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Mutagen Synergy: Hypermutability Generated by Specific Pairs of Base Analogs

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ABSTRACT

We tested pairwise combinations of classical base analog mutagens in *Escherichia coli* to study possible mutagen synergies. We examined the cytidine analogs zebularine (ZEB) and 5-azacytidine (5AZ), the adenine analog 2-aminopurine (2AP), and the uridine/thymidine analog 5-bromodeoxyuridine (5BrdU). We detected a striking synergy with the 2AP plus ZEB combination, resulting in hypermutability, a 35-fold increase in mutation frequency (to 53,000 × 10^{-8}) in the *rpoB* gene over that with either mutagen alone. A weak synergy was also detected with 2AP plus 5AZ and with 5BrdU plus ZEB. The pairing of 2AP and 5BrdU resulted in suppression, lowering the mutation frequency of 5BrdU alone by 6.5-fold. Sequencing the mutations from the 2AP plus ZEB combination showed the predominance of two new hot spots for A·T→G·C transitions that are not well represented in either single mutagen spectrum, and one of which is not found even in the spectrum of a mismatch repair-deficient strain. The strong synergy between 2AP and ZEB could be explained by changes in the dinucleoside triphosphate (dNTP) pools.

IMPORTANCE

Although mutagens have been widely studied, the mutagenic effects of combinations of mutagens have not been fully researched. Here, we show that certain pairwise combinations of base analog mutagens display synergy or suppression. In particular, the combination of 2-aminopurine and zebularine, analogs of adenine and cytidine, respectively, shows a 35-fold increased mutation frequency compared with that of either mutagen alone. Understanding the mechanism of synergy can lead to increased understanding of mutagenic processes. As combinations of base analogs are used in certain chemotherapy regimens, including those involving ZEB and 5AZ, these results indicate that testing the mutagenicity of all drug combinations is prudent.

Mutagen-induced mutations have been the subject of intensive investigation for decades (e.g., see references 1 to 4; see reviews in references 5 to 7). However, there are far fewer studies of combinations of mutagens. We are studying possible synergies between mutagens in *Escherichia coli* and initially examined a set of base analog mutagens (Fig. 1): the cytidine analogs zebularine (ZEB) and 5-azacytidine (5AZ), the adenine analog 2-aminopurine (2AP), and the uridine/thymidine analog 5-bromodeoxyuridine (5BrdU). ZEB lacks the amino group of cytidine (8) and causes C·G→T·A changes in the *rpoB*/rifampin resistance (Rif^r) system in *E. coli* (9). It is used in chemotherapy as a demethylating agent (8, 10, 11) to reverse the effects of gene-silenced tumor suppressor gene (12–15) and because the hydrated form is a potent inhibitor of cytidine deaminase (16). 5AZ, another cytidine analog that is used as a demethylating agent in chemotherapy (12, 13), possesses a unique mutagenic specificity, stimulating only C·G→G·C changes (17–19). 5AZ and ZEB have been used in combination in chemotherapy (20). 2-Aminopurine results principally in G·C→A·T and A·T→G·C changes (e.g., see references 18 and 21 to 25), as does 5BrdU (24–26). 2AP has been used to enhance the oncolytic activity of E1b-deleted adenovirus in hepatocellular carcinoma cells (27), and 5BrdU has been used in human glioma cell combination treatments with 1-nitrosourea and cisplatin (28). The initial studies of 1-nitrosourea and cisplatin focused on their direct pairing effects, but already in the early 1980s, it became apparent that for 2AP and 5BrdU, other processes, such as dinucleoside triphosphate (dNTP) pool changes, might be involved (e.g., see references 23 to 25 and references therein). It is now clear that there are several routes to mutagenesis other than direct mispairing that could emanate from the presence of some of the four compounds studied here. This includes partial induction of the SOS system in *E. coli* and related bacteria (6), the partial or complete saturation of the mismatch repair system (7, 26, 29, 30), and potential effects on altering dNTP pools, as the integrity of these pools is crucial to replication fidelity (e.g., see references 24, 25, and 31; see Discussion). Here, we report that strong synergy between certain pairs of these mutagens results in hypermutation. In particular, the combination of 2AP and ZEB creates a dramatic increase in mutation frequency that is 35-fold higher than that of either mutagen alone. The combinations of 2AP plus 5AZ and 5BrdU plus ZEB display weaker synergistic effects, and in contradistinction, the combination of 2AP and 5BrdU shows a suppressive effect on the mutation frequency of 5BrdU alone. Sequencing results for 2AP plus ZEB reveal two mutational hot spots not well represented in the spectrum of either 2AP or ZEB alone. We discuss possible explanations for this, including changes in the dNTP pools, and the potential applications and implications of these results.

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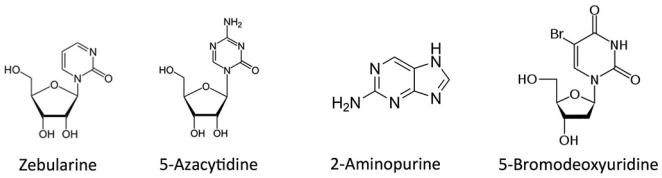

FIG 1 The structures of the four base analogs used in this study.

MATERIALS AND METHODS

***E. coli* strains.** The nucleotide diphosphate kinase (NDK)-deficient *E. coli* strain used here is from the Keio collection, described in Baba et al. (32), made from the starting strain BW25113 (33). This starting strain ($lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$ mutant) is used as the wild type (WT) in the experiments reported here, unless otherwise stated. The NDK-deficient mutant carries a complete deletion of the respective gene, with a *kan* insert in place of the gene.

Media. The media (34) used were LB (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) and minimal A [10.5 g/liter K_2HPO_4, 4.5 g/liter KH_2PO_4, 1 g/liter $(NH_4)_2SO_4$, 0.5 g/liter sodium citrate·$2H_2O$], supplemented with 10 ml of 20% glucose, 1 ml of 1 M $MgSO_4$, and 0.5 ml of 1% thiamine hydrochloride (vitamin B_1) per liter.

Growth conditions. Unless otherwise stated, all genetic methods are as described by Miller (34). Overnight cultures containing different concentrations of a given mutagen were seeded with approximately 1×10^3 cells by inoculating 2-ml cultures with 50 μl of a 10^{-4} dilution of an overday culture. After 18 h of incubation at 37°C on a rotor at 50 rpm, the cells were plated on specific media.

Determination of mutant frequencies. We inoculated 100 to 1,000 cells in a series of cultures of LB that were then grown for 18 h at 37°C with aeration, prior to plating on the appropriate media (LB plates with or without 100 μg/ml rifampin). The frequencies of Rif^r mutants were determined as described previously (35, 36). Briefly, mutation frequency (f) was determined as the median frequency of mutation from a set of cultures (the number of cultures varied from 12 to 65), and the mutation rate (μ) was determined by the formula of Drake (37). The 95% confidence limits were determined according to the method of Dixon and Massey (38).

Chromosomal DNA isolation and sequencing. The chromosomal DNA isolation and sequencing steps were carried out exactly as described by Garibyan et al. (35).

Chemicals. Rifampin (Rif), 2-aminopurine (2AP), 5-azacytidine (5AZ), and 5-bromodeoxyuridine (5BrdU) were purchased from Sigma (St. Louis, MO). Zebularine (ZEB) was a gift from Victor Marquez.

RESULTS

Mutation frequencies resulting from mutagen pairs. We used the *rpoB*/Rif^r system (35, 39) to examine mutation frequencies resulting from different pairwise combinations of the four base analog mutagens 2AP, ZEB, 5AZ, and 5BrdU. We detected a strong synergy between 2AP and ZEB. Figure 2 displays the dramatic 35-fold increase in mutation frequency compared with that of either base analog alone at the concentrations used. We also detected weak synergies between 2AP and 5AZ (2.5-fold higher than the sum of each mutagen alone) and between 5BrdU and ZEB (2.7-fold) (Table 1). On the other hand, the pair 2AP plus 5BrdU gave a 6-fold lower mutation frequency than that of 5BrdU alone, indicating that 2AP is significantly suppressing the mutagenic effects of 5BrdU. The pair 5AZ plus ZEB gave a weak suppression effect, and the pair 5AZ plus 5BrdU gave no significant effect (Table 1).

Sequence of mutational changes in *rpoB* for the 2AP plus ZEB pair. There are at least 92 base substitution mutations in *rpoB*

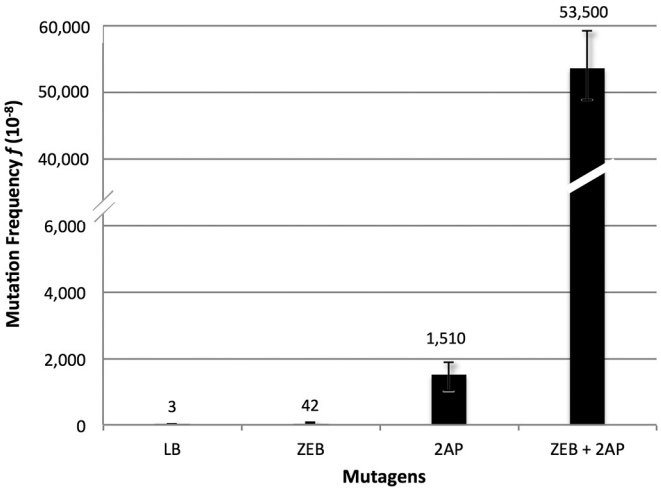

FIG 2 The frequencies of Rif^r mutants found in cultures grown with either no mutagen (LB), 5 μg/ml ZEB, 20 μg/ml 2AP, or 5 μg/ml ZEB plus 500 μg/ml 2AP. Error bars represent 95% confidence intervals.

that can lead to the Rif^r phenotype at 37°C, and each of the 6 possible base substitutions is well represented (35, 39, 40). We can detect 80 of these mutations with a single primer pair in one segment of the *rpoB* gene; we therefore analyzed this segment in this study. There are 11 A·T→G·C mutations, 17 G·C→A·T mutations, 9 A·T→T·A mutations, 10 A·T→CG mutations, 18 G·C→T·A mutations, and 15 G·C→C·G mutations included in this set. Because many mutagens have preferred hot spots resulting from aspects of the surrounding sequence (e.g., see references 2, 22, and 35), a system that monitors numerous sites allows one to define a mutagenic fingerprint for different mutagens and mutagenic pathways. Thus, we determined the sequences of the mutations resulting from the 2AP plus ZEB combination and compared them with those we found for ZEB alone (9) and for 2AP alone (this study). All mutations analyzed were of independent origin (see Materials and Methods for details). Figure 3 displays the results, showing only the two transition substitutions A·T→G·C and G·C→A·T, since these substitution sites comprise virtually all of the mutations found in these three spectra, with 90 of 90 detected mutations for 2AP, 147 of 155 detected mutations

TABLE 1 *rpoB* mutation frequencies in the BW25113 strain background with various mutagens

Mutagen(s)	Concn (μg/ml)	No. of replicates	f (10^{-8}) in *rpoB* (95% CI)[a]
None		39	2.7 (1.8–4.2)
2AP	500	62	1,505 (1,110–2,000)
ZEB	5	58	42.1 (31–56.5)
5BrdU	5	42	12,950 (11,700–14,800)
5AZ	20	20	1,045 (735–1,470)
5AZ	50	26	982 (802–1,280)
2AP + ZEB	500, 5	65	53,500 (48,800–59,300)
2AP + 5AZ	500, 20	53	6,280 (5,210–7,140)
2AP + 5BrdU	500, 5	28	2,100 (1,860–2,580)
5BrdU + ZEB	5, 5	32	34,700 (25,800–40,600)
5AZ + ZEB	50, 5	17	544 (297–669)
5AZ + 5BrdU	50, 5	15	9,680 (6,080–12,300)

[a] 95% CI, 95% confidence interval.

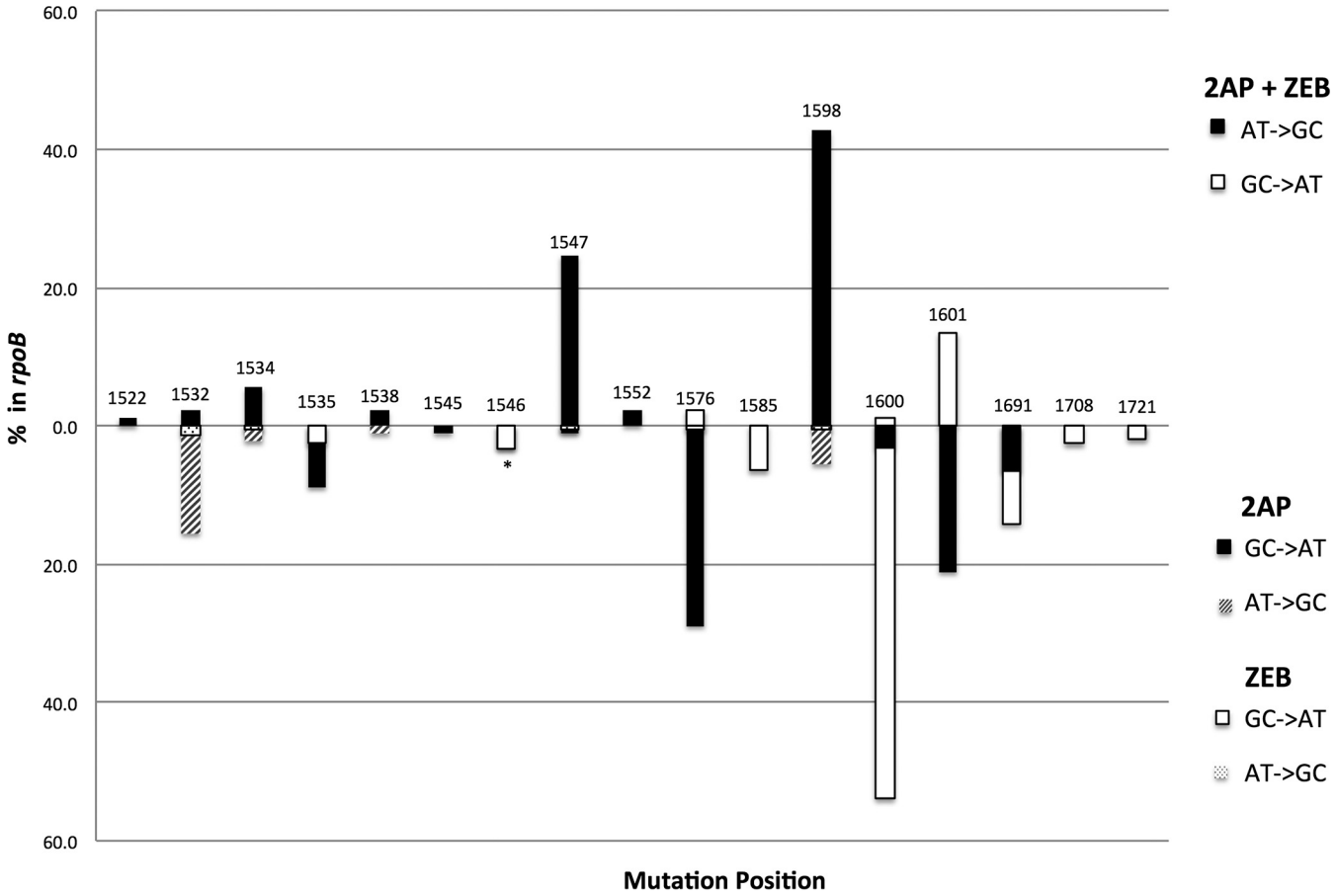

FIG 3 The spectrum of mutations in *rpoB* caused by 2AP or ZEB (bottom) or the combination of 2AP plus ZEB (top). The transitions (G·C→A·T and A·T→G·C) are shown as a percentage of the total mutations in each sample.

for ZEB, and 87 of 88 mutations for 2AP plus ZEB. The ZEB spectrum shows a major hot spot at position 1600 (G·C→A·T, 86 of 156 mutations detected [55%]) and a minor hot spot at position 1691 (G·C→A·T, 22 of 156 mutations detected [14%]). The 2AP spectrum shows a major hot spot at position 1576 (G·C→A·T, 26 of 90 mutations detected [29%]), and secondary hot spots at position 1601 (G·C→AT, 19 of 90 mutations detected [20%]) and at position 1532 (A·T→G·C, 14 of 90 mutations detected [16%]). Surprisingly, the spectrum for the combination of 2AP plus ZEB is not dominated by the 2AP or ZEB major hot spot but rather a new major hot spot at position 1598 (A·T→G·C, 38 of 88 mutations detected [43%]) and a second hot spot at position 1547 (A·T→G·C, 22 of 88 mutations detected [25%]). A minor hot spot is seen at position 1601 (G·C→AT, 12 of 88 mutations detected [14%]) that is also seen in the 2AP spectrum, although here, it is 23-fold more frequent (see below). The hot spot at position 1547 is also seen in the spectra of spontaneous mutations in wild-type mismatch repair-deficient strains, as well as for NDK- and deoxycytidine deaminase (DCD)-deficient strains (35, 36, 39, 41, 42). However, the A·T→G·C mutations at position 1598 are only rarely seen (see Discussion) and constitute only 5.5% of the mutations in the 2AP spectrum (5 of 90 mutations detected).

We can get a better picture of the increase in mutation frequencies at certain sites by multiplying the frequencies of Rif^r mutants (Fig. 2 and Table 1) by the fraction of mutants with changes at each specific site, resulting in the frequency of mutants with changes at each site. Table 2 shows these frequencies. When we discount the mutants in the 2AP plus ZEB spectrum that are represented by only one or two occurrences, we can see that although mutations at a number of sites are increased, mutations at three sites have both very high frequencies and high to very high increases in frequencies in the combination over that in the two single spectra. Mutations at the new hotspot at position 1598 (A·T→G·C) occur at a frequency of 22,900 $\times$ 10^{-8} (2.3 $\times$ 10^{-4}), 270-fold higher in the 2AP plus ZEB combination than with either 2AP or ZEB alone, those at position 1547 (A·T→G·C) occur at a frequency of 13,250 $\times$ 10^{-8} that represents at least several thousand-fold over the background single frequencies, and mutations at position 1601 (G·C→A·T) are at a frequency of 7,230 $\times$ 10^{-8}, 23-fold higher than that found in 2AP.

Sequence of mutational changes in *rpoB* for the 2AP plus 5AZ pair. We also sequenced the *rpoB* gene in mutants resulting from the combination of 2AP plus 5AZ, as well as from 2AP alone (see above) and 5AZ alone. The results are shown in Table 3, which lists all the transition sites (G·C→A·T and A·T→G·C), and the G·C→C·G sites, since these 41 sites encompass virtually all of the resulting mutations. 5AZ alone has a unique G·C→C·G specificity (17, 18), and this specificity is also seen in *rpoB* from our previous study (35) and in this study (Table 3). The combination of 2AP plus 5AZ shows much weaker synergy than that for the 2AP

TABLE 2 Distribution of mutation frequencies in *rpoB*

Site (bp)	bp change	$f(10^{-8})$ with:		
		2AP	ZEB	2AP + ZEB
1522	AT→GC	0	0	600[a]
1532	AT→GC	234	0.54	1,200[a]
1534	AT→GC	33	0.27	3,000
1538	AT→GC	17	0	1,200[a]
1547	AT→GC	0	0.27	13,250
1552	AT→GC	0	0	1,200[a]
1577	AT→GC	0	0	0
1598	AT→GC	84	0.27	22,900
1702	AT→GC	0	0	0
1703	AT→GC	0	0	0
1715	AT→GC	0	0	0
1520	GC→AT	0	0	0
1535	GC→AT	134	1.1	0
1545	GC→AT	17	0	0
1546	GC→AT	50	1.4	0
1547	GC→AT	17	0	0
1565	GC→AT	0	0	0
1576	GC→AT	435	0.27	1,200[a]
1585	GC→AT	0	2.7	0
1586	GC→AT	0	0	0
1592	GC→AT	0	0	0
1595	GC→AT	0	0	0
1600	GC→AT	50	22.6	600[a]
1601	GC→AT	318	0.81	7,230
1609	GC→AT	0	0.54	0
1610	GC→AT	0	0	0
1691	GC→AT	100	5.9	0
1708	GC→AT	0	1.1	0
1721	GC→AT	17	0.81	0

[a] Based on only one or two occurrences.

TABLE 3 Distribution of mutations in *rpoB*

Site (bp)	bp change	No. of mutations with:		
		5AZ	2AP	5AZ + 2AP
1522	AT→GC	0	0	0
1532	AT→GC	0	14	2
1534	AT→GC	0	2	0
1538	AT→GC	0	1	1
1547	AT→GC	0	0	2
1552	AT→GC	0	0	0
1577	AT→GC	0	0	0
1598	AT→GC	0	5	4
1702	AT→GC	0	0	0
1703	AT→GC	0	0	0
1715	AT→GC	0	0	0
1520	GC→AT	0	0	0
1535	GC→AT	0	8	1
1545	GC→AT	0	1[a]	0
1546	GC→AT	0	3	0
1547	GC→AT	0	1	0
1565	GC→AT	0	0	0
1576	GC→AT	0	26	5
1585	GC→AT	0	0	0
1586	GC→AT	0	0	0
1592	GC→AT	0	0	0
1595	GC→AT	0	0	0
1600	GC→AT	0	3	5
1601	GC→AT	0	19	8
1609	GC→AT	0	0	0
1610	GC→AT	0	0	0
1691	GC→AT	0	6	2
1708	GC→AT	0	0	0
1721	GC→AT	0	1	0
1527	GC→CG	1	0	2
1537	GC→CG	4	0	0
1574	GC→CG	0	0	2
1576	GC→CG	16	0	2
1578	GC→CG	4	0	0
1585	GC→CG	1	0	0
1592	GC→CG	1	0	0
1594	GC→CG	0	0	2
1597	GC→CG	0	0	0
1600	GC→CG	0	0	1
1601	GC→CG	0	0	0
1691	GC→CG	1	0	2
1709	GC→CG	0	0	0
1716	GC→CG	16	0	8
2059	GC→CG	0	0	0
Total		44	90	49

[a] Part of a two-base change with position 1576.

plus ZEB combination, so there is a significant contribution from the backgrounds of 2AP alone (e.g., positions 1576 and 1691, G·C→AT) and 5AZ alone (G·C→C·G) in the combined spectrum. Note that the sample size here for 2AP is larger than for either 5AZ or for the combination of 2AP plus 5AZ. The spectrum of the 5AZ plus 2AP combination does not show a dramatic difference from the combined spectra of 5AZ alone and 2AP alone.

Thymidine deprivation by ZEB. In mammalian cells, ZEB results in thymidine deprivation from directly inhibiting thymidylate synthase and from the inhibition of dCMP deaminase by deoxyzebularine monophosphate (dZMP) (43). This has not been determined in bacteria, so we tested the effect of adding thymidine to ZEB-treated cultures, in both minimal and rich (LB) media. As Fig. 4 shows, thymidine protects cells from killing by ZEB at a range of concentrations in both minimal and rich media, indicating that ZEB results in thymidine deprivation in *E. coli*.

Synergy in an NDK-deficient background. One explanation for the synergy between 2AP and ZEB involves altered dNTP pools, such as that seen in a number of *E. coli* mutants, for example DCD-deficient and NDK-deficient strains (41, 42, 44). We therefore decided to examine the 2AP plus ZEB combination in an NDK-deficient strain to determine whether the increased mutation frequency might be even further enhanced in this background. Table 4 shows that the frequencies of 2AP-, ZEB-, and 5BrdU-induced mutations are increased in this background, but so are the frequencies of the mutations induced by the combination of ZEB and 2AP. In the case of the combination, the levels of Rif^r mutants are 185,000 per 10^8 cells, or approximately 1 per 560 cells! We sequenced 17 Rif^r mutants from this combination and found that all 17 have A·T→G·C changes, with 6 changes at position 1534, 5 changes at position 1547, and 6 changes at position 1598. Thus, the frequency of a specific mutation at each of these three positions is approximately 60,000 per 10^8 cells, or one out of every 1,670 cells. These high mutation frequencies are not restricted to the *rpoB* region, as we found similar high levels for mutations in the *thyA* gene (after selection for trimethoprim resistance; data not shown). We also tested for auxotrophy among

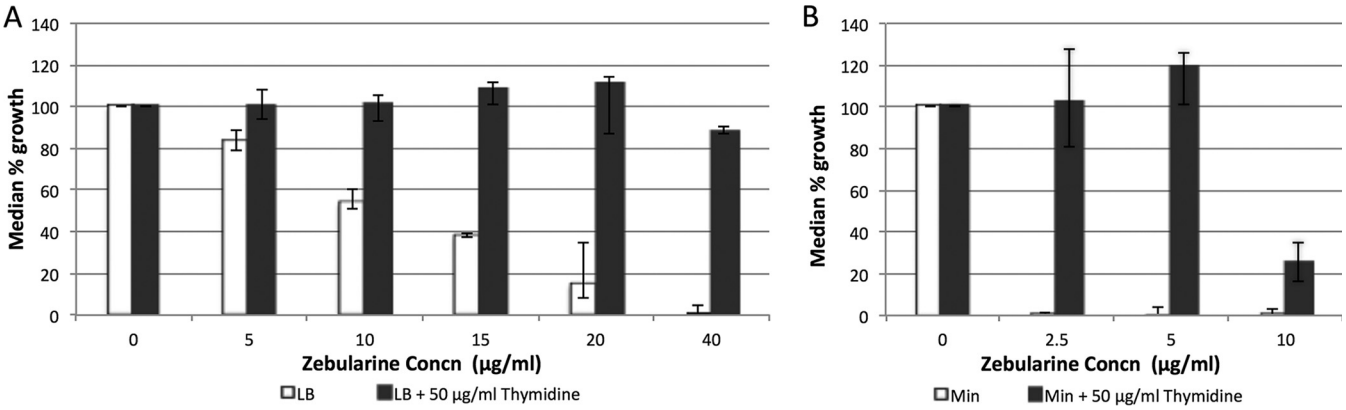

FIG 4 Growth potentiation by thymidine in the presence of different concentrations of ZEB in LB medium (A) and minimal medium (Min) (B). Error bars represent 95% confidence intervals.

100 to 200 colonies in the *ndk* background for several cultures grown on LB plates after growth in LB broth in the presence of ZEB, 2AP, and 2AP plus ZEB. We found 39% of the colonies from 2AP plus ZEB to be auxotrophs, versus 11% for 2AP alone, 1% for ZEB alone, and 0% for LB alone.

DISCUSSION

What is the effect of using more than one mutagenic treatment or mutagen at the same time? Will certain combinations of mutagens unlock unexpected pathways of mutagenesis? Many single mutagens themselves are no longer considered to have only one type of effect (e.g., see references 7 and 45). Even for base analogs, one can recognize a number of different pathways to mutagenesis that might be activated: (i) direct mispairing involving different tautomeric forms of either the analog or the conventional base (see review in reference 6), although the actual tautomers involved may be different than those originally envisioned (46, 47); (ii) up- or downregulation of different operons, such as the partial or complete induction of the SOS system (6); (iii) saturation of certain repair systems, in particular, the mismatch repair system (7, 29, 30); (iv) alteration of the dNTP pools (31); and (v) inhibition of specific enzymes (43). In this respect, there are then several possible routes to synergistic effects, including but not limited to the induction of specific pathways, dNTP pool changes, or specific mispairing between different base analogs. Here, we examined a set of four base analog mutagens, 2AP, ZEB, 5AZ, and 5BrdU, and looked for synergistic effects on mutagenesis resulting from pairwise combinations. As Table 1 and Fig. 2 show, three combinations gave higher levels of Rif^r mutants than either single mutagen alone. The increase in levels with the 2AP plus ZEB combination is striking (Fig. 2). Moreover, the analysis of the mutations occurring in *rpoB* indicates that new mutational hot spots emerge that are not prominent in the spectra of either 2AP or ZEB alone (Fig. 3 and Table 2). One possibility is that 2AP can make a specific mispair with ZEB that leads to the increased mutation rate. However, this would have to involve two rare tautomers pairing, and it appears unlikely that this could account for the very high levels of mutagen synergy we observe. A more likely explanation involves imbalances in the dNTP pools.

TABLE 4 *rpoB* mutation frequency in an NDK-deficient strain background with various mutagens

Mutagen(s)	Concn (μg/ml)	No. of replicates	$f(10^{-8})$[a] in *rpoB* (95% CI)[a]
None		18	28.2 (16–55.2)
2AP	500	18	11,650 (9,780–15,600)
ZEB	5	12	150 (85.5–535)
2AP + ZEB	500, 5	27	185,000 (144,000–250,000)
5BrdU	5	31	116,000 (96,700–134,000)

[a] 95% CI, 95% confidence interval.

There is an exquisite balance between dNTP levels and ratios and DNA replication speed (e.g., see references 48 and 49) and mutation rates. Long-standing work in both microbial and mammalian cells has shown that alterations of the dNTP pools can affect mutation rates (31, 41, 42, 44, 50–54). More recently, studies in *E. coli* have shown that perturbations in the absolute levels of all four dNTPs, even when the ratios are not altered, leads to changes in mutation rates (26, 55, 56). Increases in the levels raise mutation rates (55, 56), and decreases in the levels lead to lower mutation rates (26). (Raising dNTP levels leads to the suppression of 3′-exonuclease proofreading, resulting in increased misincorporation and increased mutagenesis; see below [57–59]). We can look to results from the analysis of different mutants with altered dNTP ratios for clues about the cause of the synergistic effects seen in the work reported here, since the spectrum of mutations in any given system (in this case *rpoB*) can be fingerprints of specific processes. Mutants defective in deoxycytidine deaminase (DCD) or nucleotide diphosphate kinase (NDK) have increased dCTP and dGTP (41, 44, 49, 50; but also see reference 60), decreased dATP (41, 44), and higher rates of certain base substitutions (36, 41, 44, 50, 51). The double mutant deficient in both DCD and NDK has a more extreme imbalance and a more extreme mutation rate increase (42). These findings are relevant, because ZEB-treated *E. coli* cells are likely to be phenotypically DCD deficient. This stems from the fact that deoxyzebularine monophosphate, generated *in vivo*, inhibits mammalian DCD (43). Moreover, zebularine itself inhibits mammalian thymidylate synthase (43). Both of these inhibitions lead to thymidine deprivation (43). Indeed, we find that *E. coli* cells grown in the presence of ZEB are severely thymidine limited, as seen in Fig. 4. In fact, Fig. 4 is similar to the respective figure we constructed for DCD-deficient cells (61). The spectrum of *rpoB* mutations in the 2AP plus ZEB com-

bination does share one hot spot with the spectra of DCD-, NDK-, or DCD- and NDK-deficient strains, the A·T→G·C mutation at position 1547 (36, 41, 42), although this site also represents a hot spot for spontaneous mutations in either a wild-type or mismatch repair-deficient background (35, 39). What really stands out in Fig. 3 is the major hot spot for 2AP plus ZEB, the A·T→G·C change at position 1598, a site that is poorly represented in most spectra (e.g., see references 35, 36, 39, 41 and 42). However, this site is well induced in only one situation described in the literature, and this allows us to pinpoint the likely source of the increased mutation level. Ahluwalia and Schaaper have reported that specific engineered mutations in the gene encoding ribonucleotide reductase (RNR) can result in a high mutation rate and a spectrum in *rpoB* that has hot spots at several of four particular A·T→G·C sites in *rpoB*, these being positions 1532, 1538, 1547, and 1598 (62; for related work in yeast, see also reference 63). Moreover, the altered RNR generates a dNTP imbalance, with increased dCTP and dGTP and reduced dATP. This favors the misincorporation of dGTP across from template T. The four sites are favored over other A·T→G·C sites in *rpoB* because the next nucleotide incorporated is either dGTP (three cases) or dCTP (in the case of position 1547) (62; see also earlier work in references 57 to 59). It is likely that the simple addition of two base analogs, 2AP and ZEB, results in a related situation, although more pronounced, yielding even higher frequencies, for at least two of these sites. The sequences surrounding these two sites are as follows: position 1547, 5′-CTG-G**T**C-CAT-3′, and position 1598, 5′-GCA-C**T**C-GGC-3′. Thus, with replication proceeding on the other strand in the 5′→3′ direction, when G is misincorporated across from T (in bold), the next nucleotides are CC and G for positions 1547 and 1598, respectively. The increased replication errors resulting from the 2AP plus ZEB combination inevitably saturate the mismatch repair system, thus resulting in the observed hypermutability. Neither analog alone fully saturates mismatch repair, as one sees a marked increase in the mutation frequency in ZEB-treated mismatch repair (MMR)-deficient cells compared to wild-type-treated cells (9), and the major hot spot seen in an MMR-deficient strain (A·T→G·C, position 1547) (35) does not appear in the 2AP spectrum among 90 sequenced mutations (Table 3). This is despite the fact that the spontaneous mutation frequencies in *rpoB* in a MutS-deficient strain and a 2AP-treated wild-type strain are similar. 2AP does appear to partially saturate MMR, however (29, 30).

One prediction of the notion that the 2AP plus ZEB combination mimics the dNTP imbalance seen in the mutant with an altered RNR is that there would be even more potent mutagenesis in an NDK strain, as the dNTP imbalance in NDK-deficient strains (41, 44) is in the same direction as the presumed imbalance generated by 2AP plus ZEB. Table 4 shows that this is indeed the case, as the *rpoB* mutation frequency increases from $53{,}000 \times 10^{-8}$ in the starting strain to $185{,}000 \times 10^{-8}$ in the NDK-deficient strain. This is, in fact, an extraordinarily high mutation frequency, particularly when the majority of mutations are at two sites (the A·T →G·C hot spots at positions 1547 and 1598; see Results). A remaining unexplained result is that in the wild-type background, we observe a large increase in a 2AP secondary hot spot in the 2AP plus ZEB combination (relative to the 2AP-alone spectrum; 23-fold), involving a G·C→A·T change at position 1601 in *rpoB* (Tables 2 and 3). This indicates that there are additional complexities in these synergies. Also intriguing is the suppression of 5BrdU mutagenesis by 2AP (Table 1). One might try to explain this either by specific mispairing models involving both 2AP and 5BrdU, or by some aspect of the pool imbalances (24, 25). However, definitive elucidation of this suppression effect is a topic for subsequent experiments.

Ongoing studies are aimed at examining a larger set of potential mutagen synergies, both with the aim of uncovering additional pathways of mutagenesis, and also of examining combinations that are currently in use in chemotherapy. For example, the agents cisplatin (CPT), ZEB, 5AZ, and brostallicin (BRC) are used in different combinations in chemotherapy (64–66). Specifically, BRC plus CPT is used to treat patients with advanced solid tumors (64), ZEB plus CPT has been used in human carcinoma cell lines (65), and ZEB plus BRC has been used in prostate cancer cell lines (66). We note that the ZEB plus 5AZ combination studied in this paper (Table 1) has been used successfully in mice with L1210 leukemia (20).

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