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N-acetylcysteine blocks SOS induction and mutagenesis produced by fluoroquinolones in *Escherichia coli*

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Background: Fluoroquinolones such as ciprofloxacin induce the mutagenic SOS response and increase the levels of intracellular reactive oxygen species (ROS). Both the SOS response and ROS increase bacterial mutagenesis, fuelling the emergence of resistant mutants during antibiotic treatment. Recently, there has been growing interest in developing new drugs able to diminish the mutagenic effect of antibiotics by modulating ROS production and the SOS response.

Objectives: To test whether physiological concentrations of *N*-acetylcysteine, a clinically safe antioxidant drug currently used in human therapy, is able to reduce ROS production, SOS induction and mutagenesis in ciprofloxacin-treated bacteria without affecting antibiotic activity.

Methods: The *Escherichia coli* strain IBDS1 and its isogenic mutant deprived of SOS mutagenesis (TLS$^-$) were treated with different concentrations of ciprofloxacin, *N*-acetylcysteine or both drugs in combination. Relevant parameters such as MICs, growth rates, ROS production, SOS induction, filamentation and antibiotic-induced mutation rates were evaluated.

Results: Treatment with *N*-acetylcysteine reduced intracellular ROS levels (by ∼40%), as well as SOS induction (by up to 75%) and bacterial filamentation caused by subinhibitory concentrations of ciprofloxacin, without affecting ciprofloxacin antibacterial activity. Remarkably, *N*-acetylcysteine completely abolished SOS-mediated mutagenesis.

Conclusions: Collectively, our data strongly support the notion that ROS are a key factor in antibiotic-induced SOS mutagenesis and open the possibility of using *N*-acetylcysteine in combination with antibiotic therapy to hinder the development of antibiotic resistance.

Introduction

Antibiotics, besides their antimicrobial action, can promote genetic variability in bacteria as an undesirable side effect.[1] In turn, genetic variability increases the chances for bacteria to acquire resistance and jeopardize the success of antimicrobial therapies. Tackling the bacterial physiological responses that promote genetic variability is thus necessary to hinder the spread of resistance.[2,3]

Most of the genetic variability produced by antibiotics has been attributed to induction of the SOS response. The SOS response is a coordinated genetic network that responds to DNA damage. Fluoroquinolones such as ciprofloxacin block DNA gyrase, generating double-strand breaks (DSBs) in DNA that are processed into single-strand DNA (ssDNA). ssDNA, together with RecA, triggers the SOS response. In *Escherichia coli*, SOS induction upregulates the expression of more than 40 genes whose functions include DNA-damage tolerance and non-mutagenic DNA repair.[4–7] When DNA damage is persistent, error-prone DNA translesion synthesis (TLS) takes place. In *E. coli*, TLS is accomplished by the specialized DNA polymerases Pol II, Pol IV and Pol V, encoded respectively by the *polB*, *dinB* and *umuDC* genes.[8] TLS polymerases are able to replicate heavily damaged DNA at the cost of reduced fidelity, therefore increasing mutagenesis. Additionally, RecA-

mediated recombination is also induced by fluoroquinolone antibiotics.[9] Hence, some antibiotics can promote mutagenesis and recombination (i.e. genetic instability) by directly inducing DNA damage and, in turn, the SOS response.

Ciprofloxacin has been shown to increase intracellular respiration, which in combination with the destabilization of the iron–sulphur clusters, leads to production of reactive oxygen species (ROS) via Fenton chemistry.[10–12] ROS are highly reactive chemical species capable of rapidly oxidizing key cellular components, including proteins, lipids and DNA. Oxidation of DNA and nucleotides by ROS produces a wide variety of lesions that, if not repaired, are mutagenic and can lead to cell death.[13–15]

In summary, previous studies have shown that there are at least two routes to antibiotic-triggered bacterial mutagenesis: SOS-mediated TLS and ROS-induced mutagenesis. Interestingly, these two routes are probably not independent but highly intertwined. For instance, oxidation of the nucleotide pool after antibiotic treatment leads to Pol IV-mediated incorporation of 8-oxo-dGTP into DNA, which creates a mutagenic lesion.[16,17] Furthermore, ROS are good SOS inducers because they directly damage DNA.[18–20] Hence, SOS-mediated TLS mutagenesis is fuelled by the presence of oxidative damage in both DNA and the nucleotide pool.[16,17,21,22]

In this study, we hypothesized that certain antioxidant molecules could reduce levels of ROS produced by antibiotic treatment and consequently inhibit SOS induction. This combined inhibition might, in turn, reduce antibiotic-induced mutagenesis. To test this idea, we focused on *N*-acetylcysteine, a well-known antioxidant that acts as a scavenger of oxidant species and as a precursor of glutathione synthesis.[23,24] *N*-acetylcysteine is clinically safe and is currently used in human therapy to treat numerous disorders.[23] Additionally, *N*-acetylcysteine does not negatively affect the activity of major antibiotic classes, except for carbapenems.[25,26] On the contrary, *N*-acetylcysteine has shown antimicrobial properties against a range of clinically relevant pathogens.[27–29] Our results show that, due to its combined antioxidant and antimicrobial properties, *N*-acetylcysteine promises to become a therapeutic complement to standard antibiotic therapy to outsmart the evolution of bacterial resistance.

Materials and methods

Bacterial strains, plasmids and media

Bacterial strains used in this study are described in Table S1 (available as Supplementary data at *JAC* Online). Mutation rate experiments, as well as growth curves and flow cytometry assays, were performed with the *E. coli* strain IBDS1 [MG1655 Δ*attλ*::*cI* (Ind$^-$) λP$_R$*tetA* Δ*ara*::FRT Δ*metRE*::FRT] and its derivative deficient in error-prone polymerases (TLS$^-$). The strain SMR14354 (MG1655 Δ*araBAD567* Δ*attλ*::P$_{BAD}$I-SceI *zfd2509.2*::P$_{N25}$*tetR* FRT Δ*attTn7*::FRT*cat*FRT P$_{N25tetO}$*gam-gfp* I-SceI cut site)[30] was used to measure the SOS induction triggered by DSBs. The plasmid pSC101-P$_{recA}$::*gfp*[31] was used to monitor SOS induction by fluorescence experiments. Bacterial strains were grown in LB broth medium or on LB agar, supplemented with ciprofloxacin at various concentrations, 30 mg/L kanamycin, 15 mg/L tetracycline, 100 mg/L rifampicin, 0.5% (v/v) *N*-acetylcysteine (Hidonac 20%; Zambon Pharma) or L-arabinose (arabinose) when needed. Unless otherwise stated, all chemicals were acquired from Sigma–Aldrich, aliquotted and stored at −20°C before use.

MIC determinations, as well as chequerboard assay, were performed according to standard susceptibility testing,[32] but using LB broth instead of Mueller–Hinton. Absorbance of the chequerboard assay was measured using a TECAN Infinite F200 spectrophotometer after 20 h of incubation at 37°C.

Induction of SOS response

Three independent overnight cultures of the IBDS1 strain containing the plasmid pSC101-P$_{recA}$::*gfp* (Table S1) were diluted 1:100 in 5 mL of LB supplemented with kanamycin and grown to late exponential phase (OD$_{600}$ = 0.5–0.6) at 37°C and 200 rpm. Subsequently, the cultures were diluted 1:50 in LB + kanamycin. Aliquots (2 mL) were treated with various concentrations of ciprofloxacin (with or without 0.5% *N*-acetylcysteine) for 8 h at 37°C and 250 rpm. The procedure was repeated with the strain SMR14354/pSC101-P$_{recA}$::*gfp* (Table S1), but adding 0.1% arabinose instead of ciprofloxacin as SOS inducer. Arabinose triggers the expression of the restriction enzyme I-SceI from the P$_{BAD}$ promoter, which targets an I-SceI restriction site located close to the chromosomal *oriC*, creating a DSB and thereby inducing the SOS response.[30] Controls without induction were also included in every experiment. Absorbance at 595 nm and green fluorescence (485/520 nm) were monitored using a TECAN Infinite F200 plate reader. SOS induction was obtained by normalizing GFP-fluorescence by the absorbance of each sample, as done previously.[31,33] Absorbance was used instead of direct estimation of cfu because ciprofloxacin leads to impaired cell septation and the production of filaments. Any estimation of the bacterial cell number based on plating and enumeration of cfu would inevitably underestimate the actual number of viable bacteria, as every filament (consisting of several viable nucleoids) would give rise to a single cfu. Therefore, plating underestimates the real number of viable bacteria after ciprofloxacin treatment.[9] To determine fold change, the average SOS induction of four biological replicates per condition was divided by the average value of untreated samples.

Flow cytometry

Intracellular ROS levels were determined by the use of the oxidation-sensitive probe 2′,7′-dichlorofluorescein diacetate (DCFDA; Sigma–Aldrich). Overnight cultures of the strain IBDS1 or its TLS$^-$ derivative were diluted 1:100 in 5 mL of LB medium containing 100 mg/L DCFDA and grown to late exponential phase (OD$_{600}$=0.5–0.6) at 37°C and 250 rpm. Controls without the probe were included to monitor autofluorescence. Aliquots (2 mL) from 1:50 dilutions from both cultures (with and without DCFDA) were treated with 0.008 mg/L ciprofloxacin or 0.1% arabinose (with or without 0.5% *N*-acetylcysteine) for 8 h at 37°C and 250 rpm. Three replicates of each condition were included in the assay. Green fluorescence emitted by the intracellular oxidation of the dye was determined using a Guava easyCyte cytometer (Millipore). Three biological replicates of 10000 events each, with a concentration of 200–400 cells/μL, were analysed for each one of the conditions. For estimation of filamentation, forward scatter (FSC) was analysed in samples without DCFDA. Data analysis was performed using custom scripts in R. Statistical significance was calculated for each set of replicate measurements using one-way ANOVA, making comparisons with the non-treated or the autofluorescence control included in each replicate. Measurements were deemed statistically significant when the corresponding *P* values were ≤ 0.05.

Microscopy

Cultures were treated with ciprofloxacin, *N*-acetylcysteine or both agents, exactly as in the fluctuation assays. After 8 h of treatment, samples were prepared as follows: 10 μL of culture was spread with a loop on a microscope slide. Samples were fixed by heat, stained with safranin (Sigma–Aldrich) for 1 min and then washed with distilled water. Slides were observed under an Olympus BX61 microscope using the ×100 objective.

Mutation rate determination

Mutation rates were determined for the strain IBDS1 and its TLS$^-$ derivative. We used a forward mutation assay which scores loss-of-function mutations in the λ *cI* (Ind$^-$) repressor gene that represses the *tetA* gene.[34] Mutational inactivation of the λ *cI* (Ind$^-$) gene confers resistance to tetracycline. We also used rifampicin to select for gain-of-function mutations in the *rpoB* gene. The experiments were conducted as follows: between 12 and 24 independent overnight cultures on at least three different days were diluted 1:100 and grown in LB medium to late exponential phase (OD_{600}=0.5–0.6) at 37°C and 200 rpm. Subsequently, 2 mL aliquots from 1:50 dilutions were treated with 0.008 mg/L ciprofloxacin (with or without 0.5% *N*-acetylcysteine) for 8 h at 37°C and 250 rpm. After treatment, 1 mL of culture was centrifuged for 6 min at 8000 rpm. Cells were then resuspended in fresh LB medium and incubated for 20 h at 200 rpm to allow resolution of filaments. As stated before, this step is necessary to avoid underestimation of viable cells. Appropriate dilutions were plated onto LB plates containing 15 mg/L tetracycline or 100 mg/L rifampicin as selective marker and LB agar plates without antibiotic for viable counting.

Plates were incubated at 37°C for 24 h. The expected number of mutations per culture (m) and 95% CIs were calculated using the maximum likelihood estimator, applying the newton.LD.plating and confint.LD.plating functions that account for differences in plating efficiency implemented in the rSalvador[35] package for R. Mutation rates (mutations per cell per generation) were then calculated by dividing m by the total number of generations, assumed to be roughly equal to the average final number of cells. Statistical significance at the 0.05 level was assessed by means of the likelihood ratio test[36] (LRT.LD.plating function in the rSalvador package) with Bonferroni-corrected *P* values when multiple comparisons were performed.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Determination of ciprofloxacin concentration producing the highest increase in mutagenesis

The mutagenic activity of antimicrobials is expected to occur within a window of concentrations around their MIC, because higher levels would kill cells or stop their growth while lower concentrations would not have a stimulatory effect.[37] To determine the concentration of ciprofloxacin that induces the highest increase in mutagenesis, we treated exponentially growing cultures of *E. coli* strain IBDS1[34] with different concentrations of ciprofloxacin ranging from 0.25× to 4× the MIC for 8 h. We then determined mutation rates using two independent selective markers. Cells treated with 0.008 mg/L ciprofloxacin (which corresponds to ½ of the MIC) showed the highest increase in the rate of mutations conferring resistance to rifampicin (Rif-R; 10-fold increase) and tetracycline (Tet-R; 2.5-fold increase) (Figure S1). Higher concentrations of ciprofloxacin barely produced any increase in mutagenesis. This probably occurred because these concentrations hampered growth of most treated cells (Figure S2), drastically reducing the effective population size and hence limiting evolvability.[38] We decided to use 0.008 mg/L ciprofloxacin thereafter to maximize antibiotic-induced mutagenesis.

N-acetylcysteine reduces ciprofloxacin-induced intracellular ROS

We then measured the levels of ROS caused by treatment with ciprofloxacin and tested whether *N*-acetylcysteine was able to reduce the levels of ciprofloxacin-generated ROS. To this end we used DCFDA, an ROS-sensitive dye that emits fluorescence when it is oxidized intracellularly.[39] DCFDA has been previously shown to be an extremely sensitive probe for the detection of ROS caused by fluoroquinolones, detecting H_2O_2, ROO· and $ONOO^-$ with great sensitivity.[39] As expected, ciprofloxacin treatment produced a massive increase in ROS levels compared with untreated cells (Tukey multiple comparisons after significant ANOVA; $P<0.000001$ for ciprofloxacin-treated versus untreated cells; Figure 1 and Figure S3a). Most importantly, ROS levels were significantly reduced after 8 h when ciprofloxacin treatment was combined with *N*-acetylcysteine (Tukey multiple comparisons after significant ANOVA; $P<0.00003$ for ciprofloxacin versus ciprofloxacin + *N*-acetylcysteine treatment; Figure 1 and Figure S3a). This result suggests that *N*-acetylcysteine, at a physiologically attainable concentration,[40] might be able to reduce DNA damage by reducing the levels of ROS upon ciprofloxacin exposure.

Finally, because it has been described previously that quinolone treatment produces autofluorescence in *E. coli* cells,[41] we assessed whether it could influence our ROS measurements. Our results with ciprofloxacin confirm such autofluorescence in all the strains used in this work. However, cells treated with ciprofloxacin consistently showed increased fluorescence levels over those produced by the antibiotic-mediated autofluorescence (Tukey multiple comparisons after significant ANOVA; $P<0.00024$ for DCFDA versus unstained; Figure S3), validating our experimental approach. Furthermore, compensation of ROS values by autofluorescence yielded qualitatively similar results.

N-acetylcysteine reduces ciprofloxacin-mediated induction of the SOS response

We then analysed the effect of ciprofloxacin and *N*-acetylcysteine on SOS induction. To this end, we used the strain IBDS1 transformed with a low-copy-number plasmid that harbours the transcriptional fusion P_{recA}::*gfp*.[31,42] As expected, ciprofloxacin strongly induced the SOS response, ∼14-fold compared with untreated controls (Tukey multiple comparisons after significant ANOVA; $P<0.001$; Figure 2a). Following the hypothesis that antioxidant compounds can be effective inhibitors of SOS induction,[43] we tested the effect of different *N*-acetylcysteine concentrations on ciprofloxacin-mediated SOS induction. *N*-acetylcysteine inhibited SOS induction caused by ciprofloxacin at all concentrations (Tukey multiple comparisons after significant ANOVA; $P<0.001$; Figure 2a), with an IC_{50} of 0.5% (Figure 2c). Again, this concentration is within the range of attainable physiological values.[40] Importantly, *N*-acetylcysteine did not reduce ciprofloxacin bactericidal activity, as shown by MIC results (Table S1), growth curves (Figure 2b) and a chequerboard assay (Figure 2d). On the contrary, *N*-acetylcysteine alone slightly inhibited bacterial growth without inducing the SOS response (Tukey multiple comparisons after significant ANOVA; $P=0.0003$). Additionally, we verified that the

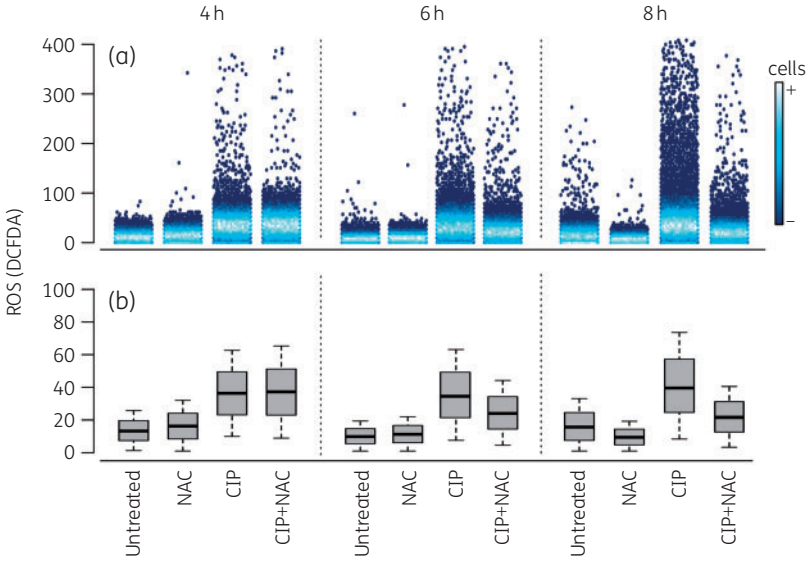

Figure 1. *N*-acetylcysteine reduces ciprofloxacin-induced intracellular ROS. ROS levels were assessed by individually capturing the fluorescence of DCFDA in 30 000 cells by flow cytometry at different timepoints after adding 0.008 mg/L ciprofloxacin, 0.5% *N*-acetylcysteine or both agents in combination. An untreated control is shown as a reference. (a) The dot plot shows the distribution of fluorescence in treated populations. At least 99% of the events recorded are shown. The colour scale displays the density of events at every fluorescence level. (b) To allow better comparison, the data are depicted as boxplots, in which the horizontal line represents the median value, the depth of the box represents the IQR (50% of the population) and whiskers extend to 0.5× the IQR. Note that both panels represent the same data. CIP, ciprofloxacin; NAC, *N*-acetylcysteine. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

differences in the final absorbance observed upon different treatments do not directly alter the measurement of SOS induction using GFP fluorescence (Figure S4).

SOS induction leads to the overexpression of *sulA* (*sfiA*), whose product inhibits FtsZ ring formation and hence cell division.[44] The phenotypic consequence of cell division inhibition is filamentation, which offers an additional SOS-dependent measurable phenotype. We assessed whether 0.5% *N*-acetylcysteine was able to inhibit ciprofloxacin-mediated cell filamentation by both flow cytometry and direct observation of safranin-stained cultures. Figure 3 shows that, as expected, ciprofloxacin treatment produces a vast increase in the fraction of the population with filamented cells compared with untreated cultures (33% versus 0.3% filamented cells). Remarkably, administration of 0.5% *N*-acetylcysteine, together with ciprofloxacin, prevented filamentation in a large fraction of cells (14% filamented with ciprofloxacin + *N*-acetylcysteine versus 33% with ciprofloxacin alone). We qualitatively confirmed these results by microscopy observation of safranin-stained cells (Figure 3b).

In summary, our results demonstrated that *N*-acetylcysteine does not decrease bacterial susceptibility to ciprofloxacin. It does, however, significantly reduce (up to 75%) ciprofloxacin-mediated induction of *recA* transcription and the production of cell filamentation, which are hallmarks of SOS induction.

N-acetylcysteine inhibits SOS response in an ROS-dependent manner

Although the above results compellingly suggest that the reduction of ciprofloxacin-induced ROS underlies SOS inhibition by *N*-acetylcysteine, we could not rule out other possibilities. *N*-acetylcysteine could potentially perturb the activity of the SOS regulatory machinery, for example inhibiting RecA-ssDNA nucleation or LexA self-cleavage. If that were the case, we reasoned that *N*-acetylcysteine would also reduce SOS induction when DNA damage is independent of ROS. To test this possibility, we used the *E. coli* strain SMR14354 (Table S1),[30] whose chromosome carries a unique cutting site for the restriction enzyme I-SceI. In the presence of 0.1% arabinose, I-SceI is produced, generating DSBs and consequently inducing the SOS response (Figure 4a). Using flow cytometry and DCFDA we first verified that generation of DSBs by I-SceI does not increase ROS levels as a side effect (Figure 4b and Figure S3c). We then measured SOS induction at different timepoints after DSB induction. Our results demonstrated that the addition of *N*-acetylcysteine causes no measurable inhibition of the SOS response (two-tailed Student's *t*-test; $t=0.64$, $df=4$ and $P=0.56$ for arabinose versus arabinose + *N*-acetylcysteine after 8 h of treatment), indicating that *N*-acetylcysteine inhibition of the SOS response is ROS-dependent (Figure 4c). Although the experimental conditions used here have been shown to cause at least a single DSB in 90% of the cells (and more than one in 50% of cells),[30] induction of the SOS response by arabinose in SMR14354 seems to be lower than that caused by 0.008 mg/L ciprofloxacin in IBDS1, even when induced with higher arabinose concentrations (Figure S5). An alternative explanation for our results could be that at lower levels of SOS up-regulation, *N*-acetylcysteine is unable to decrease SOS induction. To discard this possibility, and to match the level of induction caused by I-SceI-mediated DSB, we tested the effect of *N*-acetylcysteine in cultures treated with lower ciprofloxacin concentrations. Our results showed that *N*-acetylcysteine is able to reduce ciprofloxacin-induced SOS response in all cases (Figure S6).

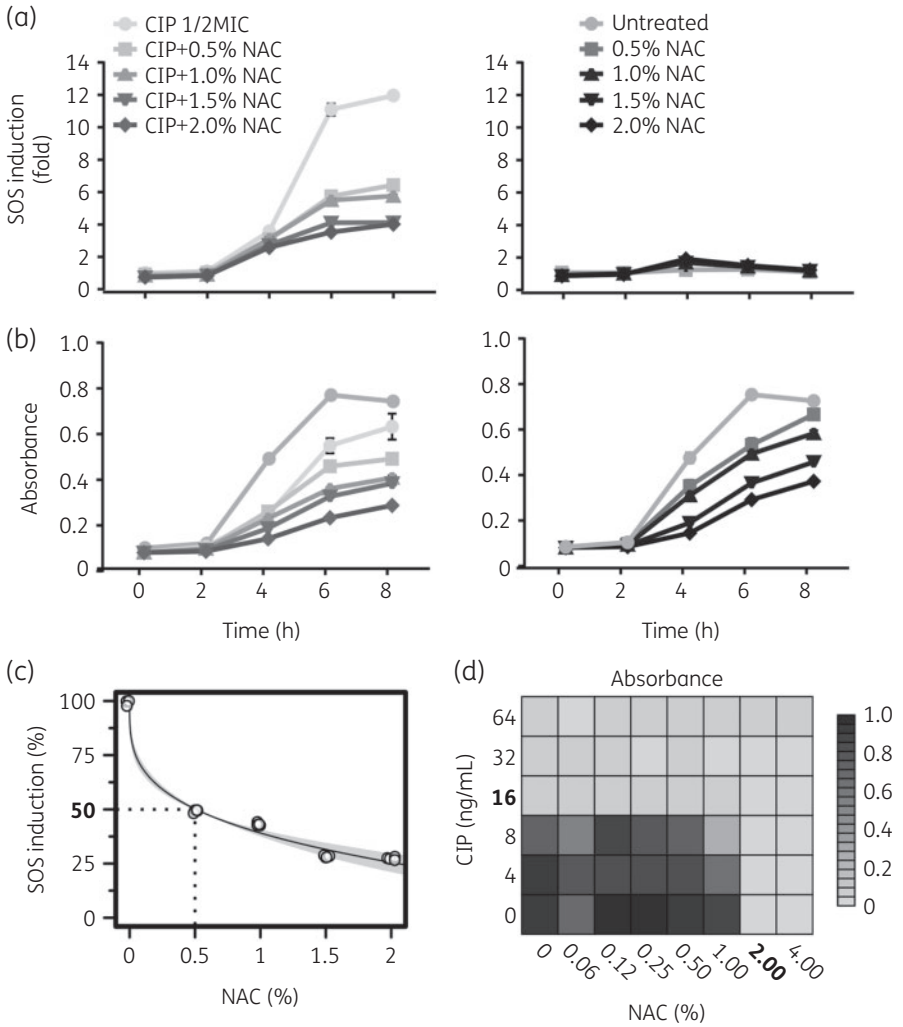

Figure 2. *N*-acetylcysteine reduces ciprofloxacin-induced SOS response. Bacterial growth and SOS induction were monitored during treatment with various concentrations of ciprofloxacin alone or in combination with *N*-acetylcysteine (left panels) or *N*-acetylcysteine alone (right panels). Samples were taken at indicated timepoints and SOS induction (a) and bacterial growth (b) were quantified. Error bars represent standard deviations and are not shown when smaller than data points. (c) Ciprofloxacin-mediated SOS induction at 8 h was assessed in combination with a range of *N*-acetylcysteine concentrations giving rise to a dose–response curve. Experimental data were fitted by a non-linear model (R^2=0.988). The concentration of *N*-acetylcysteine that inhibits 50% of SOS response (IC_{50}) was 0.5%. The area shaded light grey represents the 95% CI of the fit. (d) Potential interactions of ciprofloxacin with *N*-acetylcysteine were determined by the chequerboard method. MICs of each compound alone are shown in bold font. No synergistic or antagonistic effect was found. CIP, ciprofloxacin; NAC, *N*-acetylcysteine.

N-acetylcysteine abolishes SOS-mediated mutagenesis promoted by ciprofloxacin

The quinolone-mediated increase in mutagenesis has been attributed to the activity of TLS DNA polymerases, whose transcription is induced as part of the SOS response.[8,45] However, previous studies strongly suggest that high levels of ROS are also mutagenic.[13–15] To gain knowledge on the contribution of each of these two mechanisms we used the strain IBDS1 and its TLS$^-$ derivative, which lacks the three TLS error-prone DNA polymerases (Table S1).[34] We verified that the TLS$^-$ strain showed similar SOS induction and ROS production levels to the WT strain when treated with ciprofloxacin and *N*-acetylcysteine alone or in combination (Figure S7). Fluctuation assays showed that treatment with ciprofloxacin induced mutagenesis in both WT and TLS$^-$ strains (ciprofloxacin treated versus untreated; Bonferroni-adjusted likelihood ratio statistic $P<10^{-8}$ in all cases), although at lower levels in the TLS$^-$ strain (WT strain versus TLS$^-$ strain, both ciprofloxacin treated; Bonferroni-adjusted likelihood ratio statistic $P<10^{-4}$; Figure 5). This result indicates that a fraction of ciprofloxacin-mediated mutagenesis was not dependent on SOS TLS$^-$ polymerases and, together with recent evidence,[46] supports the existence of a TLS$^-$-independent mutagenic pathway.

The combined treatment with ciprofloxacin and *N*-acetylcysteine decreased ciprofloxacin-mediated mutagenesis in the WT strain by ~40% for both Rif-R and Tet-R selective markers, totally eliminating TLS$^-$-dependent mutagenesis (ciprofloxacin versus ciprofloxacin + *N*-acetylcysteine treatment; Bonferroni-adjusted likelihood ratio statistic $P<0.014$ for both selective

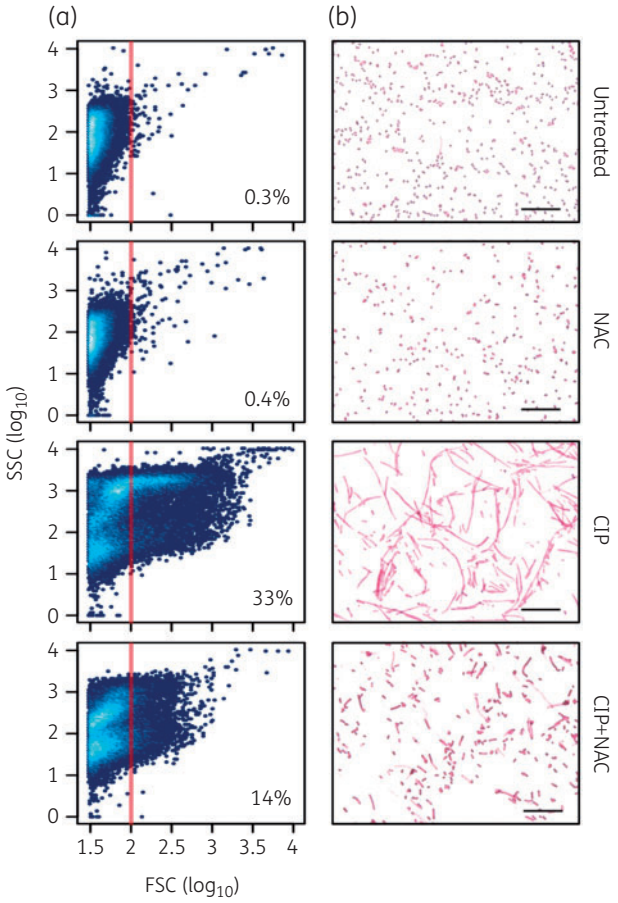

Figure 3. *N*-acetylcysteine reduces ciprofloxacin-induced filamentation. (a) The fraction of filamented cells after treatment with ciprofloxacin, *N*-acetylcysteine or both agents in combination is shown after flow cytometry analysis of 30 000 cells (proportional to cell size). The percentage on every graph represents the filamented fraction of the population [FSC ($\log_{10}$) >2; red vertical line]. (b) Representative microscopy fields of safranin-stained cells. Scale bar=20 μm. CIP, ciprofloxacin; NAC, *N*-acetylcysteine; SSC, side scatter. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

markers; Figure 5a). This highlights the importance of ROS as a major contributor to ciprofloxacin-induced mutagenesis. On the contrary, *N*-acetylcysteine was unable to alter ciprofloxacin-induced mutagenesis in the TLS$^-$ strain (ciprofloxacin versus ciprofloxacin + *N*-acetylcysteine treatment; Bonferroni-adjusted likelihood ratio statistic $P > 0.74$ for both selective markers), indicating that the TLS$^-$-independent mutagenesis shown here is also ROS-independent (Figure 5b). Together, these results indicate that, upon ciprofloxacin treatment, TLS polymerases act synergistically with ROS in a highly intertwined mutagenesis pathway. Accordingly, reduction of ROS by *N*-acetylcysteine completely abolishes SOS-mediated mutagenesis in ciprofloxacin-treated bacteria.

Discussion

Recently, there has been growing interest in the development of novel therapies designed not to kill bacteria, but to inhibit the aforementioned routes to antibiotic resistance.[2,3,47–49] These novel drugs would be administered in combination with conventional antimicrobial chemotherapy to block or at least reduce the development of antibiotic resistance. Among the possible pathways that lead to antibiotic resistance, the SOS response has received considerable attention because its inhibition may provide several benefits that go beyond reducing bacterial evolvability.[50–52] For instance, it has been shown that blocking RecA might also render bacteria more susceptible to several antibiotics,[53] in some cases leading to complete reversion of antibiotic resistance.[54] Additionally, SOS induction is tightly linked to bacterial pathogenic processes such as the production of toxins,[55] biofilm formation,[56] persistence[57] and filamentation.[58] Therefore, inhibition of the SOS response might improve the prognosis of important bacterial infections besides reducing the evolution of antibiotic resistance.

In this work, we showed that *N*-acetylcysteine, an FDA-approved drug already used for the treatment of numerous human disorders,[23,24] is able to inhibit up to 75% of the SOS induction caused by ciprofloxacin. The likely cause of this SOS inhibition is a significant reduction of intracellular ROS levels. This is supported by the observation that *N*-acetylcysteine does not inhibit the SOS response when DNA damage occurs in the absence of ROS production. It has been argued that ROS are a common cause of bacterial cell death for several antibiotic families,[11,12,17] although this notion has been further challenged.[59,60] Reducing intracellular ROS upon antibiotic treatment might thus reduce antibiotic activity as an undesired side effect. However, we consistently found that *N*-acetylcysteine does not affect ciprofloxacin antimicrobial activity, a result which agrees with previous reports.[25,26] Furthermore, *N*-acetylcysteine itself has been shown to possess antibacterial properties against *Helicobacter pylori*,[28] *Haemophilus influenzae*[25] and *Pseudomonas aeruginosa* biofilms.[29]

We also showed that *N*-acetylcysteine treatment drastically reduces two SOS phenotypes that are important to bacterial pathogenic lifestyle: filamentation and SOS-mediated mutagenesis. Filamentation has been shown to be crucial for the development of urinary tract infections[58] and hence its inhibition opens new avenues for treating this common type of infection. The finding from our work with arguably broader implications is that *N*-acetylcysteine caused a significant reduction of ciprofloxacin-induced mutagenesis in the WT strain. Most importantly, *N*-acetylcysteine treatment reduced WT mutagenesis to levels similar to those seen in the TLS$^-$ strain, suggesting that the residual ciprofloxacin-induced mutagenesis is independent of TLS repair and, probably, SOS induction. Consistent with this view, we observed an ROS-independent increase in mutagenesis in the TLS$^-$ strain when submitted to ciprofloxacin treatment. The mechanism behind this TLS- and ROS-independent mutagenesis pathway remains elusive and warrants further experimentation.

It is important to consider that even a small reduction of mutation rates may significantly influence bacterial evolution.[61] This may be particularly true when antibiotic resistance arises through the accumulation of several mutations, as in the case of fluoroquinolone resistance.[62,63] It is our view that the generalized use of 'evolution-slowing' drugs such as *N*-acetylcysteine will likely delay the development of resistance, lengthening the lifespan of our antibiotic armamentarium and providing a crucial tool to outsmart bacterial evolution.

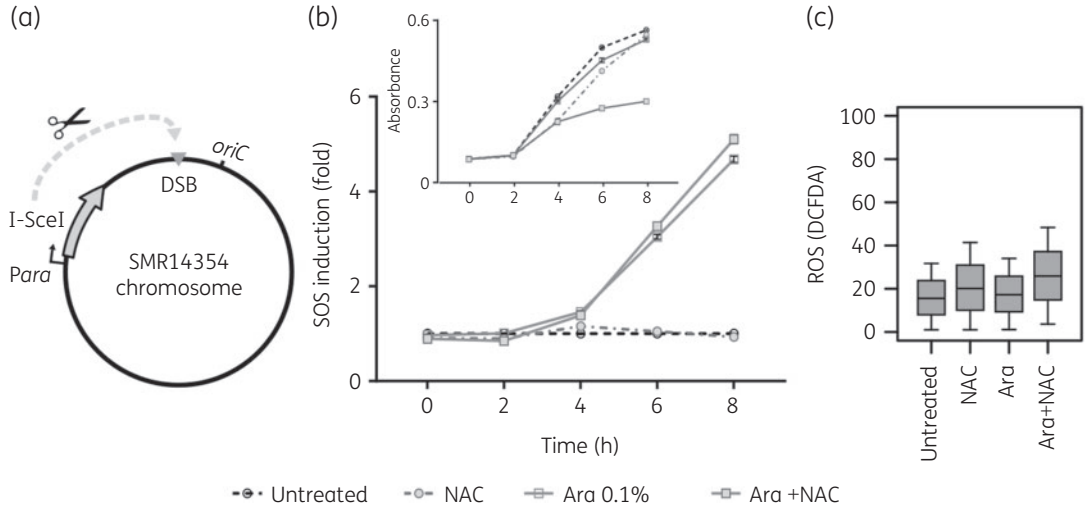

Figure 4. Artificially generated DSBs induce the SOS response, but do not generate ROS. (a) Schematic diagram of the experimental setting. The strain SMR14354 carries a unique cutting site (arrowhead) close to *oriC* of the restriction enzyme I-SceI (scissors), whose expression is induced by arabinose. (b) Addition of 0.1% arabinose generates DSBs that concomitantly induce the activation of the SOS response, measured here by means of a P_{recA}::*gfp* transcriptional fusion. The inset graph represents absorbance at 595 nm under the same conditions. Error bars (representing standard deviations) smaller than data points are not shown for the sake of clarity. (c) Boxplots showing ROS levels (DCFDA fluorescence) detected with flow cytometry analysis after DSB induction for 8 h. The depth of the box represents the IQR (50% of the population), the horizontal line represents the median value and whiskers extend to 0.5× the IQR. ARA, arabinose; NAC, *N*-acetylcysteine.

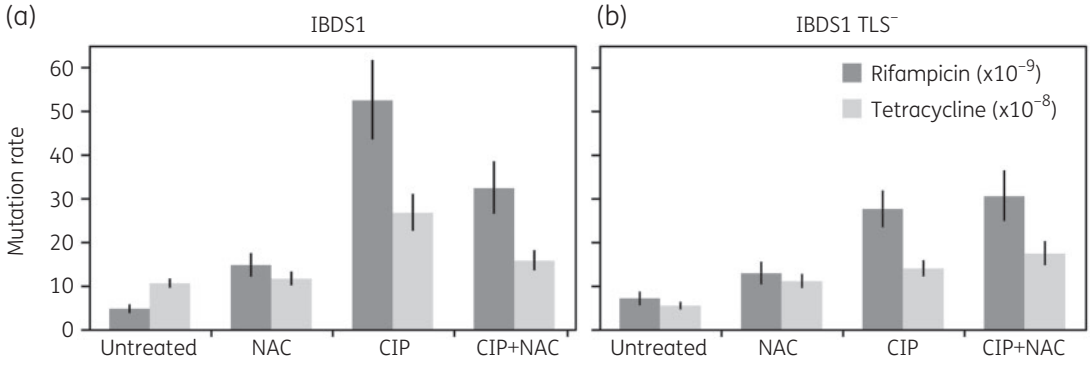

Figure 5. *N*-acetylcysteine abolishes ciprofloxacin-induced mutagenesis in the WT strain, but not in its TLS⁻ derivative. WT IBDS1 cells (a) and TLS⁻ cells (b) were treated with 0.008 mg/L ciprofloxacin and 0.5% *N*-acetylcysteine alone or in combination. After 20 h of recovery in antibiotic-free medium, mutation rates (mutations per site per generation) were calculated using rifampicin (Rif; dark grey bars) or tetracycline (Tet; light grey bars) as a selective marker. CIP, ciprofloxacin; NAC, *N*-acetylcysteine.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 to S7 are available as Supplementary data at *JAC* Online.

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