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Use of Drosophila Deoxynucleoside Kinase to Study Mechanism of Toxicity and Mutagenicity of Deoxycytidine Analogs in *Escherichia coli*

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Abstract

Most bacteria, including *Escherichia coli*, lack an enzyme that can phosphorylate deoxycytidine and its analogs. Consequently, most studies of toxicity and mutagenicity of cytosine analogs u se ribonucleosides such as 5-azacytidine (AzaC) and zebularine (Zeb) instead of their deoxynucleoside forms, 5-aza-2'-deoxycytidine (AzadC) and 2'-deoxy-zebularine (dZeb). The former analogs are incorporated into both RNA and DNA creating complex physiological responses in cells. To circumvent this p roblem, we introduced into *E. coli* the *Drosophila* deoxynucleoside kinase (*Dm*-dNK), which has a relaxed substrate specificity, and tested these cells for sensitivity to AzadC and dZeb. We find that *Dm*-dNK expression increases substantially sensitivity of cells to these analogs and dZeb is very mutagenic in cells expressing the kinase. Furthermore, toxicity of dZeb in these cells requires DNA mismatch correction system suggesting a mechanism for its toxicity and mutagenicity. The fluorescence p roperties of dZeb were used to quantify the amount of this analog incorporated into cellular DNA of mismatch repair-deficient cells expressing *Dm*-dNK and the results showed that in a mismatch correction-defective strain a high percentage of DNA bases may be replaced with the analog without long term toxic effects. This study demonstrates that the mechanism by which Zeb and dZeb cause cell death is fundamentally different than the mechanism of toxicity of AzaC and AzadC. It also opens up a new way to study the mechanism of action of deoxycytidine analogs that are used in anticancer chemotherapy.

Keywords

Anti-cancer chemotherapy; antimetabolite chemotherapy; Zebularine; Decitabine

1. Introduction

Analogs of purines and pyrimidines are some of the oldest anti-metabolities used to treat cancer. Since the introduction of the purine analogs 6-mercaptopurine and 6-thioguanine as anticancer

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agents, a number of purine and pyrimidine analogs have been developed that act upon specfic cancers [1]. These include 5-fluorouracil (5FU; commercial name Adrucil®), cytosine arabinoside (cytarabine; Cytosar -U®), 6-amino-2-fluoro-purine arabinoside 5'-monophosphate (fludarabine; Fludara®) and 5-aza-2'-deoxycytidine (decitabine; Dacogen®) and each affects multiple metabolic pathways and has complex physiological effects. It is clear that some of these analogs are phosphorylated in cells and sometimes incorporated into cellular RNA and DNA. Furthermore, their useful clinical effects are frequently due to their interactions with enzymes in nucleotide metabolism such as thymidylate synthase, DNA polymerases and DNA-cytosine methyltransferases [2].

Two cytosine analogs of this type are 5-azacytosine and 2-H pyrimidinone (Z-base). These analogs have been used in both their ribonucleoside and deoxyribonucleoside forms (Fig. 1) and their biological effects have been studied in *E. coli*, mammalian tissue culture and animals. In particular, treatment of cells with 5-azacytidine (AzaC) leads to incorporation of this cytosine analog into DNA as well as in RNA. Its incorporation into DNA causes covalent linking of the DNA-cytosine methyltransferases (DNMTases) to DNA and subsequent demethylation [3]. Additionally, presence of these protein-DNA adducts in the genome has been shown to block DNA synthesis [4] and is thought to be resposible for the cytotoxic effects of this analog. AzaC is also mutagenic in different genetic systems [5–7]. Cytotoxic and mutagenic effects of 5-aza-2'-deoxycytidine (AzadC) in mammalian cells have also been reported and these appear to be due to the formation of DNA methyltransferase-DNA adducts [8,9].

The mechanism by which 2-H pyrimidinone ribonucleoside (zebularine, Zeb) causes cell death is less well understood, but this ribonucleoside is highly toxic to *E. coli* [10]. Synthetic DNA oligomers containing Z-base form heat-stable, detergent-resistant complexes with a DNMTase [11] and crystallographic studies show that the enzyme forms a covalent complex with DNA in a manner similar to that seen with AzaC and 5-fluorocytosine [3,12]. The mechanisms by which Zeb-Mtase adducts may be repaired *in vivo* has not been studied and it is not known whether these adducts are the cause of Zeb toxicity.

Both AzaC and Zeb are mutagenic [5–7,10,13–15], but the mechanisms underlying their mutagenicity is poorly understood. AzaC is strongly mutagenic towards DNA repair -proficient *E. coli* and causes predominantly C:G to G:C transversions [5,7,15] which are dependent on SOS mutagenesis in Salmonella [13]. The mutagenicity of AzaC is somewhat higher in cells lacking nucleotide excision and mismatch repair functions, but does not depend on the presence of chromosomally coded DNA-cytosine methyltransferase, Dcm, in cells [5]. In contrast, Zeb causes mostly C:G to T:A transitions in a RecA-independent manner and its mutagenicity is significantly higher in cells defective in mismatch repair [10]. It is not known whether its mutagenicity depends on the presence of Dcm in cells.

The study of deoxy forms of these analogs, AzadC and dZeb (Fig. 1) in the simpler bacterial systems is hampered by the fact that in these organisms dNTPs are synthesized *de novo* from their ribonucleotide precursors. In particular, all dNTPs except TTP are derived from dNDPs which in turn are synthesized from the corresponding NDPs by the enzyme ribonucleotide reductase [16]. In *Escherichia coli*, *Salmonella* and most other bacteria, the salvage pathway for deoxycytidylate biosynthesis also goes through a CDP intermediate. This is because most bacteria lack a kinase that can phosphorylate dC to dCMP [Fig. 2; [17,18]]. This peculiarity of bacterial metabolism forces investigators who wish to study biological effects of incorporation of analogs of cytosine in bacterial DNA to use base analogs or ribonucleoside forms of the analogs. In both cases, the analogs are incorporated into both RNA and DNA (Fig. 1) and thus the biological effects of these analogs result from changes in both types of nucleic acids. For example, incorporation of cytosine analogs in RNA can have effects on protein

synthesis, RNA stability or RNA synthesis leading to toxic effects. Consequently, including cytosine or cytidine analogs in the growth media may not be the best way to study the effects of cytosine base modifications on bacterial DNA metabolism. We describe below a way to overcome this shortcoming using a nucleoside kinase from insects.

Drosophila melanogaster contains a single deoxynucleoside kinase (*Dm*-dNK) that is related to mammalian thymidine kinase 2 [19,20]. *Dm*-dNK has a significant preference for pyrimidine deoxynucleosides over purine deoxynucleosides, but it does phosphorylate dA and dG at significant rates [21]. The broad substrate specificity of this enzyme is also demonstrated by the fact that a variety of pyrimidine analogs compete well with normal deoxynucleosides for phosphorylation by the enzyme [21] and its expression in mammalian cells makes them [22] sensitive to a number of analogs of purine and pyrimidine deoxynucleosides including 2-chloro-2'-deoxyadenosine, and 2'-deoxy-2',2'-difluorocytidine [Gemcitabine, [22]]. Previously, human dNK gene was introduced into *E. coli* and the cells were tested for sensitivity towards some pyrimidine analogs. However, that study did not use AzadC, Zeb or dZeb and did not reach detailed conclusions regarding the mechanisms of toxicity of different analogs [23]. We demonstrate here that expression of *Dm*-dNK in *E. coli* enhances the ability of cells to incorporate AzadC and dZeb into its DNA and this helps elucidate the mechanism of toxicity of these anticancer drugs.

2. Materials and Methods

2.1 Bacterial Strains and Plasmids

All the strains used were *E. coli* K-12 derivatives and reasons for the use of specific strains in specific experiments is explained in the Results section. The relevant genotypes of the strains are- SE5000: F^- *araD139*Δ (*argF-lac*)U169 *rpsL150 recA56*; CC107: *ara* Δ (*lac-proB*)XIII/ F' *lacI lacZ461-7 proB*$^+$; GM30: *thr1 hisG4 leuB6 rpsl ara14 supE44 lacY1 tonA31 tsx78 galK2 galE2 xyl5 thi1 mtl1*; GM31 is GM30 *dcm6*; BH181 is GM30 *mutL218*::Tn*10* and BH256 is GM31 *mutL218*::Tn*10*.

The plasmid pDm-dNK [21] is derived from a pGEX vector that contains *Drosophila* deoxynucleoside kinase (dNK) gene cloned under the control of the *tac* promoter and was a generous gift from Dr. A. Karlsson (Karolinska Institute, Stockholm, Sweden). It contains the $lacI^Q$ gene and codes for carbenicillin-resistance. When pDm-dNK was used in experiments, the plasmid pGEX-4T-3 (GE Healthcare) was used as a negative control.

2.2 Toxicity Assays

All bacterial strains were grown in M9 minimal media supplemented with Casamino acids. When cells contained pDm-dNK or pGEX-4T-3, Carbenicillin was added to the liquid growth media or plates at 50 μg/ mL. Cultures were grown overnight and diluted 1000-fold and divided into three or more cultures. They were grown to OD_{600} of ~0.1 and AzaC, AzadC, Zeb or dZeb were added to the indicated final concentrations and growth was continued at 37°C for 5 hours. Cells were concentrated by centrifugation, the pellets were resuspended in LB media and various d ilutions were spread on LB plates with carbenicillin to assess cell survival. The cell survival reported below is the number of colony-forming units (cfu) per milliliter of original culture.

2.3 Rifampicin-resistance Assay

Cultures of CC107 with pDm-dNK or pGEX-4T-3 were grown in the same way as the toxicity assay described above. Preliminary experiments were done to determine toxicity of the drug at different concentrations and the results of these experiments were used to determine the volume of each culture (between 2 and 100 mL) at each concentration. The appropriate

concentration of dZeb was added to each culture and the incubation continued for five hours. Rifampicin-resistant (Rif^R) mutant frequency is the ratio, total number of cells on Rifampicin plates (100 μg/ mL) to total number of cells on Carbenicillin plates.

2.4 Preparation of plasmid DNA containing 2-H pyrimidinone

When plasmid DNA was to be isolated from cells treated with Zeb or dZeb, alkaline-SDS lysis method did not yield good results (see Results below). Consequently, a modification of the procedure described by Godson and Vapnek was used for this purpose [24]. Independent 20 milliliter cultures of GM31 (pDm-dNK) or BH256 (pDm-dNK) were treated with dZeb as described above for the toxicity assays. Following drug treatment, the cells were harvested by centrifugation and resuspended in 400 μL ice-cold 10% sucrose in 50 mM Tris-HCl (pH 8.0). Eighty microliters of freshly prepared solution of lysozyme (10 mg/ ml in 10 mM Tris -Cl, pH 8.0) was then added followed by the addition of 320 μL of 0.25 M EDTA (pH 8.0). The cell suspensions were kept on ice for 10 min and 160 μL of 10% SDS was added and mixed by stirring with a pipette tip. Two hundred microliters of 5 M NaCl was added and the mixture was kept on ice for 1 hr. The cell debris was removed by centrifugation and the supernatant was transferred to a new tube. The DNA preparations were extracted with Phenol:Chloroform followed by an extraction with Chloroform and the DNA was precipitated with ethanol. Following centrifugation the DNA pellets were dried and were dissolved in 120 μL TE (10 mM Tris-HCl, pH 7.8). The DNAs were treated further with RNase A (Sigma- Aldrich) at a final concentration of 10 μg/ mL for 45 minutes at room temperature and 10 units of RNAse H (New England Biolabs) at 37°C for 35 minutes, and passed through a Sephadex G-100 column (Sigma-Aldrich). The pDm-dNK and pGEX-4T3 plasmids were linearized with EcoRI (New England Biolabs), extracted with Phenol:Chloroform and DNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific) at 260 nm.

2.5 Fluorescence measurements

A 26 base pair synthetic DNA (**Z**-duplex) containing a single 2-H-pyrimidinone base (Z-base) was used to create a calibration curve for fluorescence measurements. The sequence of the oligomer was 5'–CCGAGTATCAGG**Z**GCTGACCCACCCCG, where **Z** is Z-base and was kindly provided by Dr. Ramon Eritja (Institute for Research in Biomedicine, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona, Spain). The complementary strand contained a G base across from **Z**. The Z-containing oligomer was annealed to its complement (molar ratio 1:3) in TE at concentration 15 μM. Several dilutions of the DNA solutions were prepared and fluorescence intensity of each was measured using a Cary Eclipse Spectrophotometer. Fluorescence measurements used the following parameters- excitation wavelength, 330 nm; emission wavelength, 415nm; excitation slit width - 10 nm; emission slit width- 10 nm, and PMT voltage- 800V. Fluorescence intensity (arbitrary units) was recorded at different DNA concentrations of **Z**-duplex.

Fluorescence of two μg of plasmid DNA from each plasmid preparation was diluted to final volume 120 μL in TE and fluorescence intensity was determined as described above. The concentration of Z-base in plasmid DNA was calculated as follows- fluorescence intensity of plasmid DNA from untreated control cells was subtracted from intensity of DNA from cells treated with Zeb or dZeb. This net intensity value was used to interpolate dZeb concentration using the standard plot created using the Z-duplex. Assuming each nucleotide to have molecular weight of 325, the total nucleotide concentration in each DNA sample of 120 μL was 51 μM. The ratio of concentration of dZeb to total nucleotide concentration was the number of Z-bases per DNA bases.

3. Results

3.1 Relative Insensitivity of *E. coli* to deoxynucleoside analogs of cytosine

We first confirmed that the lack of a deoxynucleoside kinase in *E. coli* results in a lower sensitivity to cytosine analogs in the deoxynucleoside form compared to the ribonucleoside form. It was shown previously that a *recA* strain of *E. coli*, SE5000, is highly sensitive to AzaC [25] and for this reason these cells were used for toxicity studies of 5-azacytidine and 2'-deoxy-5-azacytidine (AzaC and AzadC, Fig. 1) toxicity. The cells were grown in minimal medium and were treated with AzaC or AzadC during exponential growth and plated to determine cell survival. In separate experiments, strain CC107 was also grown in minimal media, the cells were treated with Zeb or dZeb (Fig. 1) and plated to determine cell survival. CC107 was chosen for the latter studies instead of SE5000 because this is the strain used in an earlier study involving Zeb [10]. The results are shown in Fig. 3.

SE5000 was moderately sensitive to AzadC. Treatment of cells with as little as 5 μg/ mL the cell density was lower by a factor of ~10 compared to untreated controls (Fig. 3A). As the untreated cultures double every 40–50 minutes during five hour growth, this difference in cell density could simply be a bacteriostatic effects of the drug. In any case, the cell density did not decrease any further when the concentration of AzadC was increased to 100 μg/ mL (Fig. 3A). In contrast, in AzaC treated cultures only ~1 in 10^4 cells was viable. This shows that AzaC is much more toxic to *recA E. coli* than AzadC.

A similar pattern of sensitivity for Zeb and dZeb were seen with CC107. These cells were quite sensitive to Zeb, but insensitive to dZeb. With as little as 1.5 μg/ ml Zeb in the growth medium, the cell density was reduced by a factor of ~50, while even 10 times this concentration of dZeb in the medium had little effect on cell growth (Fig. 3B). These data are consistent with previous reports that *E. coli* is unable to phosphorylate deoxycytidine and its analogs [17,18].

3.2 Effect of *Dm*-dNK on Sensitivity to Cytosine Analogs

Dm-dNK gene was introduced into SE5000 and CC107 on a plasmid, pDm-dNK, which expresses it as a fusion with Glutathione-*S*-transferase protein [21] and the sensitivity of these cells to AzaC and AzadC (strain- SE5000) or Zeb and dZeb (CC107) was studied. The *Dm*-dNK gene is expressed from a regulated promoter, but it was not induced to prevent possible toxic effects of *Dm*-dNK.

Presence of pDm-dNK in SE5000 increased the sensitivity of these cells to AzadC by two orders of magnitude. Similarly, introduction of pDm-dNK in CC107 made the cells ~1000-fold more sensitive to dZeb (Fig. 4). In contrast, *Dm*-dNK d id not increase further already high sensitivity of the two *E. coli* strains to corresponding ribonucleoside analogs (Fig. 4). Consequently, for cells containing pDm-dNK the ribo- and deoxy- forms of analogs were about equally toxic (Fig. 4; compare AzaC with AzadC and Zeb with dZeb). These results show that even basal expression of *Dm*-dNK in *E. coli* had a large effect on the toxicity of deoxycytidine analogs presumably because of their phosphorylation by *Dm*-dNK.

3.3 Mutagenicity of dZeb

Zeb is a known *E. coli* mutagen [10] and causes demethylation of DNA in mammalian cells [26], but much less is known about its mode of action compared to AzaC. For this reason, we decided to study biological effects of dZeb in greater depth. In particular, we tested dZeb for its mutagenic ability using the rifampicin-resistance (Rif^R) assay. Cells containing pDm-dNK or the empty vector were grown in the presence of different concentrations of dZeb for five hours and then plated for survival and for scoring Rif^R colonies. The mutant frequencies are shown in Table 1.

As expected, dZeb was not toxic to *E. coli* containing the empty vector, and it increased only marginally the Rif^R mutant frequency in these cells. In contrast, the deoxyribonucleoside was toxic and mutagenic towards cells expressing *Dm*-dNK in a concentration-dependent manner (Table 1). Notably, at the highest concentration tested, 0.5 μg/ ml (2.4 μM), dZeb decreased the viable cell number by ~100-fold, while increasing mutant frequency by ~7000-fold. Therefore, under conditions where cells are able to phosphorylate dZeb and incorporate it into their DNA, this analog is highly mutagenic and cytotoxic.

3.4 Role of Dcm Methylation in dZeb Toxicity

Zeb and dZeb are known methylation inhibitors, and when the Z -base is incorporated in DNA it forms covalent complexes with DNMTases [26]. Hence we wondered whether chromosomally-coded Dcm methylase in *E. coli* plays a role in sensitivity of cells to dZeb. To test this, pDm-dNK was introduced in GM30 (genotype- dcm^+) and its otherwise isogenic *dcm* derivative, GM31, and both strains were tested for sensitivity to dZeb. These two strains were chosen for these studies partly because they have been used extensively in our previous work and we already had a *mutL* derivative of GM31 available for some of the work (see below).

GM30 carrying pDm-dNK was no more sensitive to dZeb than GM31 carrying the same plasmid. When cells were treated with 0.5 μg/ mL of the drug, cell survival was reduced to 1.9% (S.D.± 4.1%; Zeb) or 1.0% (± 0.02%; dZeb) compared to untreated controls. Therefore, the state of cytosine methylation or presence of Dcm in cells does not play a significant role in toxicity of dZeb towards cells that are capable of phosphorylation of this analog.

3.5 Role of Mismatch Correction in dZeb Toxicity

As DNA methylation d id not appear to play a role in dZeb toxicity, we examined next whether dZeb was being rapidly excised from DNA by repair processes creating strand breaks and these breaks were the cause underlying high toxicity of this analog. We tested this possibility in the context of DNA mismatch repair (MMR) by comparing sensitivity of strains GM31 (genotype- $mutL^+$, phenotype MMR^+) and BH256 (*mutL*; MMR^-) to dZeb when *Dm*-dNK was present in cells. Both strains are *dcm* eliminating any complicating issues involving Dcm.

Like CC107, GM31 cells were insensitive to dZeb unless pDm-dNK plasmid was present in cells. Adding dZeb to the growth media at as low a concentration as 0.5 μg/ mL reduced viability of GM31 cells containing *Dm*-dNK by a factor of ~18 (Fig. 5). At a concentration of 1.0 μg/ mL even greater cytotoxic effects were seen (data not shown). In contrast, BH256 cells carrying pDm-dNK were quite insensitive to dZeb (Fig. 5). In this case the cell density was lowered by only ~25% following dZeb treatment and this was within the variation of the data set. Similar results were also seen at higher dZeb concentrations (data not shown) and demonstrate that MMR plays a key role in causing cell death following dZeb treatment.

3.6 Quantification of dZeb in DNA

In order to directly demonstrate that dZeb is incorporated into cellular DNA, we made several unsuccessful attempts to isolate plasmid DNA from CC107 or GM31 cells containing pDm-dNK following dZeb treatment. When standard alkaline-SDS method of plasmid purification (Qiagen QIAprep Spin Miniprep Kit) was used, the yields were low and the DNA was seen to be degraded when separated by agarose gel electrophoresis (data not shown). Consequently, we switched to a plasmid preparation method that did not involve alkaline denaturation of DNA [24] and obtained consistently high quality DNA. We prepared plasmid DNA from analog-treated GM31 or BH256 cells carrying pDm-dNK using this method and used this DNA for quantification of dZeb incorporation in cellular DNA. One concern regarding subsequent quantification of dZeb in DNA was that if this nucleoside was broken down to Z-base inside

cells, it could get incorporated into RNA and this may complicate data interpretation. To avoid this, plasmid DNA was treated extensively with ribonucleases to degrade any contaminating RNA and then subjected to gel filtration to remove small RNA molecules.

To quantify dZeb, we used fluorescence properties of this base analog [27]. First, fluorescence intensity of different concentrations of a 26-mer duplex (**Z**-duplex) containing a single zebularine base was used create a calibration curve. The fluorescence intensity was linearly related to concentration of **Z**-duplex (see Supplementary data). Subsequently, fluorescence intensities of two micrograms of each DNA were determined for three independent preparations of plasmid DNAs and these data are shown in Figure 6.

The fluorescence intensity was higher than untreated controls only for DNA from cells expressing *Dm*-dNK and which had been treated with dZeb. Furthermore, this was true only in cells defective in MutL function; i.e. defective in mismatch repair. In a $MutL^+$ strain there was no statistically significant increase in DNA fluorescence following dZeb treatment (Fig. 6). These results confirm that incorporation of dZeb in cellular DNA requires phosphorylation of this analog by Dm-dNK and MMR process efficiently excises it from DNA. Based on the fluorescence intensity of dZeb in DNA extracted from MMR-defective cells expressing *Dm*-dNK, 1 in 8.6 bases in this DNA was found to be replaced with the Z -base. Despite this very high level incorporation of this base analog in the MMR^- strain there was no great loss of viability of these cells following dZeb treatment (Fig. 5).

4. Discussion

4.1 Utility of *Dm*-dNK for Studying Deoxycytidine Analogs

We showed that introduction of a plasmid containing gene for the *Drosophila* deoxynucleoside kinase, *Dm*-dNK, makes *E. coli* sensitive to deoxycytidine analogs AzadC and dZeb (Fig. 4). This demonstrates that *Dm*-dNK expression confers upon this bacterium an ability it normally lacks; i.e. phosphorylation of deoxycytidine analogs. We used this bacterial strain to show that dZeb is highly mutagenic when incorporated into cellular DNA and studied the mechanism by which it causes cell death in DNA repair -proficient *E. coli*. Our results show that cell death is unlikely to be caused due to formation of Dcm-DNA adducts. Instead, it is likely to be due to cellular mismatch repair system nicking the DNA in response to incorporation of the analog. Furthermore, we were able to show that in a mismatch repair -deficient strain, dZeb accumulates to a very high level- better than 1 in 10 bases- without significant loss of cellular viability. Together, these results show that dZeb principally acts as a base-analog mutagen which is effectively acted upon by MMR and that it has fundamentally different mechanisms for causing mutations and cell death than AzaC and AzadC (see below). They also demonstrate usefulness of expressing *Dm*-dNK in *E. coli* as a way to study mechanism of action of bioactive analogs of pyrimidine deoxynucleosides.

4.2 Differences in the mechanism of action of AzaC/AzadC and Zeb/dZeb

A number of studies have shown that addition of AzaC to the growth media has only moderate effects on the viability of DNA repair -proficient *E. coli*. For example, at 20 μg/ mL, this analog reduced cell survival by factors of 1.4 or 3.6 in two different repair -proficient strains [25]. However, AzaC is highly toxic for *recA* strains defective in recombination function and this effect depends on the presence of active Dcm protein [6,25,28]. Additionally, several C5-cytosine DNMTs have been shown to form presumed covalent (i.e. detergent - and heat-stable) adducts with DNA containing 5-azacytosine [see Gowher and Jeltsch for a recent review; [3]]. Consequently, the current model regarding how AzaC causes cytotoxic effects stipulate that the AzaC-DNMT covalent adducts block DNA replication and other essential cellular processes causing cell death [4].

In contrast, as little as 1.0 μg/ mL Zeb reduced cell survival of DNA repair -proficient *E. coli* by 100-fold or more (Fig. 3B and 4, and Supplementary Data). Furthermore, Zeb was toxic regardless of whether Dcm was present in cells (Supplementary data). Same pattern of toxicity was seen with dZeb, when *Dm*-dNK was introduced in cells. Treating repair-proficient cells with less than 1 μg/ mL dZeb reduced cell survival by 100- to 1000-fold (Fig. 4 and Table 1). Additionally, dZeb was equally toxic to dcm^+ and *dcm* cells (see above). This was not expected because both an X-ray crystal structure [12] and biochemical studies [11] suggest C5-cytosine DNMTs form a stable covalent complex with DNA containing zebularine. Regardless, our data strongly suggest that Zeb and dZeb cause cell death by a mechanism that is quite different than that caused by AzaC/ AzadC.

Alkaline-SDS lysis-based method for plasmid isolation requires that the plasmids are present inside the cell in a covalently closed circular form [29]. The low yield of plasmid DNA from cells containing *Dm*-dNK that have been treated with dZeb u sing this method suggests that plasmids in the cell were nicked (open circle) or were in a linear form. Furthermore, the lack of sensitivity of MutL-defective strain to dZeb (Fig. 5) and Zeb (Supplementary data) shows that MMR plays a key role in cell killing caused by Zeb and dZeb. No such role for MMR has been reported in the toxicity of AzaC or AzadC in *E. coli* or mammalian cells [30].

Together, these data suggest strongly that when dZeb is incorporated by polymerases into DNA (presumably across a G) the DNA mismatch repair system of *E. coli* recognizes Zeb:G as a mispair and tries to repair it. When the level of analog incorporation goes up, the frequent excision of the base by MMR results in nicks and double-strand breaks that lead to cell death. This mode of cell killing is similar to the toxicity of adenine analog, 2-aminopurine. MMR-dependent excision of 2-aminopurine from DNA is known to lead to extensive DNA degradation and cell death in *E.coli* [31–33].

Some of the previous studies of Zeb toxicity have suggested that inhibition of enzymes in pyrimidine biosynthesis pathway such as thymidylate synthase and dCMP deaminase by dZeb monophosphate may lead to reduction in cell growth and inhibition of DNA synthesis [34, 35]. However, it is likely that such metabolic disturbances lead to bacteriostatic rather than bactericidal effects in our experiments. We treated growing cells with dZeb for a few hours and then spread them for survival on nutrient rich plates lacking dZeb. Our data suggest that this protocol allows cells to recover from a growth lag or pause that may be induced by the inhibition of pyrimidine biosynthesis pathways. In contrast, degradation of DNA due to extensive repair of Z-base in DNA is unlikely to be reversible.

Our model for the involvement of MMR in cell death caused by Zeb/ dZeb, but not by AzaC/ AzadC, is consistent with differences in the mutation spectra of these analogs. Zeb predominantly causes G:C to A:T transitions which are characteristic of base analog mutagens [10]. In contrast, AzaC predominantly causes C:G to G:C transversions [7,15]. Furthermore, while the mutagenicity of Zeb is substantially higher in a *mutS* (i.e. MMR-defective) strain [10], MMR modulates mutagenicity of AzaC by smaller amounts [30].

4.3 Level of DNA incorporation of dZeb in MMR-defective cells

We did not expect that treatment of cells for five hours with as little as 0.5 μg/ mL dZeb would result in replacement of about 1 in 10 DNA bases with Z -base (Fig. 6) and that such high level incorporation of this mutagenic analog would not result in death of a vast majority of MMR^- cells (Fig. 5). There are several possible explanations for the lack of significant killing of these cells. First, it is possible that our estimate of dZeb in DNA is significantly in error. We used one synthetic DNA duplex containing a single Z-base as the standard in fluorescence measurements of plasmid DNA. If Z-base fluorescence of the standard was somehow lower than average- for example, due to sequence context effects- then this would result in

overestimation of Z-base in plasmid DNA where the analog is expected to be present in many different sequence contexts. We cannot eliminate this possibility, but we did create a second fluorescence standard using a different synthetic duplex in which the Z -base was present in a very different sequence context. Use of this standard did not lead to significantly different estimation of Z-base incorporation in plasmid DNA (data not shown). Second, *Dm*-dNK is catalytically much more efficient than previously reported nucleoside kinases [19] and hence it may create high levels of dZeb monophosphates in cells. Additionally, treatment of cells with Zeb or dZeb is thought to inhibit a number of enzymes in pyrimidine synthesis pathways including *E. coli* thymidylate synthase [35] and human dCMP deaminase [34]. Consequently, it is possible that treatment of cells with Zeb or dZeb inhibits synthesis of normal dNTPs increasing the likelihood of incorporation of dZeb in DNA. Third, it is likely that when cellular DNA and RNA polymerases copy a Z-base they do so with relatively high fidelity. It should be noted that Z-base can form two Watson-Crick H-bonds with guanine and this probably the reason why DNA polymerase I Klenow fragment was found to insert a G across it more often than an A [36]. For similar reasons, Z-base may be better tolerated compared to many other base analogs by cellular proteins that interact with nucleic acids.

4.4 Applications to study of anti-cancer pyrimidine analogs

A number of deoxycytidine analogs such as AzadC (Decitibine), Gemcitabine, Troxacitabine, Tezacytibine are used in anti-metabolite anticancer chemotherapy, but in many cases their mechanism of action remains poorly understood [2,37]. Some of these drugs are known inhibitors of nucleotide biosynthesis enzymes and may act by changing cellular pools of normal nucleotides. However, it is also known that analogs such as 2'-deoxy-2',2'-difluorocytidine (Gemcitabine) are incorporated into cellular DNA and this incorporation plays a key role in their ability to kill tumor cells [38]. It is likely that these abnormal nucleotides are acted upon by DNA repair enzymes and this alters their clinical effectiveness. *E. coli* shares with mammalian cells nearly all DNA repair pathways and most human DNA repair proteins act in a manner similar to their bacterial homologs. For example, all *E. coli* DNA glycosylases have orthologs or functional homologs in mammalian cells. In particular, all *E. coli* glycosylases that excise damaged pyrimidines (namely Ung, Mug and Endonuclease III) have orthologs in mammalian cells (UDG, TDG and NEIL1/ NEIL2, respectively; [39]). Another example of strong evolutionary conservation is found in proteins required for mismatch repair. The structure of the human MutSα dimer with DNA shows that this protein recognizes a G• T mismatch in the same manner as the *E. coli* MutS protein [40,41]. Consequently, our finding that introducing *Dm*-dNK into *E. coli* helps understand the mechanisms of toxicity of AzadC and dZeb opens up the possibility that the mechanism of action of Gemcitabine and other anticancer drugs could be studied using *E. coli* as a model system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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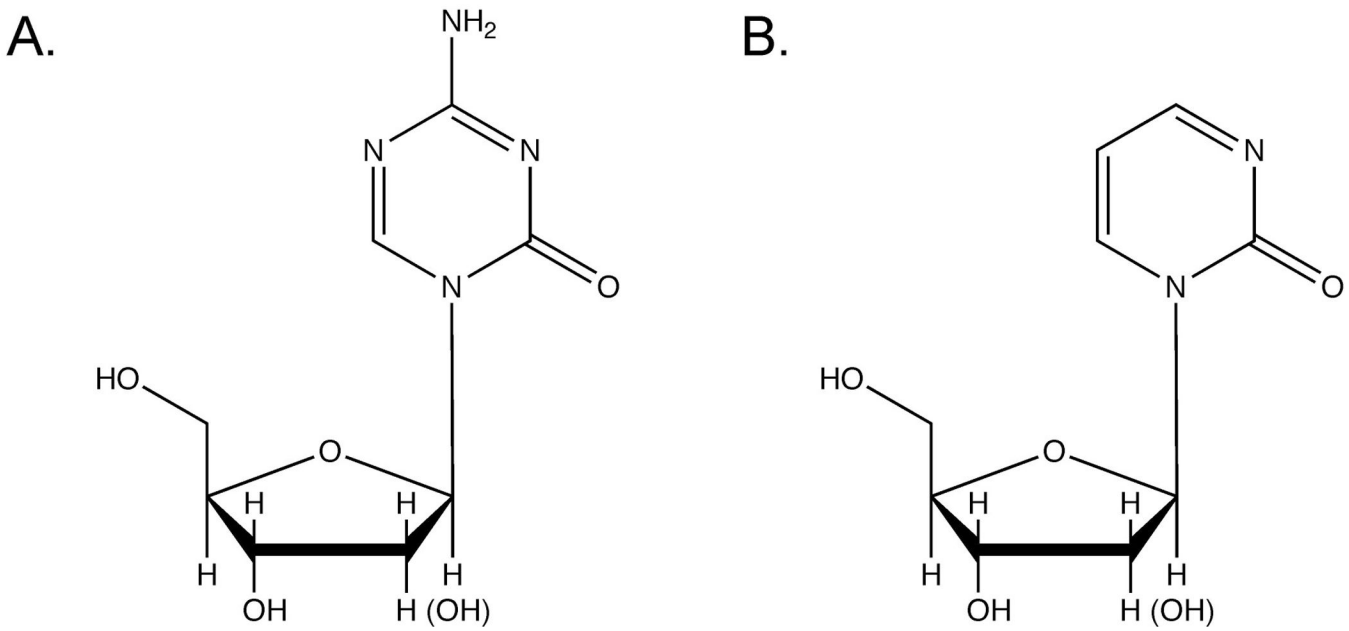

Figure 1. Structures of nucleoside analogs

A. AzadC (5-aza-2'-deoxycytidine). The corresponding ribonucleoside (AzaC) has a hydroxyl at 2' p osition (shown in parethesis). B. dZeb (2-H pyrimidinone deoxyribonucleoside) and Zeb.

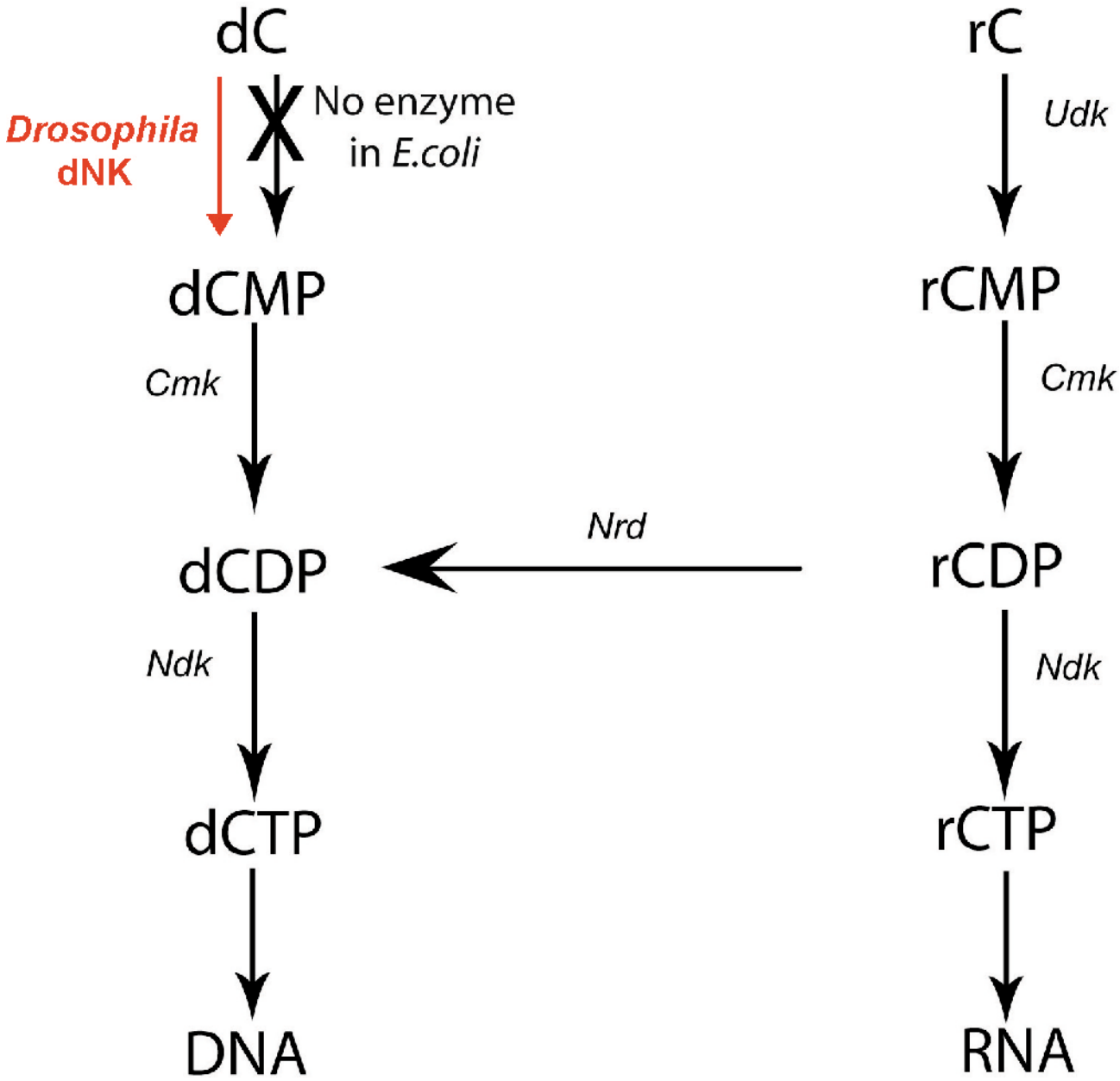

Figure 2. Pathway for the phosphorylation of cytidine and deoxycytidine

The biochemical pathway by which cytidine or deoxycytidine may be converted to nucleoside triphosphates and incorporated into RNA or DNA are shown. The figure points out that a deoxycytidine kinase is missing in *E. coli* and that *Drosophila* deoxynucleoside kinase (Dm-dNK) can perform this step. Udk- Uridine/ Cytidine kinase, Cmk- CMP kinase, N rd- Ribonucleotide Reductase, Ndk- Nucleotide Diphosphate Kinase.

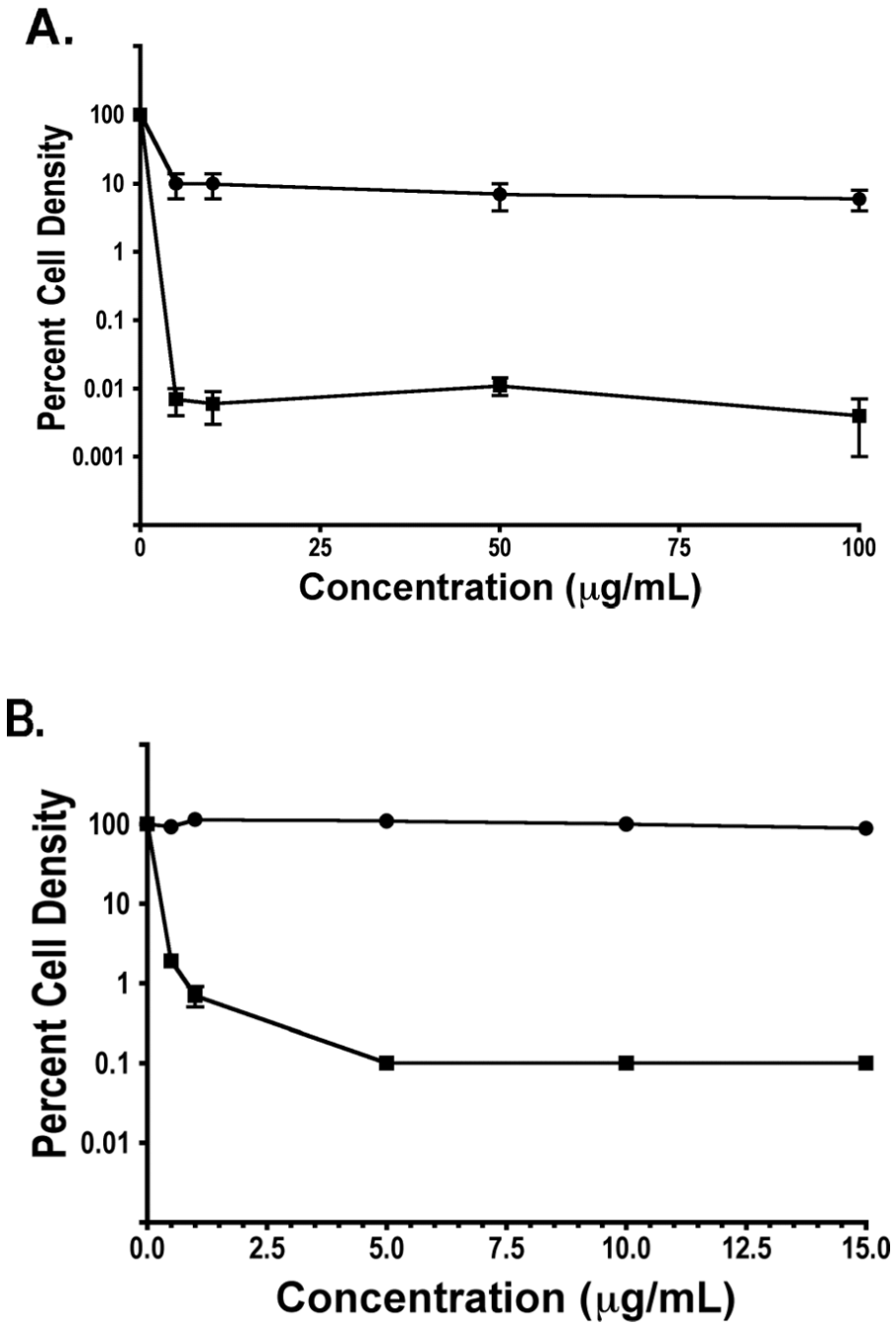

Figure 3. Sensitivity of *E. coli* to pyrimidine analogs

The Y-axis shows colony-forming units (cfu)/ mL of cells treated with the drug as percent of cfu/ mL of untreated cells. Averages from three or more independent cultures and standard deviation are shown. A. Host strain- SE5000. AzaC (squares), AzadC (circles). B. Host strain- CC107. Zeb (squares), dZeb (circles).

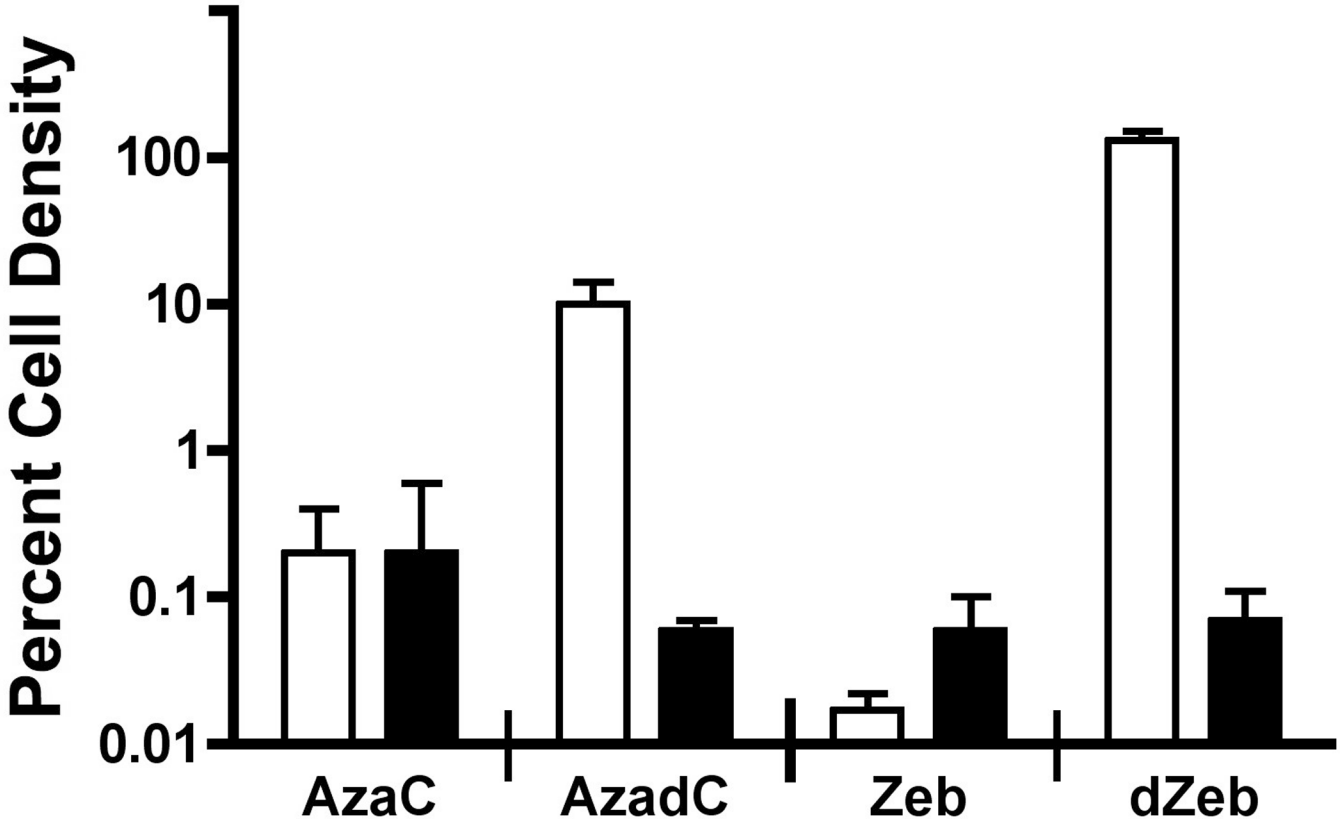

Figure 4. Effect of Dm-dNK on drug sensitivity

The Y-axis shows cfu/ mL of cells treated with the drug as percent of cfu/ mL of untreated cells containing pDm-dNK (solid bars) or vector plasmid (empty bars). AzaC and AzadC were used at 100 μg/ ml, while Zeb and dZeb were used at 0.5 μg/ ml.

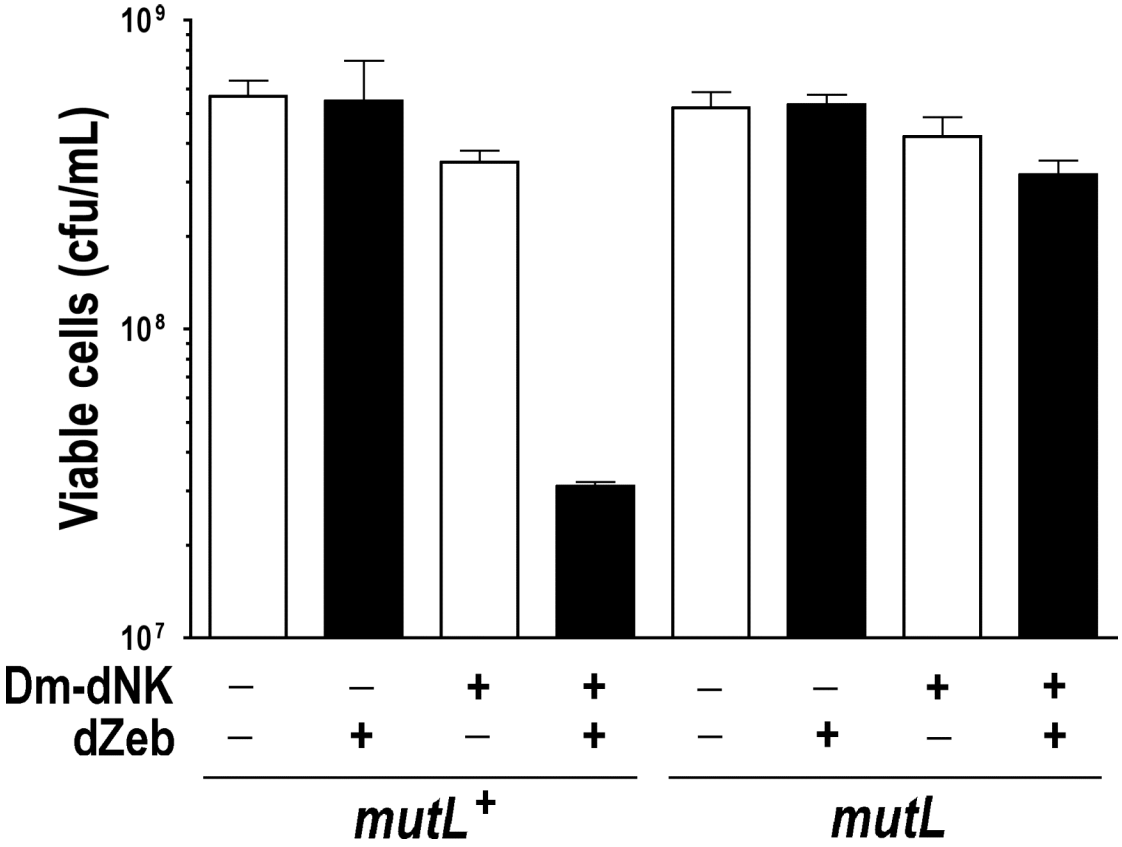

Figure 5. Effect of mismatch repair on dZeb toxicity

Cell survival is shown as cfu/ mL following treatment of *mutL*$^{+}$ (GM31) and *mutL* (BH256) cells containing either pDm-dNK or vector plasmid with dZeb. Mean and standard deviation of three parallel cultures is shown.

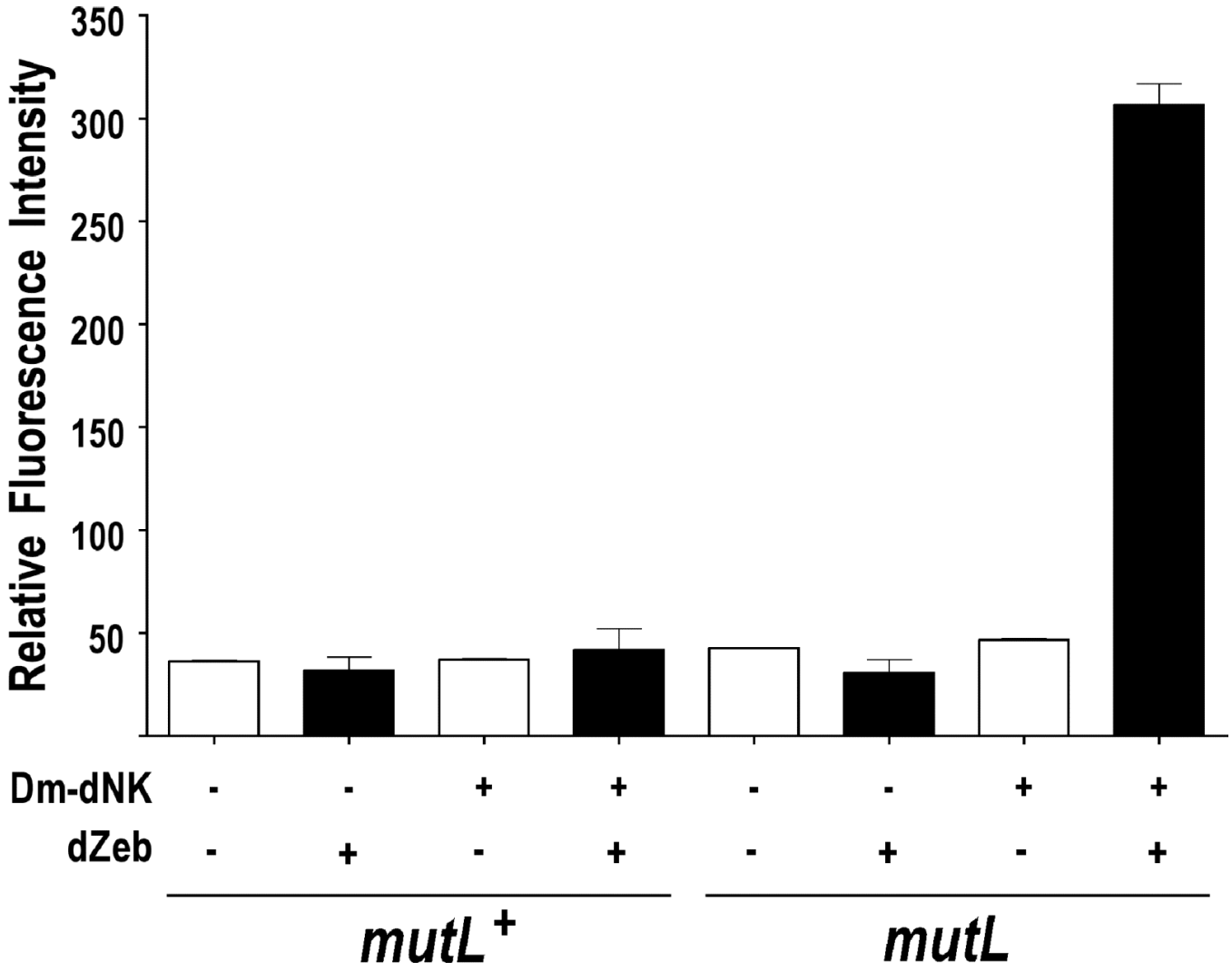

Figure 6. Fluorescence intensities of plasmid DNA

Relative fluorescence intensities of plasmid DNAs isolated from GM31 and BH256 cells carrying pDm-dNK or vector plasmid are presented. Mean and standard deviation of intensities of three independent plasmid preparations are shown.

Table 1

Plasmid	dZeb Concentration (μg/ml)	Cell density (Percent control)	Mutant Frequency ($\times 10^{8}$)
Empty vector	0.0	(100)	6.8 ± 9.3
	0.5	135.1 ± 14.3	20.8 ± 3.2
pDm-dNK	0.0	(100)	1.4 ± 0.4
	0.01	96.9 ± 4.9	4.6 ± 1.3
	0.05	5.2 ± 2.5	415 ± 66
	0.5	0.9 ± 0.7	6688 ± 1030

The host was CC107 and three independent cultures were treated with indicated concentrations of dZeb. Mean and standard deviation of percent survival and mutant frequency are shown.