxacin was tested. We found that curcumin abrogated various cytotoxic effects of ciprofloxacin against *Salmonella* Typhi and *Salmonella* Typhimurium, giving them a survival advantage. Our study highlights the effect of diet on the outcome of typhoid disease and its treatment, especially considering the prevalence of typhoid in Asia, where consumption of curcumin is 0.03–0.12 g/person/day.[30]

Materials and methods

Bacterial strains, medium and growth conditions

Salmonella Typhimurium strain 12023/14028 and *Salmonella* Typhi Ty2 were used as wild-type (WT) strains. All the strains were gifts from Professor M. Hensel (Institut für Klinische Mikrobiologie, Erlangen, Germany). The strains were grown at 37°C in Luria broth (LB), with respective antibiotics. Curcumin (Sigma) and ciprofloxacin (Sigma) were added to the medium wherever required.

Eukaryotic cell lines and growth conditions

RAW 264.7 and U937 cells, gifts from Professor Anjali Karande (Department of Biochemistry, IISc, Bangalore, India), were grown in Dulbecco's modified minimum essential medium or RPMI 1640 supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Intracellular survival assay

A total of 1.5×10^5 RAW 264.7 or U937 cells per well were seeded in 24-well plates. The U937 cells were stimulated with 10 ng/mL phorbol 12-myristate 13-acetate 2 days prior to infection. These cells were infected with *Salmonella* Typhimurium (RAW 264.7 cells) or *Salmonella* Typhi (U937 cells), in stationary phase, at a multiplicity of infection of 10 and the intracellular survival was assayed.[31] For the ciprofloxacin susceptibility assay, 0.5 mg/L ciprofloxacin was added along with 25 mg/L gentamicin. For the interferon γ (IFNγ)-related study, RAW 264.7 cells were pre-treated with IFNγ for 22–24 h. The cells were lysed with Triton X-100 at different timepoints. The lysates were plated to determine the cfus.

Antibiotic susceptibility assay

Salmonella Typhimurium was grown at 37°C for 12–16 h in LB. Overnight cultures were subcultured into fresh medium (1:50). After 2 h the cultures (optical density at 600 nm of 0.25–0.4) were used for the determination of antibiotic susceptibility. Ciprofloxacin was added to a final concentration of 0.5 and 2 mg/L. The cultures were then incubated at 37°C at 180 rpm. After 3 h the cultures were diluted and plated to estimate the percentage of survivors with respect to the initial pre-inocula. The bacteria were also observed with a confocal microscope to determine the change in the shape, if any. The bacteria with a length >5 μm were considered filamentous. The percentage of such bacteria in the population of bacteria was calculated for each experiment.

For the membrane potentiation assay the bacteria were treated with 0.5 and 2 mg/L ciprofloxacin for 2 h and were then incubated with 1 mg/L of bis-(1,3-dibutylbarbituric acid)-trimethine oxonol [DiBAC$_4$(3); Invitrogen] for 10 min and washed twice with PBS. The samples were further analysed in an FACS scanner (BD Biosciences) to test for metabolically active bacteria.

Animal experiments

Animal experiments were carried out with prior approval from the Institutional Animal Ethics Committee (approval numberCAF/Ethics/189/2010), Indian Institute of Science, Bangalore. The guidelines stipulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India, New Delhi (registration number 48/1999/CPCSEA) were followed while carrying out all animal experiments. BALB/c mice, 6–7 weeks old (obtained from the Central Animal Facility, Indian Institute of Science, Bangalore), were maintained under specific pathogen-free conditions. All the procedures with mice were carried out according to institutionally approved protocols. Mice were infected intragastrically with 10^7 cfu of *Salmonella* Typhimurium for the organ infiltration study. Mice were either fed with 80 mg/kg body weight/day of curcumin for 4 days before or after infection. One set of mice were fed with curcumin 5 days pre- and post-infection. Ciprofloxacin treatment (50 mg/kg body weight every 12 h) was started 3 days post-infection. On the fifth day post-infection, liver, spleen, mesenteric lymph nodes and Peyer's patches were isolated under aseptic conditions, weighed and homogenized in a tissue homogenizer with 1 mL of PBS. The homogenate was plated at different dilutions. The number of cfu per gram weight of the organ was calculated.

Confocal microscopy

RAW 264.7 and U937 cells, grown on coverslips, were infected with WT-green fluorescent protein (GFP) (*Salmonella* Typhimurium and *Salmonella* Typhi, respectively) as described in the intracellular survival assay protocol. The cells were fixed with 3.5% paraformaldehyde at different timepoints (24 h, IFNγ; 8 h, ciprofloxacin). Extracellular bacteria were labelled with primary antibody against *Salmonella* (*Salmonella* polyflagellar antibody, Difco) followed by cy3 conjugated secondary antibody. Samples were observed under a confocal microscope (Carl Zeiss) and analysed for filamentous *Salmonella* as described previously.[32] The bacteria that had a length >5 μm were considered as filamentous and the percentage of filamentous bacteria per experiment was calculated.

Thymidine release assay

The assay was done as described previously.[33] Briefly, bacteria from an overnight culture were subcultured at a 1:50 ratio in LB containing 4 μCi/mL [methyl-^{3}H]thymidine. The cultures were incubated at 37°C at 180 rpm for 3 h. The cells were washed three times with PBS (centrifugation at 7000 rpm). The washed cultures were resuspended in LB without [methyl-^{3}H]thymidine. The cultures were either treated or untreated with ciprofloxacin (2 mg/L) for 4 h. The samples were processed to calculate the thymidine release. The fold increase in the percentage was calculated as follows: % thymidine released (treated)/% thymidine release (untreated).

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TUNEL assay

The cells were treated as described for the thymidine release assay. The TUNEL assay was done as described previously[34] and as per the manufacturer's protocol (DeadEnd Fluorometric TUNEL System, Promega).

Bacterial RNA extraction and RT–PCR analysis

RNA was isolated from the log-phase bacteria grown in LB in the presence and absence of curcumin and exposed or un-exposed to ciprofloxacin. cDNA was prepared from bacterial RNA using reverse primers of the *ftsZ, umuC* and *16s rRNA* reverse transcription system in accordance with the manufacturer's protocol (Thermo Fischer). The cDNA thus obtained was stored at −20°C. cDNA was amplified (35 cycles or 20 cycles for *16S rRNA*) using primers (*ftsZ* forward: tgaagaccgtgcggaagaag, *ftsZ* reverse: gatgcaaaggcacggatggt; *umuC* forward: cagggcaaagggtga tgt, *umuC* reverse: cgtaccgtgagagagcttcg; *16S rRNA* forward: gatcatggctcagattgaacgctggcgg, *16S rRNA* reverse: caccgctacacctggaat tatacccccctc) that amplify the intergenic region of the specific genes.

Immunoblot

The ciprofloxacin treatment of *Salmonella* Typhimurium was done as described for the antibiotic susceptibility assay. After 3 h of ciprofloxacin treatment the cells were lysed and processed for immunoblotting with affinity-purified anti-*Escherichia coli* FtsZ antibody[35] at 1:5000 dilution and anti-sera against *E. coli* Rrf (a kind gift from Professor Umesh Varshney, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India).

RAW 264.7 cells treated with IFNγ (20 U/mL, 12 h) and curcumin (10 μM), were infected with *Salmonella* Typhimurium. The cells were lysed and processed for immunoblotting with anti-ERK and p-ERK antibodies 2 h post-infection.

Determination of ROS

Measurement of extracellular H_2O_2 by a phenol red assay

A method devised by Wautier *et al*.[36] was used with some modification. Infected RAW 264.7 cells were incubated in solution containing NaCl (0.14 M), potassium phosphate (0.01 M; pH 7.0), glucose (0.0055 M), phenol red (200 mg/L) and horseradish peroxidase (19 U/mL; Genei). After 8 h the medium obtained, after pelleting down the cells, was assayed in a spectrophotometer at 610 nm. Total protein from each well was estimated using the Bradford method and the data recorded as the amount of H_2O_2 produced per milligram of total protein. A standard curve with H_2O_2 concentrations ranging from 0 to 10 mg/L in phenol red solution was generated.

Nitroblue tetrazolium (NBT) reduction

Bacteria were treated as mentioned for the antibiotic susceptibility assay. The assay was done as described previously.[37] Briefly, 375 μL of NBT (1 mg/mL) was added to 75 μL of treated or untreated culture. This mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 75 μL of 0.1 N HCl. The tubes were centrifuged at 5000 rpm for 10 min. The blue colour of the aqueous phase (culture supernatant) was measured at 575 nm (ROS extracellular). The blue crystals in bacterial pellets were dissolved with 300 μL of DMSO followed by the addition of 300 μL of PBS and the optical density was determined at 575 nm (ROS intracellular).

ROS detection using luminol

Overnight cultures were inoculated into fresh LB (1:50). After 2 h the cultures (optical density at 600 nm of 0.25–0.4) were used for the determination of ROS. Ciprofloxacin was added at a concentration of 0.5 mg/L or 2 mg/L. The assay was done as described previously with some modifications.[37] Immediately, 0.1 mL of culture was incubated with 0.1 mL of luminol (500 μM) and 19 U/mL horseradish peroxidase. The light emission was measured by chemiluminescence using a luminometer (Turner Designs, Sunnyvale, CA, USA). ROS produced by infected RAW 264.7 cells treated with IFNγ (20 IU/mL, 12 h pre-treatment) and ciprofloxacin (0.5 mg/L, 1 h post-treatment) were measured in a similar manner 1 h post-infection. The cells were infected as mentioned for the intracellular survival assay.

DNA cleavage assay

Individual subunits of *E. coli* DNA gyrase were purified and the holoenzyme reconstituted as described earlier.[38] *E. coli* DNA gyrase (100 nM) was incubated with 300 ng of supercoiled pUC18 plasmid and curcumin (1–5 μM) in a supercoiling reaction buffer (35 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$, 25 mM potassium glutamate, 2 mM spermidine, 2 mM ATP, 50 μg/mL BSA, 90 μg of yeast tRNA and 5% glycerol) for 10 min at 4°C followed by the addition of 2.5 mg/L of ciprofloxacin to each reaction. The reactions were shifted to 37°C for 30 min followed by the addition of 0.1% SDS to terminate the reaction and the addition of 90 μg/mL proteinase K to digest the covalently bound protein. The incubation was continued for another 1 h followed by heat inactivation at 75°C for 10 min. The reaction products were resolved by agarose (1.2%) gel electrophoresis and visualized on an UV trans-illuminator.

DNA binding assay

The mobility shift assay was done as described previously.[39–41] A 240 bp DNA from pBR322[42] was PCR amplified using ^{32}P-labelled primer. *E. coli* DNA gyrase (100 nM) was incubated with 240 bp of DNA in the presence of increasing concentrations of curcumin for 15 min. The binding reactions were carried out in supercoiling reaction buffer. The DNA-bound complexes were resolved on 5% native PAGE and visualized using a PhosphorImager.

Statistical analysis

Each experiment was performed in triplicate and repeated a minimum of three times. Means ± SEM were plotted. Data were analysed using Student's *t*-test or the Mann–Whitney *U*-test in GraphPad Prism software (version 5; GraphPad Software, La Jolla, CA, USA). The data were considered statistically significant for P values ≤0.05.

Results

Curcumin improved the survival of Salmonella Typhi and Salmonella Typhimurium treated with ciprofloxacin

Bactericidal antibiotics act by inducing the generation of ROS in bacterial cells.[25,26] The oxidative radicals thus formed damage proteins, lipids and nucleic acids, poisoning the cells.[43] Iron chelators and antioxidants are known to attenuate the bactericidal effects of antibiotics.[25] It has been demonstrated that antioxidants protects *E. coli* from the lethal effects of norfloxacin,[24,25] and probably ciprofloxacin.[23] In our study, we have tested the ability of curcumin, a commonly used antioxidant, to rescue *Salmonella* Typhi/Typhimurium from the bactericidal activity of

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ciprofloxacin. Intracellular proliferation and *in vitro* bacterial susceptibility assays were performed in the presence and absence of ciprofloxacin and curcumin. We found that curcumin increased the fold proliferation of both *Salmonella* Typhi and *Salmonella* Typhimurium by 2- to 4-fold in RAW 264.7 (murine macrophage like cell line) and U937 (human macrophage cell line) cell lines, respectively (Figure 1a), and protected them from the detrimental effects of ciprofloxacin (0.5 mg/L; Figure 1a). Hereafter, all the *in vitro* experiments were carried out with *Salmonella* Typhimurium.

An *in vitro* bacterial survival assay (based on the ability to form colonies on a drug-free agar plate following drug treatment) was performed to assess the ability of curcumin to protect *Salmonella* against the lethal action of ciprofloxacin (0.5 and 2 mg/L, the concentration of ciprofloxacin reached in the serum of humans/mice fed with recommended dose ciprofloxacin).[44] Curcumin prevents ciprofloxacin-mediated killing of bacteria as demonstrated by a cfu-based assay (Figure 1b).

The quinolone-treated bacteria, though unable to form colonies, are metabolically active.[45,46] To assess for metabolically active bacteria, a membrane potentiation assay using $DiBAC_4(3)$ was performed.[47] The metabolically active bacteria are assumed to maintain an intact membrane potential through energy-dependent mechanisms and exclude dye, whereas the depolarized cells will take up the dye and fluoresce green.[47,48] We found that the percentage of *Salmonella* Typhimurium positive for fluorescence greater than that of cells not exposed to ciprofloxacin was 2- to 4-fold less in curcumin-treated cells compared with curcumin-untreated cells (Figure 1c). Thus, these results demonstrate that curcumin protects *Salmonella* against the bactericidal activity of ciprofloxacin.

The bactericidal action of ciprofloxacin was abrogated by curcumin in a mouse model of typhoid

The protective effect of curcumin against the microbicidal activity of ciprofloxacin was tested in the murine model of typhoid fever. BALB/c mice were fed with curcumin alone, ciprofloxacin alone, or both. The infected mice subjected to different treatment regimens were either dissected (on the fifth day) or monitored for survival. To determine the change in infection, if any, under different treatment conditions various organs of mice were isolated, homogenized and processed to calculate the bacterial burden. As shown in Figure 2(a), cohorts of mice fed with curcumin alone had significantly high (2- to 7-fold) bacterial burden in all organs tested. Treatment of infected mice with ciprofloxacin reduced the bacterial burden in all organs tested (Figure 2a). However, the bacterial burden was significantly high in organs of mice fed with curcumin and ciprofloxacin (Figure 2a). A reduction in survival and body weight is the characteristic of *Salmonella* infection. When analysed for survival and alteration in body weight, we found that infected mice fed with both curcumin and ciprofloxacin showed significantly lower survival rates as compared with the mice fed with ciprofloxacin alone (Figure 2b). Infected mice fed with curcumin had lower body weights than the infected mice not fed with curcumin (Figure S1, available as Supplementary data at *JAC* Online). Thus, the *in vivo* data further affirms that curcumin inhibits the microbicidal activity of ciprofloxacin, thus increasing the bacterial burden. We next analysed whether curcumin can rescue ciprofloxacin-mediated changes in bacteria.

Curcumin reduced ciprofloxacin-induced filamentation in Salmonella

Ciprofloxacin treatment is known to induce filamentation in a Gram-negative bacterium like *E. coli*.[49] We tested the ability of ciprofloxacin and curcumin to induce and inhibit filamentation in *Salmonella*, respectively. Two kinds of curcumin treatments were given. In one set, curcumin was present throughout the experiment [STM+Cur (Con)]; in the other set it was added along with ciprofloxacin [STM+Cur (2 h)]. In both these cases, curcumin reduced the amount of ciprofloxacin-induced filamentation by ∼15% (Figure 3a). The length of the ciprofloxacin-induced filaments was significantly shorter in bacteria grown in the presence of curcumin (Figure 3a). The ciprofloxacin-induced filamentation was also observed in intracellular bacteria, *Salmonella* Typhimurium (RAW 264.7) or *Salmonella* Typhi (U937 cells), where the host cells were treated post-infection with ciprofloxacin (0.5 mg/L) (Figure 3b). The extent of ciprofloxacin-induced filamentation was reduced with treatment with curcumin by ∼30%–60% (30% *Salmonella* Typhimurium and 60% *Salmonella* Typhi) (Figure 3b).

Curcumin reverses the downstream effects of ciprofloxacin

Ciprofloxacin is known to arrest the supercoiling reaction of DNA gyrase at the ternary complex state, inducing double-stranded breaks in bacterial chromosomes.[14,15] Consequently, there is fragmentation of chromosomes[50] followed by the death of cells. ROS induced upon ciprofloxacin treatment[23] further increases the DNA damage.[51] The status of DNA damage was examined using various assays, i.e. by thymidine release and TUNEL assays. Both the experiments demonstrated that curcumin could curtail the ciprofloxacin-induced DNA damage (Figure S2, available as Supplementary data at *JAC* Online).

The cleaved DNA complexes lead to induction of the SOS response.[45,52] Curcumin is known to inhibit induction of the SOS response.[29] Hence, expression of the *umuC* gene, a widely used marker for SOS induction, was assessed in bacterial cells treated with ciprofloxacin and curcumin. It was found that curcumin indeed suppressed the ciprofloxacin-induced expression of the *umuC* gene (Figure 4), indicating inhibition of the SOS response. Induction of the SOS response further leads to the inhibition of cell division, making the bacteria filamentous. The evidence that the increased *ftsZ* gene dosage delayed the commencement of SOS-induced filamentation[53] prompted us to evaluate the levels of FtsZ protein in ciprofloxacin-exposed and curcumin-treated *Salmonella* Typhimurium. We found that curcumin-treated, ciprofloxacin-exposed *Salmonella* Typhimurium had elevated levels of FtsZ protein (Figure 4). However, *ftsZ* mRNA did not show any significant difference (Figure 4). Relatively high levels of FtsZ protein in curcumin-treated, ciprofloxacin-exposed cells can be correlated with inhibition of the SOS response by curcumin. Therefore, the reduction in the

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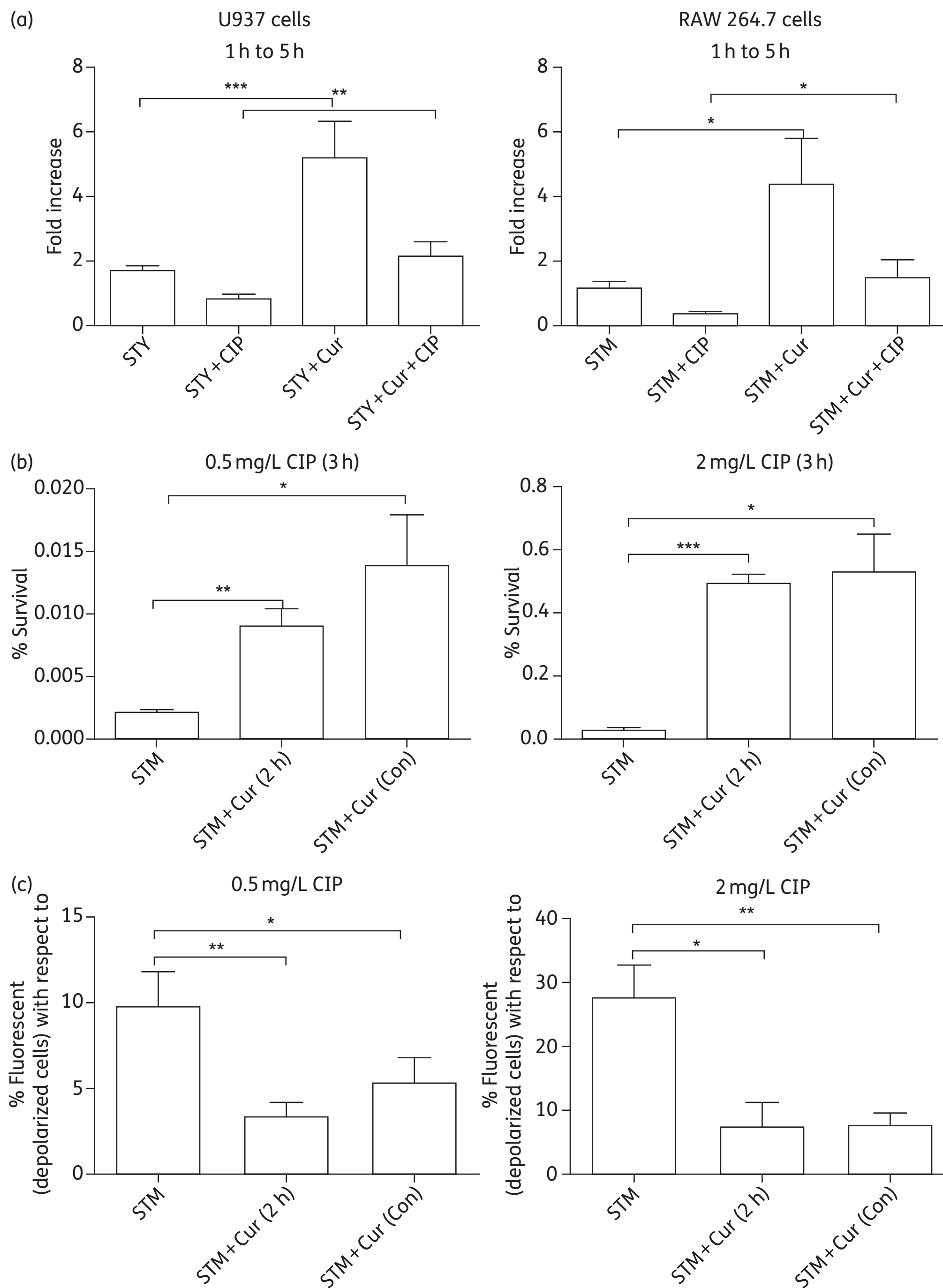

Figure 1. Survival of *Salmonella* against ciprofloxacin. (a) The fold proliferation of *Salmonella* Typhi (STY) and *Salmonella* Typhimurium (STM) in U937 and RAW 264.7 cells, respectively. The cells infected with STM or STY were either untreated or treated with 0.5 mg/L ciprofloxacin and 10 μM curcumin 1 h post-infection for 4 h. The surviving bacteria were enumerated by cfu analysis and the fold increase, from 1 to 5 h, was calculated. STY and STM, infection in cells not treated with curcumin or ciprofloxacin; STY+CIP and STM+CIP, ciprofloxacin treatment in infected cells; STY+Cur and STM+Cur, infected cells treated with curcumin alone; STY+Cur+CIP and STM+Cur+CIP, infected cells treated with curcumin and ciprofloxacin. (b) Effect of curcumin on the viability of *Salmonella* Typhimurium in the presence of ciprofloxacin. The bacteria in log phase were treated with ciprofloxacin in the presence and absence of curcumin (20 μM) for 3 h and surviving bacteria measured by plating on drug-free medium. The percentage survival was assessed relative to ciprofloxacin-untreated bacteria. (c) Percentage of metabolically active bacteria after ciprofloxacin treatment. The bacteria in log phase were treated with ciprofloxacin in the presence and absence of curcumin (20 μM) for 2 h. The cells were then stained with $DiBAC_4(3)$ (1 mg/L) for 10 min and processed for flow cytometric analysis. STY and STM, untreated bacteria; STY+Cur (2 h) and STM+Cur (2 h), bacteria exposed to curcumin and ciprofloxacin simultaneously for 2 h; STY+Cur (Con) and STM+Cur (Con), bacteria continuously treated with curcumin throughout the experiment until plated on drug-free agar. ***$P<0.001$, **$0.001\leq P<0.01$ and *$0.01\leq P<0.05$.

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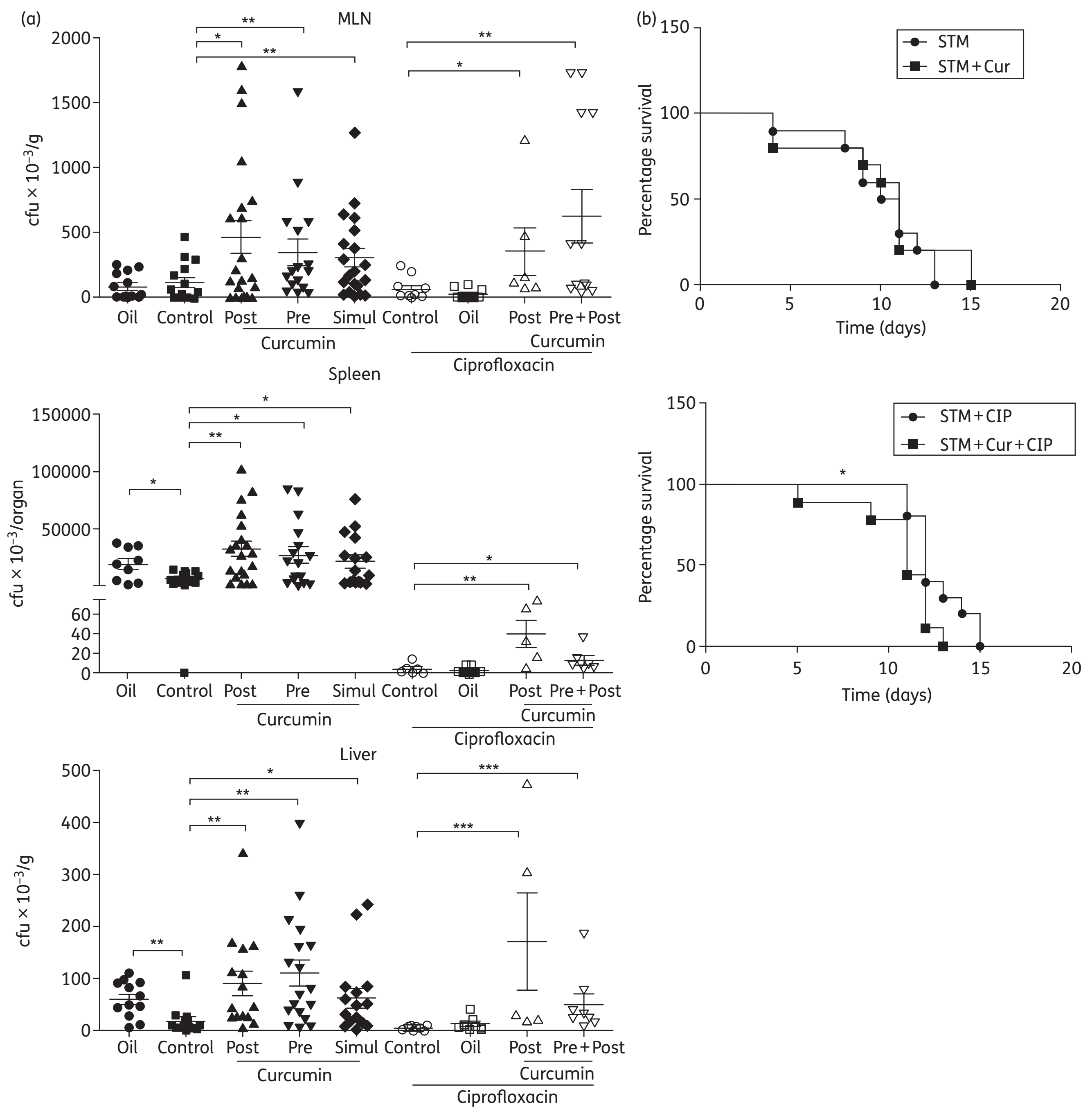

Figure 2. Curcumin confers protection to *Salmonella* Typhimurium under *in vivo* conditions. (a) Bacterial burden in different organs of *Salmonella* Typhimurium–infected mice fed with curcumin and treated with ciprofloxacin. The mice were fed with curcumin under different regimens and were infected with *Salmonella* Typhimurium (10^7 bacteria/mice). One set of infected mice was treated with ciprofloxacin on the third and fourth days post-infection. All the mice were dissected on the fifth day post-infection and the bacterial burden in different organs was determined by plating the organ lysates on LB agar plates. Control, curcumin untreated; Oil, vehicle control (peanut/olive oil); Post, mice fed with curcumin (4 days) after infection; Pre, mice fed with curcumin (4 days) before infection; Simul, mice fed with curcumin immediately after infection; Pre+post, curcumin fed before (4 days) and after (4 days) infection. MLN, mesenteric lymph node. (b) Survival of *Salmonella* Typhimurium–infected mice fed with curcumin and treated with ciprofloxacin. The mice were fed with curcumin under different regimens and were infected with *Salmonella* Typhimurium (10^8 bacteria/mice). One set of infected mice was treated with ciprofloxacin on the third and fourth days post-infection. The death of mice was recorded and the percentage survival plotted. STM, mice infected with *Salmonella* Typhimurium; STM+Cur, mice fed with curcumin before (4 days) and after (4 days) infection and infected with *Salmonella* Typhimurium; STM+CIP, mice infected with *Salmonella* Typhimurium and treated with ciprofloxacin on the third and fourth days post-infection; STM+Cur+CIP, *Salmonella* Typhimurium-infected mice fed with curcumin before (4 days) and after (4 days) infection and treated with ciprofloxacin on the third and fourth days post-infection. ***$P<0.001$, **$0.001\leq P<0.01$ and *$0.01\leq P<0.05$.

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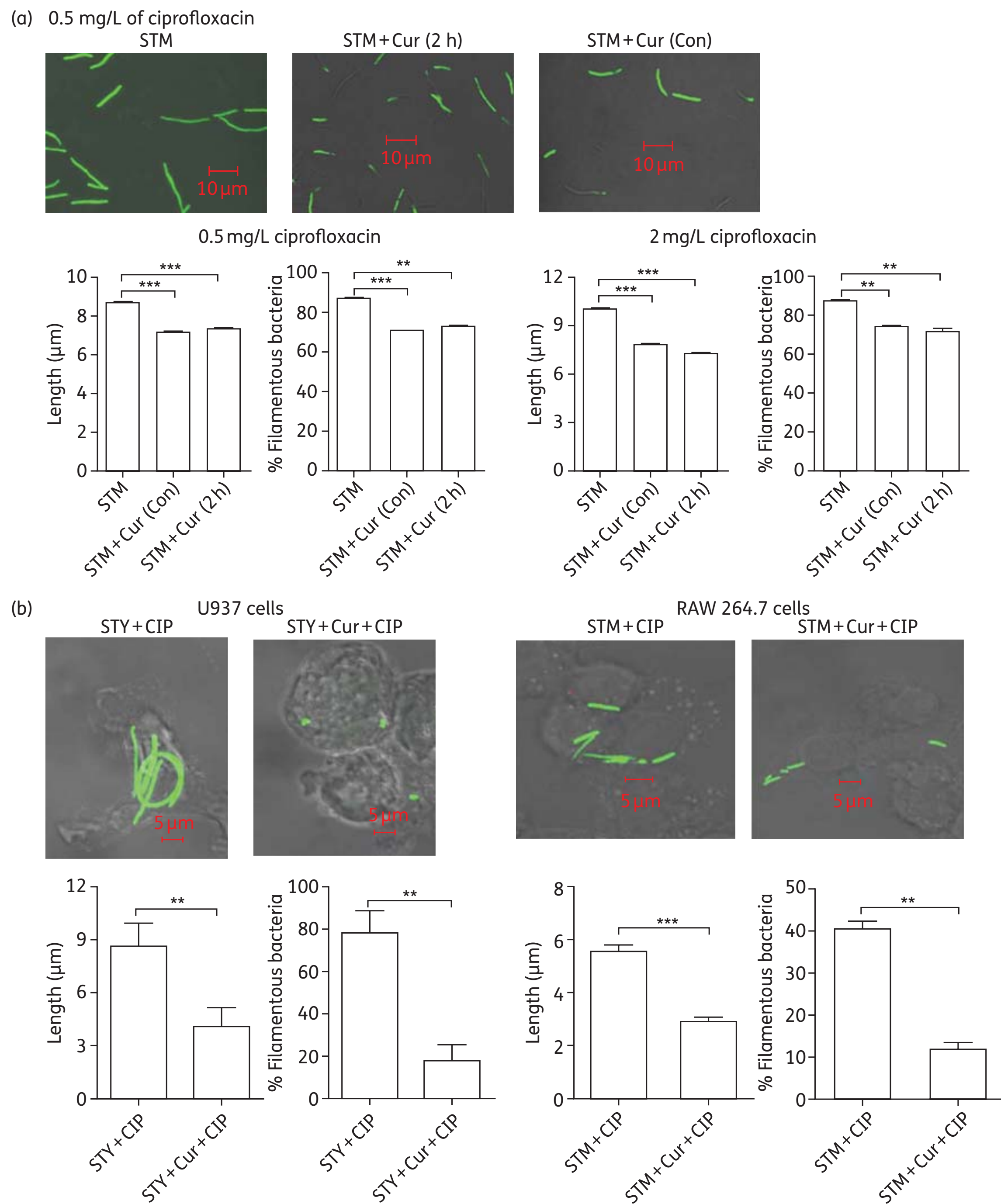

Figure 3. Curcumin reduces the amount of ciprofloxacin-induced filamentation in *Salmonella*. (a) Fluorescent images of bacteria exposed to ciprofloxacin. *Salmonella* Typhimurium, treated or untreated with curcumin (20 μM) in log phase were exposed to ciprofloxacin and images captured with a confocal microscope. More than 300 bacteria were analysed in each case for their length. The percentage of filamentous bacteria (>5 μM in length) was calculated. STM, curcumin-untreated *Salmonella* Typhimurium; STM+Cur (2 h), *Salmonella* Typhimurium exposed to curcumin and ciprofloxacin simultaneously for 3 h; STM+Cur (Con), *Salmonella* Typhimurium continuously treated with curcumin throughout the experiment. (b) Fluorescent images of *Salmonella* containing cells exposed to ciprofloxacin. The macrophages infected with *Salmonella* and treated with 0.5 mg/L ciprofloxacin and 10 μM curcumin for 8 h post-infection were fixed and observed with a confocal microscope for filamentous bacteria (>5 μM in length). More than 170 bacteria were analysed in each case. STY+CIP and STM+CIP, ciprofloxacin treatment in cells infected with *Salmonella* Typhi or *Salmonella* Typhimurium; STY+Cur+CIP and STM+Cur+CIP, cells infected with *Salmonella* Typhi or *Salmonella* Typhimurium treated with curcumin and ciprofloxacin. ***$P<0.001$ and **$0.001\leq P<0.01$.

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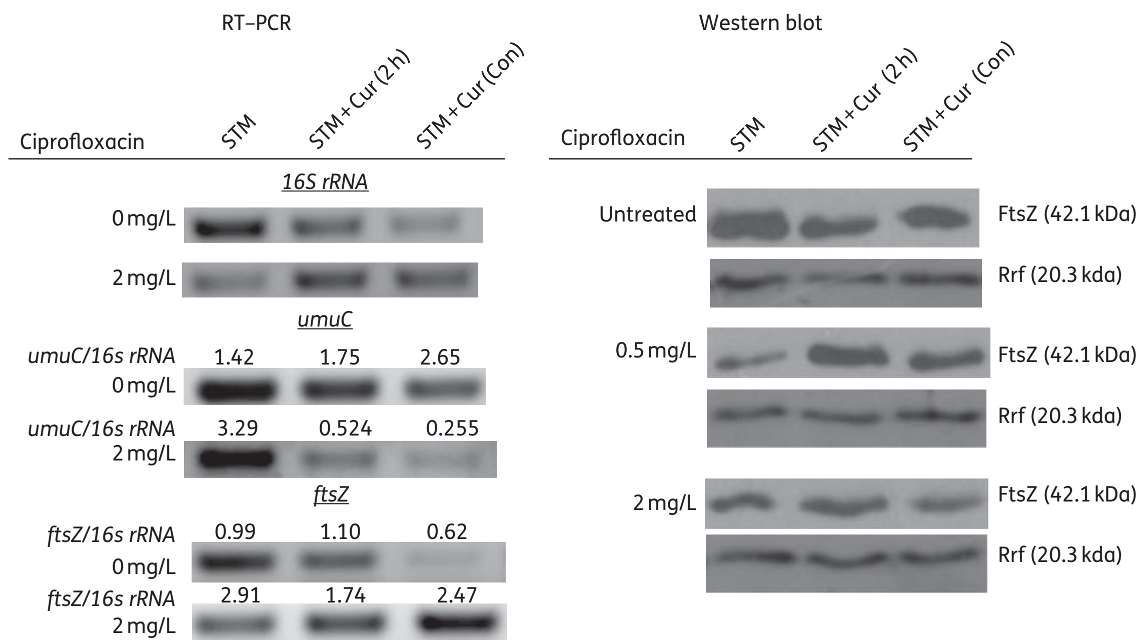

Figure 4. Curcumin inhibits downstream effects of ciprofloxacin. Curcumin inhibits the SOS response and FtsZ degradation caused by ciprofloxacin. The bacteria in log phase were treated with ciprofloxacin in the presence and absence of curcumin (20 μM) for 3 h. The bacteria were processed for RT–PCR (a) or western analysis (b). The induction of *umuC*, an early SOS response gene, was monitored by (a) RT–PCR and (b) FtsZ degradation was analysed by western blot. The induction of *umuC* was measured by densitometric analysis of the PCR bands (numbers above the bands) relative to a constitutive protein, Rrf (ribosome release factor). STM, untreated *Salmonella* Typhimurium; STM+Cur (2 h), *Salmonella* Typhimurium exposed to curcumin and ciprofloxacin simultaneously for 3 h; STM+Cur (Con), *Salmonella* Typhimurium continuously treated with curcumin throughout the experiment.

amount and extent of ciprofloxacin-induced bacterial filamentation by curcumin can be attributed to its ability to inhibit the SOS response and the degradation of FtsZ protein.

Role of the antioxidant property of curcumin in protecting Salmonella against the action of ciprofloxacin

The inhibition of ciprofloxacin-mediated downstream effects by curcumin can be either due to curcumin's antioxidant property, its ability to reverse the ciprofloxacin-mediated gyrase inhibition, or its ability to prevent the access of gyrase to DNA by intercalating in the DNA, thereby preventing the access of ciprofloxacin to DNA-bound gyrase. We checked for the induction of ROS in *Salmonella* Typhimurium by ciprofloxacin and the ability of curcumin to suppress this induction. Chemiluminescence and the NBT reduction assay demonstrated a 1.5- to 3-fold induction of oxidative stress in bacteria on treatment with ciprofloxacin, whereas curcumin suppressed this induction (Figure 5a,b). Ciprofloxacin also induced the production of ROS in both the infected and uninfected macrophages (RAW 264.7) (Figure 5c) and curcumin suppressed this induction (Figure 5c). When assessed for the possibility that curcumin can rescue ciprofloxacin-induced gyrase inhibition, we found that curcumin did not prevent the access of gyrase to DNA (Figure 5d), nor does it prevent the ciprofloxacin-induced cleavage of DNA by gyrase (Figure 5e). This implies that the protection offered by curcumin to *Salmonella* against the bactericidal action of ciprofloxacin is a consequence of its antioxidant activity and ability to inhibit the SOS response rather than direct action at the arrested ternary complex, gyrase–DNA–ciprofloxacin.

Treatment of host macrophages with curcumin inhibits filamentation in Salmonella Typhimurium in IFNγ-primed cells through the inhibition of reactive oxygen and nitrogen species

ROS, produced by the host cells, restricts infection by pathogens.[32] There is an induction of ROS and MEK kinase cascade in the cells either primed with IFNγ or infected with *Salmonella*.[32] This inhibits bacterial replication, inducing filamentation.[32] Curcumin acts as an antioxidant[27] and an inhibitor of the ERK pathway.[54,55] Thus, we tested the ability of curcumin to suppress ROS and MEK kinase cascade induced by *Salmonella* infection or IFNγ. When tested for the intracellular proliferation of *Salmonella* Typhimurium in RAW 264.7 cells stimulated or unstimulated with IFNγ (20 IU/mL, 20–22 h), a 2-fold increase in bacterial proliferation was observed in the cells pre-treated with curcumin (10 μM) (Figure 6a). Curcumin treatment also reduced (by ∼36%) the amount of IFNγ-induced filamentation in *Salmonella* Typhimurium inside RAW 264.7 cells (Figure 6b). To test whether curcumin has any effect on MEK kinase cascade, the activation of ERK was monitored in RAW 264.7 cells infected with *Salmonella* or induced with IFNγ. It was observed that curcumin did not affect the activation of ERK at the concentration tested (Figure 7a). However, curcumin decreased ROS production in RAW 264.7 cells primed or infected with IFNγ and *Salmonella*,

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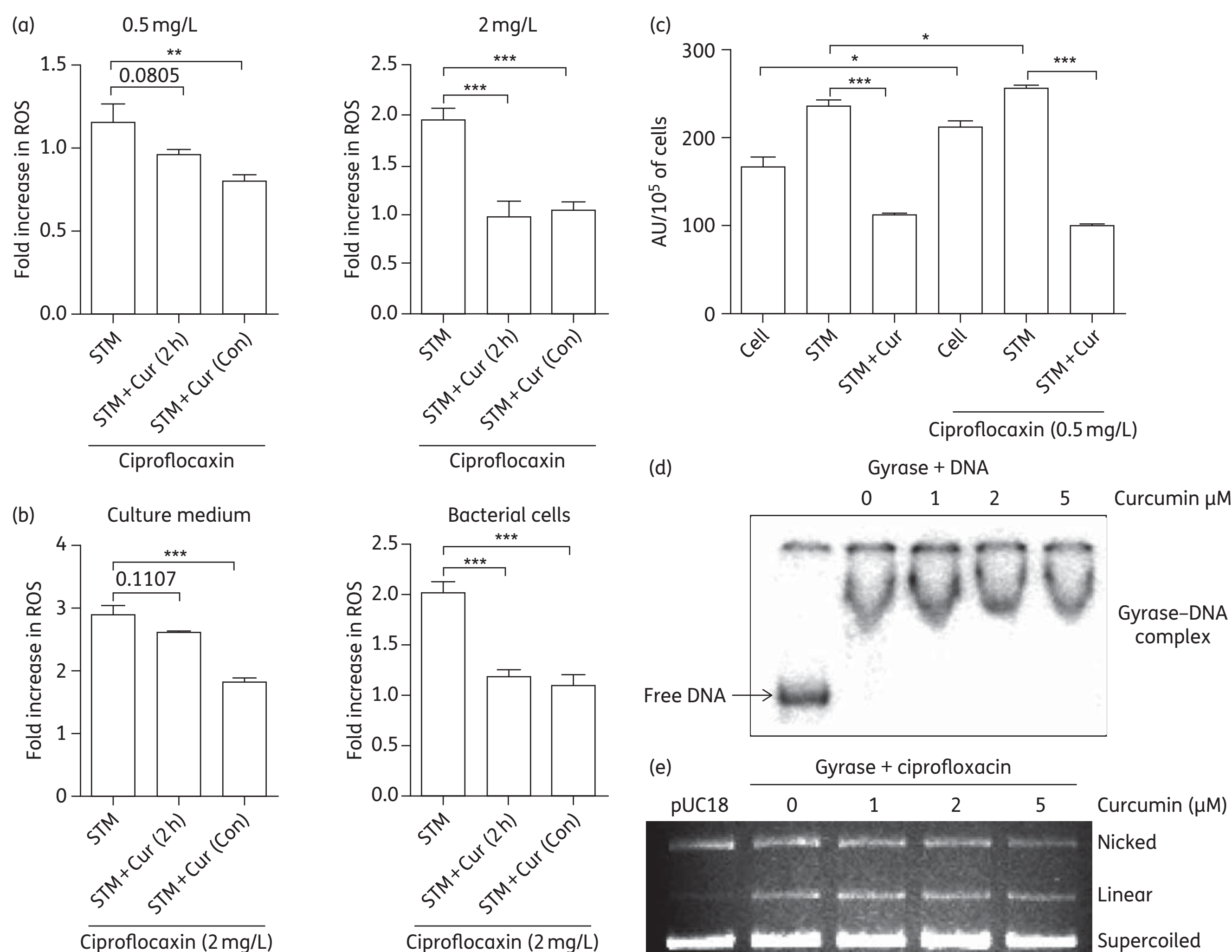

Figure 5. Curcumin partially reverses ciprofloxacin-induced oxidative stress, but not gyrase inhibition. Fold increase in ROS on ciprofloxacin treatment is plotted using (a) chemiluminescence and (b) NBT reduction assays. The bacteria in log phase were treated with ciprofloxacin in the presence and absence of curcumin. The induction of ROS was then determined immediately by (a) a luminol-based assay or (b) after 3 h of incubation in an NBT reduction assay. STM, untreated *Salmonella* Typhimurium, STM+Cur (2 h), *Salmonella* Typhimurium exposed to curcumin and ciprofloxacin simultaneously for 2 h; STM+Cur (Con), *Salmonella* Typhimurium continuously treated with curcumin throughout the experiment. (c) ROS produced by cells infected and treated with ciprofloxacin. RAW 264.7 cells infected with *Salmonella* Typhimurium were treated with 0.5 mg/L ciprofloxacin and 10 μM curcumin. After 1 h of treatment the ROS produced was determined by a luminol-based assay and the readings expressed as arbitrary units (AU) per 10^5 cells. Cell, untreated and uninfected cells; STM, cells infected with *Salmonella* Typhimurium; STM+Cur, cells infected with *Salmonella* Typhimurium and treated with curcumin. (d) DNA binding activity of DNA gyrase in the presence of curcumin. ^{32}P-labelled DNA was incubated with DNA gyrase in the presence of increasing curcumin concentrations (1–5 μM). The reaction mixture was resolved on native PAGE to check for the change in the shift of the gyrase–DNA complex. (e) Ciprofloxacin-induced cleavage of pUC18 plasmid DNA gyrase in the presence of curcumin. DNA gyrase, pUC18 plasmid and curcumin (1–5 μM) were mixed and then incubated with ciprofloxacin for 30 min. The covalently bound gyrase was digested by proteinase K. The reaction products were resolved by agarose gel electrophoresis. ***$P<0.001$, **$0.001\leq P<0.01$ and *$0.01\leq P<0.05$.

respectively (Figure 7b). We can thus conclude that the antioxidant property of curcumin is important in reducing the ROS-induced filamentation in intracellular *Salmonella* Typhimurium.

Curcumin also inhibited (by 2-fold) the induction of nitric oxide· in IFNγ-primed and unprimed RAW 264.7 cells infected with *Salmonella* Typhimurium (Figure S3, available as Supplementary data at *JAC* Online). The amount of inducible nitric oxide synthase was also less in cells (infected or induced with IFNγ/lipopolysaccharide) pre-treated with curcumin compared with that present in untreated cells (data not shown). Thus, we speculate that both the anti-inflammatory and antioxidant property of curcumin might have a role in improving the survival of *Salmonella* Typhimurium inside the macrophages.

Discussion

Bactericidal antibiotics, like β-lactamases, aminoglycosides and quinolones possess a ROS-dependent killing mechanism.[24,25]

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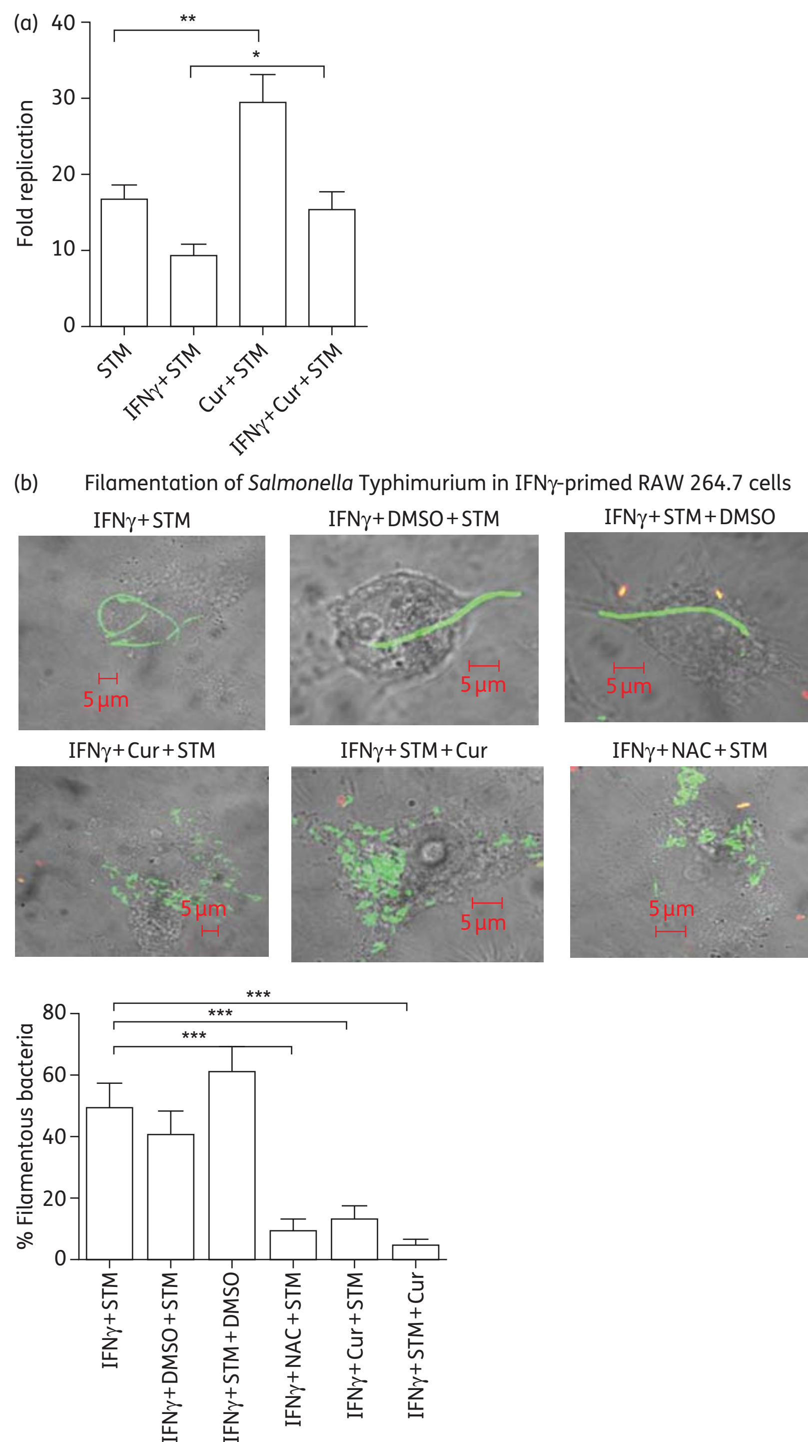

Figure 6. Curcumin treatment partially reverses the action of IFNγ. (a) Fold proliferation of *Salmonella* Typhimurium in RAW 264.7 cells. RAW 264.7 cells treated with IFNγ (20 U/mL, 12 h) and curcumin (10 μM) were infected with *Salmonella* Typhimurium. The infected cells were lysed at 2 and 16 h and the fold proliferation calculated by cfu analysis. (b) Filamentation of *Salmonella* Typhimurium in IFNγ-primed (20 U/mL, 12 h) RAW 264.7 cells. RAW 264.7 cells treated with IFNγ (20 U/mL, 12 h) and curcumin (10 μM) were infected with *Salmonella* Typhimurium. The cells were fixed and observed 16 h post-infection under a confocal microscope for filamentous bacteria (>5 μM in length). STM, untreated cells infected with *Salmonella* Typhimurium; IFNγ+STM, IFNγ-primed and infected cells; Cur+STM, curcumin treatment before infection; IFNγ+Cur+STM, IFNγ-primed, curcumin-pre-treated and infected cells; IFNγ+DMSO+STM, IFNγ-primed, DMSO-pre-treated and infected cells; IFNγ+STM+DMSO, IFNγ-primed, infected and DMSO-post-treated cells; IFNγ+STM+Cur, IFNγ-primed, infected and curcumin-post-treated cells; IFNγ+NAC+STM, IFNγ-primed, antioxidant (*N*-acetyl cysteine)-pretreated and infected cells. Pre-treatment and post-treatment are with respect to *Salmonella* infection. ***$P<0.001$, **$0.001\leq P<0.01$ and *$0.01\leq P<0.05$.

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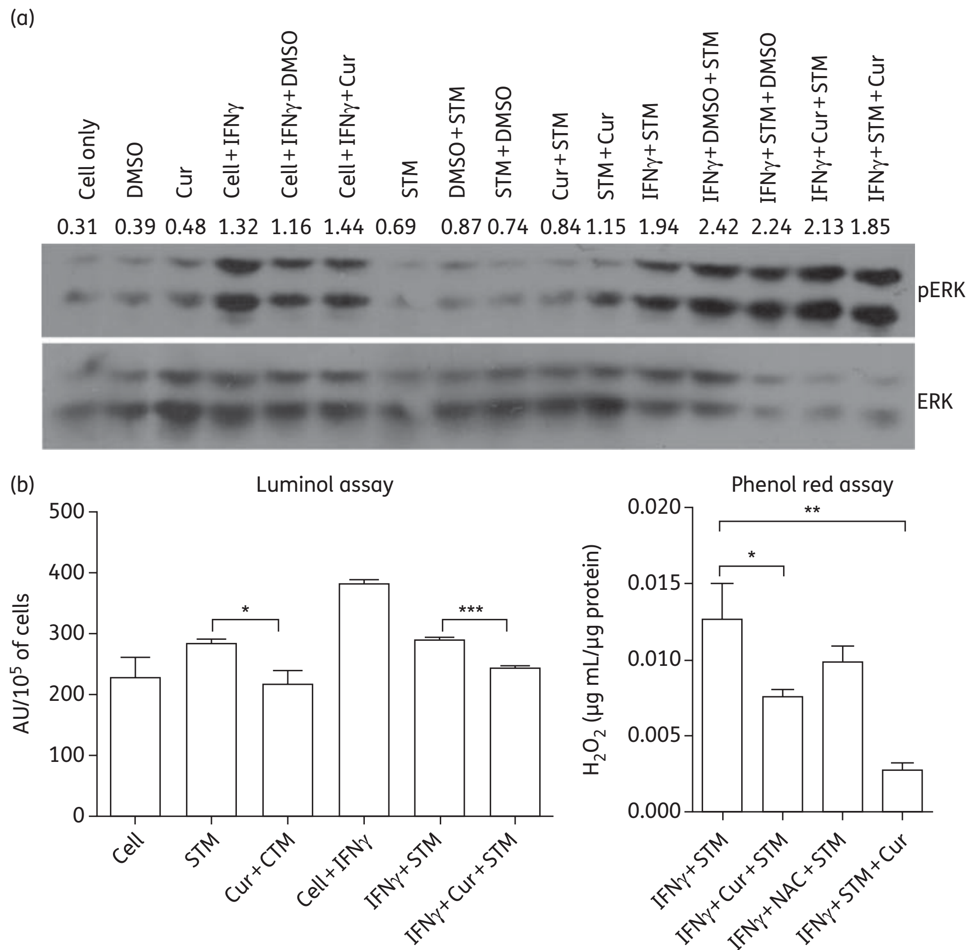

Figure 7. Antioxidant property of curcumin is responsible for reducing IFNγ-induced filamentation in *Salmonella* Typhimurium. (a) Curcumin did not inhibit the activation of ERK1/2 in RAW 264.7 cells (infected or stimulated with IFNγ). RAW 264.7 cells treated with IFNγ (20 U/mL, 12 h) and curcumin (10 μM) were infected with *Salmonella* Typhimurium. Cells were lysed 2 h post-infection and the lysates probed against ERK and pERK. The numbers above the bands describe the ratio of pERK to ERK. (b) Curcumin reduced the ROS induced by IFNγ or *Salmonella* Typhimurium. RAW 264.7 cells treated with IFNγ (20 U/mL, 12 h) and curcumin (10 μM) were infected with *Salmonella* Typhimurium. The ROS produced were assessed by phenol red and luminol assays. Cell, untreated and uninfected cells; Cell+IFNγ, IFNγ-treated cells; STM, untreated cells infected with *Salmonella* Typhimurium; IFNγ+STM, IFNγ-primed and infected cells; Cur+STM, curcumin treatment before infection; IFNγ+Cur+STM, IFNγ-primed, curcumin-pre-treated and infected cells; IFNγ+STM+Cur, IFNγ-primed, infected and curcumin-post-treated cells; IFNγ+NAC+STM, IFNγ-primed, antioxidant (*N*-acetyl cysteine)-pre-treated and infected cells. Pre-treatment and post-treatment are with respect to *Salmonella* infection. ***$P<0.001$, **$0.001\leq P<0.01$ and *$0.01\leq P<0.05$. AU, arbitrary units.

Hence, the presence of antioxidants can interfere with the action of these antibiotics, rendering them less effective. We have tested the effect of curcumin, a well-known therapeutic agent with antioxidant properties,[27] on the bactericidal activity of ciprofloxacin, a member of the quinolone group of antibiotics. Ciprofloxacin is one of the few drugs that is effective against multidrug-resistant typhoid strains,[4–6] though strains resistant to ciprofloxacin have been identified.[7–9] It poisons bacteria by blocking the gyrase-mediated supercoiling reaction, inducing double-stranded DNA breaks.[14,15] This leads to the inhibition of DNA synthesis, induction of the SOS response, fragmentation of chromosomes and generation of ROS.[50,52,56]

Curcumin is consumed on a regular basis in Southeast Asia,[30] where the prevalence of typhoid is high.[57] Its intake during antibiotic therapy may interfere with the efficacy of antibiotics, delaying the recovery of typhoid patients. Our findings suggest that curcumin protected *Salmonella* against the ciprofloxacin-mediated killing both *in vitro* and *in vivo*, thereby increasing the bacterial (*Salmonella*) burden in infected RAW 264.7 macrophages and reticuloendothelial organs of the infected mice. Our results are similar to those obtained by Moghaddam *et al.*,[58] who showed that curcumin counteracted the antibacterial activity of nalidixic acid, belonging to the same class of antibiotics as ciprofloxacin, against *Staphylococcus aureus*. However, the authors did not see any interference of curcumin with the antibacterial activity of ciprofloxacin.[58]

The presence of iron chelators like 2,2′-dipyridyl reduces the hydroxyl radical formed due to the action of bactericidal antibiotics and rescues the bacteria from antibiotic-induced death.[25,51] Curcumin, being a good iron chelator[59] and an antioxidant, can

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reduce the oxidative stress induced by bactericidal antibiotics (e.g. ciprofloxacin) and reverse their action, as is the case in our study. We can thus speculate that curcumin may reverse the oxidative stress induced by bactericidal antibiotics. However, when curcumin is used at a higher concentration it can act as a pro-oxidant,[60,61] aggravating ROS production in antibiotic-treated bacterial cells.

Ciprofloxacin induced filamentation in extracellular as well as intracellular *Salmonella*. In the extracellular environment, the effect of ciprofloxacin is direct, whereas it can be direct or indirect on intracellular bacteria. Previous studies have illustrated that ciprofloxacin penetrates and accumulates inside the macrophage cells.[62,63] According to Gurbay *et al*.,[64] ciprofloxacin induced free radical formation in a time- and dose-dependent manner in liver microsomes, leading to lipid peroxidation. Thus, the induction of filamentation in intracellular bacteria might be due to the direct effect of ciprofloxacin on *Salmonella* through endogenous ROS,[18–20] or the indirect effect of free radicals formed in ciprofloxacin-treated RAW 264.7 cells. We did see a reduction in the ciprofloxacin-induced oxidative stress in infected RAW 264.7 cells on curcumin treatment. This might contribute to the increased survival/replication of *Salmonella* inside the macrophages primed or unprimed with ciprofloxacin. Hartog *et al.*[63] demonstrated that ciprofloxacin ($<$1 mg/L) had a very mild effect on the physiology of intracellular bacteria. However, in our study we saw a decline in the *Salmonella* numbers at a concentration of 0.5 mg/L ciprofloxacin. The assay used by Hartog *et al.*[63] to check for an effect of ciprofloxacin on intracellular bacteria was based on the ability of the bacteria (with luciferase reporter plasmid) to emit light. However, we used cfus as a measure for the surviving bacteria against ciprofloxacin toxicity. The treatment in the Hartog *et al.*[63] study lasted for 1 h, whereas the treatment in our study lasted for 5 h. We too did not observe any cytotoxic effect of ciprofloxacin (0.5 mg/L) on intracellular bacteria up to 3 h (data not shown). The reduction in bacterial load was at the fifth and seventh hours of ciprofloxacin treatment.

Different assays demonstrated that curcumin inhibited ciprofloxacin-induced filamentation of *Salmonella*, generation of ROS, DNA damage and the SOS response (induction of the *umuC* gene). The induction in filamentation due to ciprofloxacin was a result of the inhibition of cell division. Almost all the cells treated with ciprofloxacin had FtsZ dispersed throughout the cytoplasm (data not shown). Curcumin did not affect the gyrase activity or accessibility of gyrase to DNA.

Our result of the inhibition of ciprofloxacin-induced filamentation is in contrast to that of Rai *et al*.,[65] who showed that curcumin induces filamentation in *Bacillus subtilis* by inhibiting the GTPase activity of FtsZ.[65] This discrepancy might be due to the difference in the bacteria used (Gram-negative versus Gram-positive), concentration of curcumin (10–20 versus 20–100 μM) and the medium used for growing bacteria (LB, pH 7 versus LB+ascorbic acid, pH 6.5). Our experimental conditions were very close to the physiological conditions, while pH 6.5 (used by Rai *et al.*[65]) was below the physiological pH. The concentration of curcumin used in our study was non-toxic to peritoneal macrophages, RAW 264.7, Intestine 407 and $CaCo_2$ cells for at least 24 h (data not shown). We observed that curcumin was not cytotoxic to *Salmonella* Typhimurium up to 80 μM ($\sim$30 mg/L; data not shown), which is consistent with the results of Oda,[29] who showed that curcumin does not inhibit the growth of *E. coli* up to 30 mg/L.

The production of ROS is one of the innate immune mechanisms (type I response) that macrophages use against invading pathogens like *Salmonella*. The production of ROS is further stimulated by IFNγ secreted by natural killer (NK) and T cells in response to *Salmonella* infection. This potentiates the killing of pathogens like *Salmonella* by macrophages. Inhibition of this induced inflammatory response and oxidative burst might be dangerous, as it can aggravate the disease.[32,66] Our results of suppression of inflammatory response and oxidative stress induced on *Salmonella* infection (in IFNγ-stimulated and unstimulated RAW 264.7 cells), and Adapala and Chan[67] provide evidence for the same. Apart from its effect on type I immune response, curcumin suppressed the action of ciprofloxacin against *Salmonella* (Typhimurium and Typhi), increasing their burden in host cells. These results caution us to be vigilant against the repercussions associated with the consumption of antioxidants like curcumin during antibiotic (ciprofloxacin or oxidative burst-inducing antibiotics) therapy against typhoid. This might interfere with the microbicidal activity of antibiotics, rendering them less effective.

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Transparency declarations

None to declare.

Supplementary data

Figures S1, S2 and S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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