Reactive Oxygen Species

Deutsche Ausgabe: DOI: 10.1002/ange.201509340
Internationale Ausgabe: DOI: 10.1002/anie.201509340

Reactive Oxygen Species Play an Important Role in the Bactericidal Activity of Quinolone Antibiotics

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Abstract: *Recent studies posit that reactive oxygen species (ROS) contribute to the cell lethality of bactericidal antibiotics. However, this conjecture has been challenged and remains controversial. To resolve this controversy, we adopted a strategy that involves DNA polymerase IV (PolIV). The nucleotide pool of the cell gets oxidized by ROS and PolIV incorporates the damaged nucleotides (especially 8oxodGTP) into the genome, which results in death of the bacteria. By using a combination of structural and biochemical tools coupled with growth assays, it was shown that selective perturbation of the 8oxodGTP incorporation activity of PolIV results in considerable enhancement of the survival of bacteria in the presence of the norfloxacin antibiotic. Our studies therefore indicate that ROS induced in bacteria by the presence of antibiotics in the environment contribute significantly to cell lethality.*

The mode of action of different types of bactericidal antibiotics generally falls into one of three classes: inhibition of DNA replication, inhibition of protein synthesis, and inhibition of cell-wall turnover.[1] In the recent past, the induction of reactive oxygen species (ROS) has also been implicated in the antimicrobial function of antibiotics.[2] All three major classes of bactericidal antibiotics, namely quinolones, β-lactams, and aminoglycosides, were shown to affect respiration, thus supporting a mechanistic hypothesis for the formation of superoxide and subsequently, lethal hydroxyl radicals.[3]

Despite a growing volume of supportive evidence, the view that the induction of ROS plays an important role in the action of antibiotics has been challenged and remains controversial.[4] Two studies failed to detect an increase in ROS upon antibiotic treatment and came to the conclusion that there is no relation between oxidative stress and cell lethality as a result of antibiotics.[4a,b] To resolve this controversy, we have utilized an approach that exploits the role of DNA polymerase IV (PolIV) in mediating the ill effects of ROS.

One hypothesized adverse effect of hydroxyl radical generation is oxidation of the nucleotide pool (dNTPs and rNTPs) present in the bacterial cell. Foti and colleagues showed that incorporation of the oxidized nucleotide pool—especially 8oxodGTP (8odGTP)—into the genome by PolIV contributes substantially to the deleterious effects of ROS on cellular physiology.[5] The resulting dA:8odGTP base pairs are promutagenic and lead to deleterious transversion mutations. In addition, 8oxodG lesions in newly synthesized genomic DNA lead to double-strand breaks due to aberrant repair of closely spaced 8oxodG lesions.[5b,6] The 8odGTP incorporation activity of PolIV is in addition to previously observed roles in stress-induced mutagenesis or translesion synthesis that aid survival of the cell.[7]

PolIV has significant ability to incorporate 8odGTP opposite dA but not dC, even at high concentrations of 8odGTP (Figure 1). The catalytic efficiency (k_{cat}/K_M) for the incorporation of 8odGTP opposite dA is 200-fold less than that for the incorporation of dTTP opposite dA (Table 1). The lower catalytic efficiency suggests that the incorporation of 8odGTP into genomic DNA by PolIV will be minimal in the

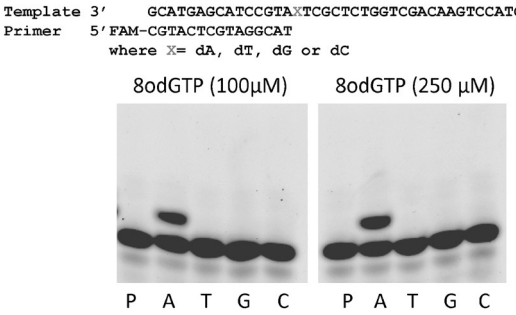

Figure 1. The ability of PolIV to incorporate 8odGTP opposite different template nucleotides was compared. P stands for only primer, A for template dA, T for template dT, G for template dG, and C for template dC. Even at very high concentrations of 8odGTP, PolIV incorporates the damaged nucleotide only opposite template dA and not dC.

Table 1: Catalytic efficiency of incorporation by PolIV of dTTP, dGTP, and 8odGTP opposite template dA, dC, and dA, respectively.[a]

	k_{cat} [min^{-1}]	K_M [μM]	k_{cat}/K_M [$mM^{-1} min^{-1}$]
dA:dTTP	0.48 ± 0.04	2.10 ± 0.10	229
dC:dGTP	0.43 ± 0.03	2.07 ± 0.02	208
dA:8odGTP	0.11 ± 0.01	102.3 ± 5.46	1.08

[a] The value after the ± is the standard deviation ($n = 3$).

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presence of baseline levels of 8odGTP and PolIV and in the absence of oxidative stress.

To elucidate the mechanism utilized by PolIV to incorporate 8odGTP, the structure of PolIV in complex with DNA presenting template dA and incoming 8odGTP was determined (Table 2 and Figure S1 in the Supporting Information).

Table 2: Data collection and Refinement statistics.[a]

Data Collection	$PolIV_{dA:8odGTP}$
Wavelength [Å]	1.0
Space group	$P2_1$
Cell [Å] dimensions	86.5, 57.4, 110.6
[°]	90.00, 94.51, 90.00
Resolution [Å]	2.1 Å (2.21–2.10 Å)[b]
Rpim	3.7 (32.5)
$I/\delta I$	11.2 (2.4)
Completeness [%]	99.8 (100)
Redundancy	4.4 (4.4)
Refinement	
Resolution [Å]	41.07–2.1
No. Reflections	63 388
R_{work}/R_{free}	23.1/27.3
No. of atoms	
Protein	5376
DNA	1356
8odGTP	32
Ion	1
Water	442
RMSD	
Bond lengths [Å]	0.003
Bond angles [°]	0.850

[a] Coordinates and structure factors have been deposited in the PDB with accession code 5C5J ($PolIV_{dA:8odGTP}$). [b] Highest resolution shell is shown in parentheses.

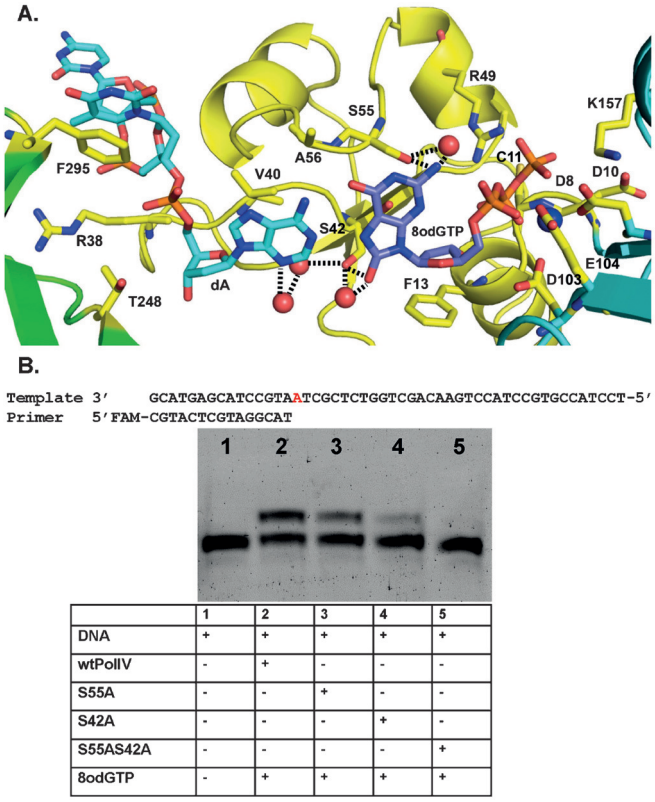

Figure 2. A) A close-up of the structure of the active site shows that the dA:8odGTP base pair adopts an *anti*:*syn* Hoogsteen configuration. The S42 and S55 residues form critical interactions with the 8odGTP to stabilize the base in the *syn* conformation. B) Primer-extension assays clearly show that the mutations S42A and S55A reduce the ability of PolIV to add 8odGTP opposite template dA. In the case of the double mutant, the ability to add 8odGTP opposite template dA is completely abolished.

The $PolIV_{dA:8odGTP}$ shows the presence of a Hoogsteen base pair in the active site (Figure 2A and Figure S2). The base moiety of 8odGTP adopts the *syn* conformation and presents its Hoogsteen edge for base pairing with the adenine base of the template nucleotide. Hydrogen bonds are formed between the N7 and N3 atoms and the N6 and O4 atoms of dA and 8odGTP, respectively. The C1′–C1′ distance between the two nucleotides in the nascent base pair is 10.8 Å and is similar to that observed for canonical Watson–Crick base pairs (Figure S4). The 8odGTP:dATP base pair is known to adopt a *syn*:*anti* Hoogsteen base configuration in the active site of different DNA polymerases.[8] However, the *anti*:*syn* Hoogsteen base pair for dA:8odGTP has been observed previously only in the case of DNA polymerase beta.[9]

The *anti* to *syn* transition occurs to prevent repulsion between the oxygen atom at the 8 position of the base and the α-phosphate of 8odGTP. Due to this transition, the C1′–C1′ distance of the nascent dA:8odGTP base pair is similar to that for a canonical Watson–Crick base pair and hence this mismatched base pair can fit in the polymerase active site in a conformation compatible with productive catalysis.

In the *syn* conformation, the N2 atom of 8odGTP is oriented towards the major groove and the O8 atom is present in the minor groove. The N2 atom forms direct (3.4 Å) and water-mediated hydrogen bonds with the side-chain hydroxy group of the S55 residue. Also, the O8 atom forms direct (3.4 Å) and water-mediated hydrogen bonds with the side chain hydroxy group of the S42 residue (Figure 2A). These two interactions, one present towards the major groove and another in the minor groove, stabilize the oxidized base in the *syn* conformation and ensure productive base pairing with template dA. S42 and S55 are conserved in all close orthologues of the PolIV enzyme (including those from pathogenic bacteria) and this suggests that the ability and mechanism utilized to incorporate 8odGTP into DNA may be common to these enzymes (Figure S5).

Primer extension assays were carried out with mutant enzymes to validate the importance of the S55 and S42 residues. Three mutant enzymes were utilized, one with an S42A mutation, another with an S55A mutation, and the third harboring both these mutations. The assays showed that the ability of PolIV to add 8odGTP to the primer was reduced in the single mutants and nearly abolished in the double mutant (Figure 2B). These experiments show that the interactions formed between S42 and S55 with the oxidized base are vital for the ability of PolIV to incorporate 8odGTP into the genome.

The observation that mutation of S42 and S55 to Ala drastically reduced the ability of PolIV to incorporate

8odGTP into DNA provided us with an opportunity to shed light on the role of ROS in antibiotic action. If antibiotics promote the production of ROS and incorporation of the oxidized nucleotide pool into genomic DNA by PolIV factors, thereby leading to lethality, then selective perturbation of this activity of PolIV should lead to enhanced survival in the presence of oxidizing agents and antibiotics. PolIV is coded for by the *dinB* gene in *E. coli*. To test this hypothesis, a *dinB*-deleted strain of *E. coli* was transformed with a plasmid bearing wild type (wt) and mutant versions of the *dinB* gene. The transformed bacterium was grown in the presence and absence of oxidizing agents such as H_2O_2 and a quinolone antibiotic such as norfloxacin. Three mutant versions of the *dinB* gene were tested, one bearing the S42A mutation, another with the S55A mutation, and a third with both S42A and S55A mutations.

Treatment with oxidizing agents such as H_2O_2 is known to enhance the concentration of ROS within the cell and cause damage to cellular physiology.[10] We used H_2O_2 as a source of oxidative stress to test the role of PolIV in mediating the deleterious effects of ROS. Our results clearly show that cell survival is reduced when PolIV is expressed in the presence of H_2O_2 (Figure 3 and Figure S6). The observation that perturbation of the 8odGTP incorporation activity through site-specific mutations (S42A and S55A) considerably improves survival in the presence of oxidative stress further substantiates the role of PolIV in cell lethality owing to ROS.

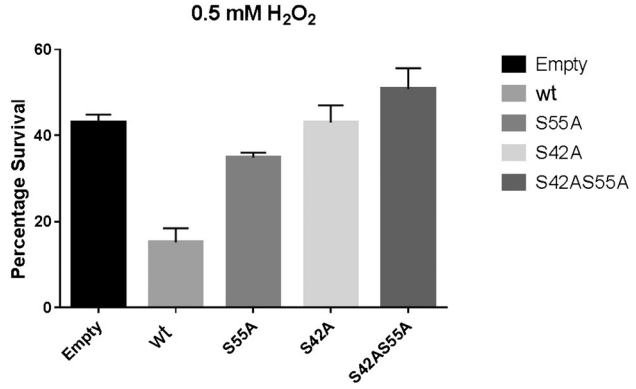

Figure 3. Survival of the *dinB*$^-$ strain in the presence of oxidative stress. The bacteria were transformed with empty plasmid, plasmid bearing wt *dinB*, or mutant versions of *dinB* (S42A, S55A, and S42AS55A).The data are shown for 0.5 mM H_2O_2 with standard deviation ($n=3$). The growth measurements were taken at 3 hours post-treatment. The ANOVA statistical test for this dataset gave $p<0.0001$ and $F=49.3$.

Based on these observations, we tested the effect of norfloxacin antibiotic on the survival of bacteria bearing wt and mutant versions of *dinB* (Figure 4 and Figure S7). Norfloxacin is a quinolone antibiotic that exerts its bactericidal effect through the inhibition of DNA gyrases.[1] The survival of bacteria transformed with wt *dinB* is reduced considerably in the presence of norfloxacin. It is known that PolIV can incorporate oxidized nucleotides into the genome and that this can cause death in bacteria.[5b] The observed

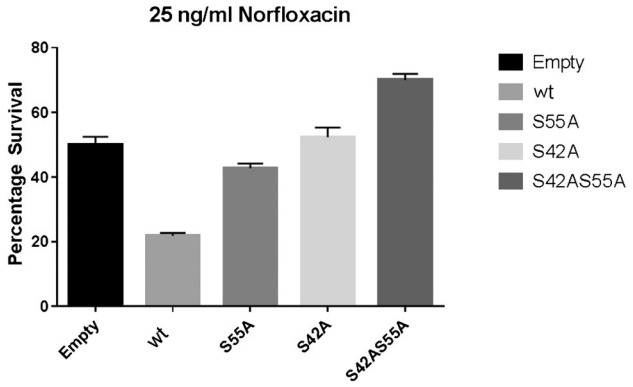

Figure 4. Survival of the *dinB*$^-$ strain in the presence of norfloxacin. The bacteria were transformed with empty plasmid, plasmid bearing wt *dinB* or mutant versions of *dinB* (S42A, S55A, and S42AS55A). The data is shown for 25 ng mL^{-1} norfloxacin with standard deviation ($n=3$). The growth measurements were taken at 3 hours post-treatment. The ANOVA statistical test for this dataset gave $p<0.0001$ and $F=206.6$.

reduction in cell survival upon norfloxacin treatment therefore occurs in part due to an increase in the oxidized nucleotide pool within the cell, and this can only happen due to an increase in ROS levels. Norfloxacin treatment therefore leads to a rise in ROS levels in bacteria. The increased survival of the bacteria transformed with mutant genes of *dinB* clearly shows that elimination of the ability of PolIV to incorporate the oxidized nucleotide pool results in a considerable reduction in the antimicrobial activity of this antibiotic. These observations corroborate the view that ROS contribute substantially to cell death in response to antibiotics.

Overall, our results provide strong evidence that ROS play an important role in the antimicrobial activity of quinolone antibiotics. In addition, the reduction in cell survival of bacteria lacking the *dinB* gene in the presence of norfloxacin (Figure 4) maybe due to the incorporation of 8odGTP by DNA Polymerase V ($UmuCD_2'$)[5b,11] and inhibition of the gyrase enzyme by the antibiotic. Therefore, different activities of the norfloxacin antibiotic contribute to cell death and the ability to induce ROS represents one of the major mechanisms to generate cell lethality in prokaryotes. Our studies also provide a platform for identifying small-molecule ligands that selectively enhance 8odGTP incorporation by PolIV and thus potentiate the antimicrobial activity of known antibiotics.

Acknowledgements

We thank the X-ray diffraction facilities located in the Molecular Biophysics Unit of the Indian Institute of Science and at the Regional Centre for Biotechnology (RCB). DTN thanks Dr. Hassan Belrhali and Dr. Babu Manjashetty (BM14 beamline, ESRF). The research was funded by RCB and NCBS-TIFR. JK is a recipient of Senior Research Fellowship (SRF) from CSIR (Govt. of India). Data collection at the BM14 beamline of ESRF (Grenoble, France) was funded by

the BM14 project, a collaboration between DBT, EMBL, and ESRF.

Keywords: antibiotics · DNA damage · DNA replication · oxidation · reactive oxygen species

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 2397 – 2400
Angew. Chem. **2016**, *128*, 2443 – 2446

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Received: October 6, 2015
Revised: November 23, 2015
Published online: January 12, 2016

