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Potential roles for DNA replication and repair functions in cell killing by streptomycin

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Abstract

The aminoglycoside streptomycin binds to ribosomes to promote mistranslation and eventual inhibition of translation. Streptomycin kills bacteria, whereas many other non-aminoglycoside inhibitors of translation do not. Because mistranslation is now known to affect DNA replication, we asked if hydroxyurea, a specific inhibitor of DNA synthesis, affects killing, and find that hydroxyurea significantly attenuates killing by streptomycin. We find that the hydroxyl radical scavengers D-mannitol and thiourea have either no effect or only a modest protective effect. The iron chelator 2,2'-dipyridyl eliminated killing by streptomycin, but further investigation revealed that it blocks streptomycin uptake. Prior treatment of cells with low-levels of methyl methanesulfonate to induce the adaptive response to alkylation leads to a significant attenuation of killing, which, together with the hydroxyurea effect, suggests roles for DNA replication and repair functions in cell killing by streptomycin.

Keywords

mistranslation; DNA replication and repair; dipyridyl; antibiotic uptake; hydroxyl radicals; DNA alkylation damage; alkB

1. Introduction

Streptomycin, like other aminoglycoside antibiotics, binds to the 30S subunit of the bacterial ribosome [1], and leads initially to mistranslation, and ultimately to a complete inhibition of translation. Many non-aminoglycoside antibiotics such as chloramphenicol also lead to a complete inhibition of translation. However, even a brief exposure to streptomycin is lethal [2–5] to *Escherichia coli*, whereas prolonged exposure to chloramphenicol or spectinomycin does not lead to significant loss of viability. The mechanisms for cell killing by streptomycin have remained elusive despite a number of hypotheses such as the membrane-damage and ribosomal blockade hypothesis [6], and more recently, the hydroxyl radical hypothesis [7, 8]. While hydroxyl radical generation as a universal mechanism of killing by antibiotics is

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an attractive model, follow-up investigations failed to demonstrate an unequivocal role for hydroxyl radicals in killing by aminoglycosides and other antibiotics [9, 10].

We became interested in potential mechanisms of cell killing by streptomycin because of our previous work on the unexpected effects of mistranslation on DNA replication [11–22]. *E. coli* cells in which mistranslation is elevated due to expression of missense mutant tRNA genes, ribosomal ambiguity mutations, or exposure to sublethal levels of streptomycin display an intriguing mutator phenotype termed translational stress-induced mutagenesis (TSM). According to current understanding, TSM is mediated by a corruption of DNA synthesis apparatus by recruitment of mistranslated protein subunits of DNA polymerase III, the replicative polymerase of *E. coli*. Cells undergoing aberrant DNA synthesis are prone to concurrent replication fork collapse as indicated by SOS induction in recombination-proficient cells [12], and inviability in recombination-deficient cells [12, 20]. We therefore asked if compromised DNA synthesis could conceivably play a role in cell killing by streptomycin. We found evidence consistent with roles for misreplication and DNA damage in cell killing by streptomycin.

2. Materials and methods

E. coli K12 strains strain MG1655 was from W. Reznikoff. MZH472 [MG1655 *metEo-3079::Tn10* (Tet^R) *polA1* (Am)] was constructed by P1 transduction from AM134 [17] into MG1655, selecting for Tet^R followed by screening for increased UV sensitivity (30% of Tet^R transductants). MZH473 [MG1655 *metEo-3079::Tn10* (Tet^R) *polA*⁺] was constructed as described for MZH472, except that strains with wild type level of UV sensitivity were chosen (70% of Tet^R transductants). All cell growth was in minimal medium A [23] at 37°C with shaking. For MZH472 and MZH473, the medium was supplemented with methionine (40 µg/ml). Hydroxyurea was from AK Scientific (Mountain View, CA); except as noted below, all other chemicals were from Fisher Scientific.

To measure killing by streptomycin, fresh overnight culture was diluted 1:100 in fresh medium and cells allowed to reach the midlog phase (2.5 to 3 h at 37°C with aeration). Streptomycin (Duchefa Biochemie) from frozen stock solution was added to a final concentration of 100 µg/ml (except when stated otherwise), and cultures were incubated at 37°C with aeration. Aliquots (0.1 ml) were withdrawn at the indicated time intervals, diluted immediately in sterile saline, and plated in duplicate on LB agar plates (or minimal A agar plates supplemented with methionine for the *polA1* strain MZH472). Colonies were counted after overnight growth at 37°C (LB plates) or 48 h (minimal A agar plates). Data were averaged from 3 to 5 experiments, with error bars representing standard deviation.

To evaluate streptomycin uptake, midlog cells were incubated with 100 µg/ml streptomycin spiked with ³H labeled dihydrostreptomycin (2.5 µCi/ml; 12–15 Ci/mmol; Vitrox Radiochemicals, Placentia, CA), followed by filtration and washing of cells to measure streptomycin accumulation as described below. At indicated time intervals, 1 ml aliquots were filtered on a Millipore Millex-GV (SLGV013SL) 0.22µ filters pre-wetted with 2 ml of a 2.5 mg/ml solution of streptomycin in minimal A medium, washed with 5 ml of 3% NaCl, and eluted with 1 ml of 5% trichloroacetic acid, followed by scintillation counting of 0.5 ml of the eluate. [Dihydrostreptomycin, whose bactericidal action and uptake are identical to those of streptomycin, has been extensively used to measure streptomycin uptake [24]].

3. Results and Discussion

To test if cell killing by streptomycin is affected in the presence of hydroxyurea, an inhibitor of DNA synthesis (but not of RNA or protein synthesis), we carried out assays in which *E. coli* MG1655 cells grown to midlog phase were pre-incubated (30 min at 37°C with

aeration) with hydroxyurea (200 mM) before adding streptomycin to 100 µg/ml. Hydroxyurea blocks DNA synthesis by inhibiting ribonucleotide reductase, which in turn leads to an eventual depletion of deoxyribonucleotides required for DNA synthesis. Fig. 1 shows that hydroxyurea led to a significant attenuation of killing by streptomycin over a 2 hour period. Adding hydroxyurea at the same time as streptomycin was less effective. Continued incubation with streptomycin beyond 2 hours resulted in a resumption of killing (data not shown). Prolonged exposure to hydroxyurea by itself was reported to kill cells by several proposed mechanisms [25–27]; however, we did not observe significant killing by hydroxyurea alone in the timeframe used in these studies (Fig. 1).

Because of recently hypothesized role of hydroxyl radicals in cell killing by aminoglycosides [7, 28, 29], and because hydroxyurea could conceivably attenuate streptomycin killing by acting as a weak hydroxyl radical scavenger, we also tested the effects of the much more efficient hydroxyl radical scavengers D-mannitol [30, 31] and thiourea [7, 30], as well as the iron chelator 2,2 -dipyridyl [7] on cell killing by streptomycin. In addition, because *polA1* cells (defective for DNA polymerase I, the “repair polymerase”) are known to be exquisitely sensitive to killing by hydroxyl radical-mediated DNA damage [32], we also examined the effect of streptomycin exposure in *polA1* (Am) cells. Pre-incubation (30 /37°C) or co-incubation with D-mannitol (10 mM) had little effect on cell killing (Fig. 2) even though at this concentration D-mannitol was previously shown to essentially eliminate oxidative DNA damage [30]. Pre-incubation (30 /37°C) with 200 mM thiourea led to a small reduction in killing (Fig. 2). Fig. 3 shows that exposure to streptomycin (100 µg/ml or 10 µg/ml) induced comparable killing in *polA1* and *polA*⁺ cells, indicating a lack of enhanced killing. We also did not observe enhanced sensitivity of *polA1* cells at streptomycin concentrations of 1 µg/ml (data not shown).

In contrast to the above results, pre-incubation (or co-incubation) with 2,2 -dipyridyl (1 mM), a reagent known to block Fenton chemistry because of its ability to chelate iron, effectively stopped streptomycin killing (Fig. 2). At the same time, 4,4 -Dipyridyl (CAS No. 553-26-4), a closely related chemical that is not a chelator [33, 34], did not attenuate streptomycin killing (data not shown), suggesting a correlation between chelating ability and protection against killing. Iron chelation by 2,2 -dipyridyl is hypothesized to block the Fenton reaction, disrupting the generation of lethal intracellular hydroxyl radical formation [35, 36]. As such, 2,2 -dipyridyl has been used by a number of investigators to evaluate the role reactive oxygen species in cell killing by antibiotics [7, 37, 38]. While the strong protection against streptomycin killing by 2,2 -dipyridyl is consistent with this idea, the limited or absent protective effect by the efficient hydroxyl radical scavengers thiourea and D-mannitol, and the lack of enhanced streptomycin sensitivity in *polA1* cells prompted us to ask if the protection afforded by 2, 2 -dipyridyl could be due to a non-specific factor such as interference with streptomycin uptake.

Fig. 4 show that streptomycin accumulation in *E. coli* MG1655 is eliminated in the presence of 1 mM 2,2 -dipyridyl, but not in the presence of 4,4 -dipyridyl, suggesting that the protection by 2,2 -dipyridyl against streptomycin killing may be mediated largely at the level of blocking uptake (or enhancing efflux). Thus, the ability to act as a chelating agent is strongly correlated with the ability to block streptomycin accumulation, and protection against killing. Although addition of 2,2 -dipyridyl to growth medium is known to trigger the iron limitation response [39] as well as certain multidrug resistance efflux pathways [40], how iron-chelation by 2,2 -dipyridyl blocks streptomycin uptake (or enhances efflux) remains to be investigated. These data warrant caution in using 2,2 -dipyridyl to evaluate the role of oxygen radicals in cell killing by antibiotics. Whereas 2,2 -dipyridyl essentially abolishes streptomycin uptake, pre-incubation with hydroxyurea leads to a reduced but still substantial accumulation of streptomycin (Fig. 4): at 2 hours in the presence of hydroxyurea,

92.8 ng of streptomycin accumulated in 4×10^7 cells, which is equivalent to 48 molecules of streptomycin per ribosome (assuming 20,000 ribosomes per cell), suggesting a different mechanism for protection by hydroxyurea.

Since thiourea appeared to partially protect from streptomycin killing, and the effect of 2,2-dipyridyl as a hydroxyl radical scavenger could not be tested in cells due to its effect on streptomycin uptake, we sought to address the question of a role for oxygen radicals by a different approach. Exposure to hydrogen peroxide is known to induce a large number of genes with disparate functions that are collectively believed to defend against oxygen radical toxicity. If oxygen radicals contributed to killing by aminoglycosides, one anticipates pre-treatment of cells with low-levels of hydrogen peroxide might protect them from killing by streptomycin. Fig. 5 shows that pretreatment with 0.1 mM hydrogen peroxide does reduce killing by streptomycin to some extent. Hydrogen peroxide induces a large number of genes that are *oxyR*-dependent as well as *oxyR*-independent [41]. Due the pleiotropic effects of hydrogen peroxide exposure, it is hard to rule out non-specific causes for the observed moderate levels of protection, but these results are also consistent with a contributory role for oxygen radicals in killing, as considered elsewhere below.

While this work was in progress, Kang *et al.* reported that *E. coli* cells deficient for *alkB*, a DNA repair gene, are much more sensitive to the aminoglycoside kanamycin compared to wild type cells [42], suggesting a potential role for DNA damage in cell killing by aminoglycosides. Since *alkB* is a part of the adaptive response to alkylation [43, 44], prior exposure to very low-levels of alkylating agents induces the expression of *alkB* as well as other member genes of the adaptive response regulon [45]. We therefore asked whether such pretreatment could attenuate killing by streptomycin. Fig. 5 shows that pre-treatment of cells with 0.04% methyl methanesulfonate (MMS) significantly attenuates killing by streptomycin. This finding confirms and complements the results of Kang *et al.* in implicating a role for DNA damage in aminoglycoside killing. Exocyclic DNA adducts such as ethenoadenine are known to be induced by products of lipid peroxidation [46], leading Kang *et al.* to speculate that aminoglycosides promote membrane lipid peroxidation resulting in the formation of exocyclic adducts targeted by AlkB [42]. However, it should be noted that the range of target lesions repaired by AlkB includes the potent replication-blocking methylated bases 1-methyladenine and 3-methylcytosine, which are formed when endogenous (or exogenous) alkylating agents attack single-stranded DNA [47–49]. AlkB, which oxidatively excises N-alkyl groups without cutting the DNA strand (“direct repair activity”), is a dioxygenase that uses non-heme iron(II) as a co-factor and α -ketoglutarate as a co-substrate, and preferentially binds to, and acts on, single-stranded DNA [47, 50, 51]. A protective effect of AlkB implies that structural changes in DNA must occur in streptomycin-exposed cells such as to enable infliction of single-strand specific DNA damage regardless of the source of the chemical agents responsible for the damage.

Hydroxyurea, which specifically blocks DNA synthesis without affecting protein or RNA synthesis [52, 53], attenuates cell killing by streptomycin (Fig. 1). This effect of hydroxyurea is consistent with the possibility that active DNA synthesis in the initial stages of exposure is a prerequisite for cell killing by streptomycin. Integrating the observations in this report with published information on cellular effects of streptomycin and other aminoglycosides, current understanding of DNA damage and repair pathways, as well as the hitherto under-appreciated effects of mistranslation on DNA replication [11–22], we suggest the following minimal model. Because DNA synthesis continues for a significant period of time after exposure to streptomycin, as recognized early [54–56], it is conceivable that as mistranslation continues, ongoing DNA synthesis is increasingly corrupted by the recruitment of mistranslated replication protein subunits leading to replication fork collapse and accumulation of unreplicated single-stranded DNA [12], which becomes vulnerable to

lethal damage. The damaging agents could be both lipid peroxidation products (regardless of whether their formation occurs at basal or enhanced levels), and endogenous alkylating agents such as S-adenosylmethionine or products of enzymatic nitrosation of amino acid derivatives [47–49]. Such a possibility has the potential to reconcile paradoxes such as partial protection by thiourea [7], by glutathione [29], or by induction of the oxidative response (Fig. 5) in the absence of consistent evidence for the induction of oxygen radicals by aminoglycosides [9, 10, 29]. Although complete blockade of protein synthesis does occur in streptomycin-exposed cells in the end, it is unlikely to be the critical determinant in cell killing because complete inhibition of protein synthesis also occurs in *E. coli* cells exposed to chloramphenicol (a non-aminoglycoside inhibitor of translation), but chloramphenicol blockade does not kill, merely leading to cell stasis (i.e., complete recovery when the chloramphenicol is removed). Because chloramphenicol blocks replication re-initiation without affecting ongoing DNA synthesis, the critical difference in streptomycin-exposed cells would be a disruption of DNA elongation leading to an accumulation of unreplicated single-stranded DNA that becomes vulnerable to lethal DNA damage.

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Fig. 1.

Effect of hydroxyurea on streptomycin killing of *E. coli* MG1655 cells. Str (diamonds), midlog cells incubated with 100 μ g/ml streptomycin; HU+Str (squares), cells pre-incubated for 30 at 37°C with 200 mM hydroxyurea (freshly made or from a frozen stock) before addition of streptomycin to 100 μ g/ml; HU (triangles), as in HU+Str, except that no streptomycin was added at the end of the pre-incubation period.

Fig. 2. Effect of anti-oxidants on streptomycin killing of *E. coli* MG1655 cells. Str+DP (diamonds), midlog cells pre-incubated with 1 mM 2,2 -dipyridyl and 100 µg/ml streptomycin; Str+TU (squares), midlog cells pre-incubated for 30 at 37°C with 200 mM thiourea (freshly made) before addition of streptomycin to 100 µg/ml ; Str+Man (triangles), midlog cells pre-incubated for 30 at 37°C with 10 mM D-mannitol before addition of streptomycin to 100 µg/ml.

Fig. 3. Streptomycin killing in *E. coli* cells defective for DNA polymerase I. Cells were grown in minimal A medium (supplemented with methionine for MZH472 and MZH473) to midlog phase, and were incubated at 37°C with either 10 µg or 100 µg/ml streptomycin as indicated. Diamonds, MZH473 (*polA*⁺, 10 µg); squares, MZH472 (*polA*⁻, 10 µg); circles, MG1655 (*polA*⁺, 10 µg); triangles, MZH472 (*polA*⁻, 100 µg); asterisks, MG1655 (*polA*⁺, 100 µg).

Fig. 4. Streptomycin accumulation in *E. coli* MG1655 cells the presence of 1 mM 2,2 -dipyridyl (DP+Str), 1 mM 4,4 -dipyridyl (4,4 DP+Str), or 200 mM hydroxyurea (HU+Str); Str, control with no additives. Midlog cells were pre-incubated with the indicated additives for 30 at 37°C before adding streptomycin to 100 µg/ml and ³H-dihydrostreptomycin (see text). Whereas 2,2 -dipyridyl is a strong iron chelator, 4,4 -dipyridyl is a non-chelator due to the unfavorable placement of the Nitrogen atoms.

Fig. 5. Streptomycin killing in *E. coli* MG1655 cells induced for adaptive response to alkylation (MMS) or for oxidative response to hydrogen peroxide (H_2O_2). Str (diamonds), midlog cells incubated with 100 μ g/ml streptomycin; MMS+Str (squares), overnight MG1655 cultures were diluted 1:100 in minimal medium A containing 0.04% MMS and cells allowed to grow with aeration at 37°C to midlog phase (about 3 h) before adding streptomycin to 100 μ g/ml. The concentration of MMS and exposure times were chosen for optimal induction of AlkB protein with minimal effects on growth [57, 58]. H_2O_2 +Str (triangles), overnight MG1655 cultures were diluted 1:100 in minimal medium A containing 0.1 mM H_2O_2 and cells allowed to grow with aeration at 37°C to midlog phase (about 3 h) before adding streptomycin to 100 μ g/ml. At an H_2O_2 concentration of 0.1 mM, a slight reduction in growth was observed, whereas at 1 mM H_2O_2 , growth was noticeably reduced, and there was much less protection against streptomycin killing (not shown) as compared to protection conferred by 0.1 mM H_2O_2 .