HEALTH AND MEDICINE

Comment on "A commensal strain of *Staphylococcus epidermidis* protects against skin neoplasia" by Nakatsuji *et al*.

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A recent article in *Science Advances* described the striking discovery that the commensal *Staphylococcus epidermidis* strain MO34 displays antimicrobial and antitumor activities by producing a small molecule, identified as the nucleobase analog 6-*N*-hydroxylaminopurine (6-HAP). However, in contradiction to the literature, the authors claimed that 6-HAP is nonmutagenic and proposed that the toxic effect of 6-HAP results from its ability to inhibit, in its base form, DNA synthesis. To resolve the discrepancy, we proved by genetic experiments with bacteria and yeast that extracts of MO34 do contain a mutagenic compound whose effects are identical to chemically synthesized 6-HAP. The MO34 extract induced the same mutation spectrum as authentic 6-HAP. Notably, the toxic and mutagenic effects of both synthetic and MO34-derived 6-HAP depended on conversion to the corresponding nucleotide. The nucleobase 6-HAP does not inhibit DNA synthesis in vitro, and we conclude that 6-HAP exerts its biological activity when incorporated into DNA.

INTRODUCTION

Synthetic analogs of the nucleic acid bases are widely used in medicine as anticancer, anti-inflammatory, and antiviral agents (*1*). Bases per se are prodrugs and have to be activated to nucleotides to exert their biological effect primarily by interfering with the synthesis of nucleic acids (*2*). A recent publication in *Science Advances* (*3*) is at stark contrast with the current knowledge. The authors found that a strain of the commensal bacterium *Staphylococcus epidermidis* MO34, which colonizes human skin, produces a base analog, 6-*N*-hydroxylaminopurine (6-HAP), which was shown responsible for strong bactericidal activity against group A *Streptococcus*, antiproliferative activity against tumor cell lines, and protection of mice colonized with this bacterium from ultraviolet-induced skin tumors. The effects were attributed to an ability of 6-HAP, in its base form, to inhibit DNA polymerase reactions. Overall, these findings may be of great importance for medical studies, but the proposed mechanism of action of 6-HAP, including its reported nonmutagenicity, starkly contradicts an extensive body of literature data on 6-HAP (*4*–*6*). To resolve the apparent discrepancies, we contacted the authors, and they sent us the extracts of strain MO34, as well as preparations of synthetic 6-HAP produced in their laboratory. Using these reagents and commercial 6-HAP, we conducted extensive genetic experiments in bacteria and yeast to test the mutagenicity of these reagents and also conducted DNA polymerase reactions to address its mode of action. We document that the MO34 extract is highly mutagenic in a manner identical to authentic 6-HAP, including the production of a similar mutational spectrum. We also demonstrate that the base 6-HAP per se does not interfere with DNA polymerase reactions. Instead, conversion to the nucleoside triphosphate is a prerequisite for its biological activity. We conclude that the toxic and antitumor effects of the 6-HAP production reported in (*3*) are caused, such as is the case for most classical antitumor agents, by its deoxyribonucleoside triphosphate [d(6-HAP)TP], which is incorporated into DNA, causing genotoxicity.

RESULTS

The metabolism of 6-HAP, the compound produced by the MO34 strain of *S. epidermidis* (*3*), has been extensively studied (Fig. 1A). In bacteria, intracellular 6-HAP, after import by a specific permease (*7*), is subject to efficient reduction to adenine by two molybdenum cofactor (Moco)–dependent enzymes encoded by the *ycbX* and *yiiM* genes (*8*). Defects in genes controlling the synthesis of Moco or the 6-HAP reductases, shown in blue in Fig. 1A, lead to hypersensitivity to 6-HAP–induced killing and mutagenesis (*8*–*10*). If not detoxified, 6-HAP can become activated by conversion first to the corresponding ribonucleoside monophosphate and, subsequently, to deoxy- and ribonucleoside triphosphates (Fig. 1A) (*11*). The deoxyribonucleoside-5′-triphosphate d(6-HAP)TP is a good yet base-pairing ambiguous substrate for DNA polymerases (*12*–*14*), which incorporate it into DNA (*15*), thereby engendering toxicity and inducing mutations of the transition type (*5*, *16*, *17*). In contrast, defects in genes responsible for activation and incorporation of 6-HAP, as shown in red in Fig. 1A, diminish or abolish 6-HAP lethality and mutagenesis (*5*, *18*).

We first examined the toxic and mutagenic effects of extracts of *S. epidermidis* and two preparations of synthetic 6-HAP using spot tests (see Materials and Methods) in *Escherichia coli* strains carrying diagnostic mutations in the 6-HAP activation/deactivation pathways (Fig. 1, B and C). As in previous studies (*8*–*10*), in this assay, no toxicity was detected for the wild-type strain (Fig. 1B, upper row,

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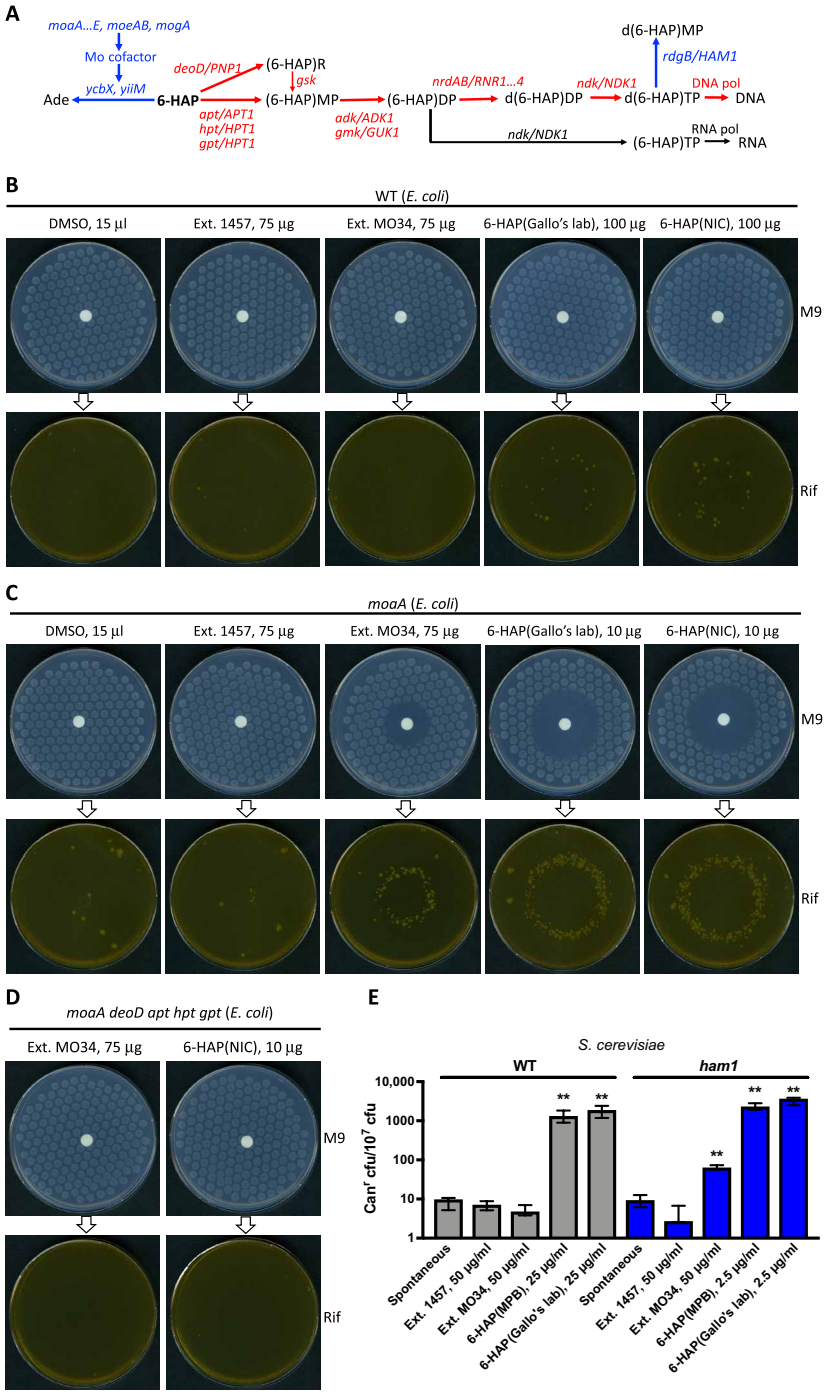

Fig. 1. Same genetic control of toxicity and mutagenicity effects of 6-HAP and *S. epidermidis* MO34 extract. (**A**) Schematic representation of the genetic control of 6-HAP metabolism in *E. coli* and yeast (*S. cerevisiae*). Genes responsible for the detoxification pathways are indicated in blue, and genes responsible for activation are in red. *E. coli* genes are listed first, yeast genes are in capital letters after the slash. Note that the Moco-dependent defense against 6-HAP is not present in yeast. Ade, adenine; (6-HAP)R, 6-HAP-riboside; (6-HAP)MP, 6-HAP-riboside monophosphate; (6-HAP)DP, 6-HAP-riboside diphosphate; d(6-HAP)DP, 6-HAP-deoxyriboside diphosphate; (6-HAP)TP, 6-HAP-riboside triphosphate; d(6-HAP)TP, 6-HAP-deoxyriboside triphosphate; d(6-HAP)MP, 6-HAP-deoxyriboside monophosphate. (**B** and **C**) Hypersensitivity of *E. coli* Δ*moa* mutants to 6-HAP or to extract of *S. epidermidis* strains MO34 or 1457. The compounds to be tested were spotted on the disc at the center of the plate. The upper row shows the growth on minimal M9 plates; the lower row shows the ability of compounds spotted in the center to induce Rif^r mutations. Ext., extract; NIC, Natland International Corporation. (**D**) 6-HAP by itself does not produce any toxic or mutagenic effects because mutations blocking 6-HAP conversion to the ribonucleotide monophosphate level prevent both 6-HAP–induced toxicity and mutagenicity as seen in (C). (**E**) The extract of *S. epidermidis* strain MO34 is mutagenic in the *ham1* yeast strain unable to deactivate d(6-HAP)TP. Canavanine resistance (Can^r) is measured (see Materials and Methods). Medians with 95% confidence intervals are shown. Double asterisks indicate mutant frequencies significantly higher than for the spontaneous sample (*U* test, $P < 0.003$). Note the 10-fold difference in the 6-HAP (MPB, MP Biomedicals) concentration used for the WT strain and *ham1* mutant.

M9 minimal plates) (no circle of inactivation around the disc), but when these plates were subsequently replica plated to medium containing the antibiotic rifampicin, modest mutagenicity was seen for both preparations of synthetic 6-HAP (antibiotic-resistant colonies in the center of the plate, where the concentration of the test compound is sufficiently high for the recovery of mutants). The corresponding experiment with the Δ*moaA* mutant (lacking Moco) showed (Fig. 1C) that both preparations of 6-HAP exert a high level of toxicity as well as a potent mutagenic effect. Importantly, the experiment also indicates that the MO34 extract is likewise toxic and mutagenic in this assay. We conclude that 6-HAP contained in the MO34 extract is toxic and mutagenic such as chemically synthesized 6-HAP, with slightly smaller effects noted for the extract being attributable to the lesser amount of 6-HAP in the extract (see below). The extract of the control strain 1457 did not show any toxicity or mutagenicity (Fig. 1C).

To address the issue whether the active compound in MO34 extract could be 6-HAP nucleobase per se, as reported in (*3*), we combined the Δ*moaA* defect with a set of mutations blocking all possible pathways for conversion of 6-HAP to its nucleoside monophosphate form (see Fig. 1A). In this new strain (*moaA deoD apt gpt hpt*), both 6-HAP and the MO34 extract completely lost their toxicity and mutagenic potential (Fig. 1D), indicating that the 6-HAP base per se is inactive. If 6-HAP toxicity were due to inhibition of replication by the 6-HAP base, as proposed in (*3*), then an opposite effect (6-HAP hypersensitivity) might be predicted for such a nonmetabolizing strain.

Yeast *S. cerevisiae* does not have Moco. Instead, most 6-HAP detoxification in this organism occurs at the level of the d(6-HAP)TP triphosphate by inosine triphosphate pyrophosphatase (ITPA) (*19*), encoded by the *HAM1* gene (Fig. 1A) (*20*–*22*). Using a strain with a *HAM1* deletion gave us an opportunity to further characterize the mutagenic mechanism of the 6-HAP contained in the MO34 extract. In these experiments, we assayed the production of canavanine-resistant mutants during growth in 6-HAP–containing liquid medium (see Materials and Methods). As shown in Fig. 1E, both synthesized 6-HAP preparations were much more efficient mutagens in the yeast *ham1* mutant than in the wild-type strain. The MO34 extract was nonmutagenic in the wild-type strain (because of the limited amount of 6-HAP in the extract) but was mutagenic in the *ham1* mutant. These results are fully consistent with d(6-HAP)TP being the ultimate biologically active metabolite of 6-HAP.

Consistent with the requirement for 6-HAP to be converted to the triphosphate level, we also demonstrated that 6-HAP in its base form did not have an inhibitory effect on a DNA polymerase reaction, as was asserted in (*3*). As seen in Fig. 2, no effect on DNA polymerase activity was seen at 6-HAP concentrations 100- to 10,000-fold higher than the normal DNA precursor dATP, the first nucleotide to be incorporated opposite template "T." As further illustrated in fig. S1, no suppression of DNA synthesis by 6-HAP was seen with a template where the first incorporation is that of dCTP opposite template "G," or for both templates with all four deoxyribonucleoside triphosphates (dNTPs) provided. At present, we cannot find a ready explanation for the result reported in (*3*), in which, in a similar setup, 6-HAP, at an unspecified concentration, completely inhibited incorporation of dATP opposite template T.

To further characterize the mutagenic properties of the 6-HAP present in the MO34 extract, we first quantitated the amounts of extract needed to produce the same mutagenic activity as synthetic 6-HAP. For this, we used the Δ*moaA* bacterial strain and quantitated

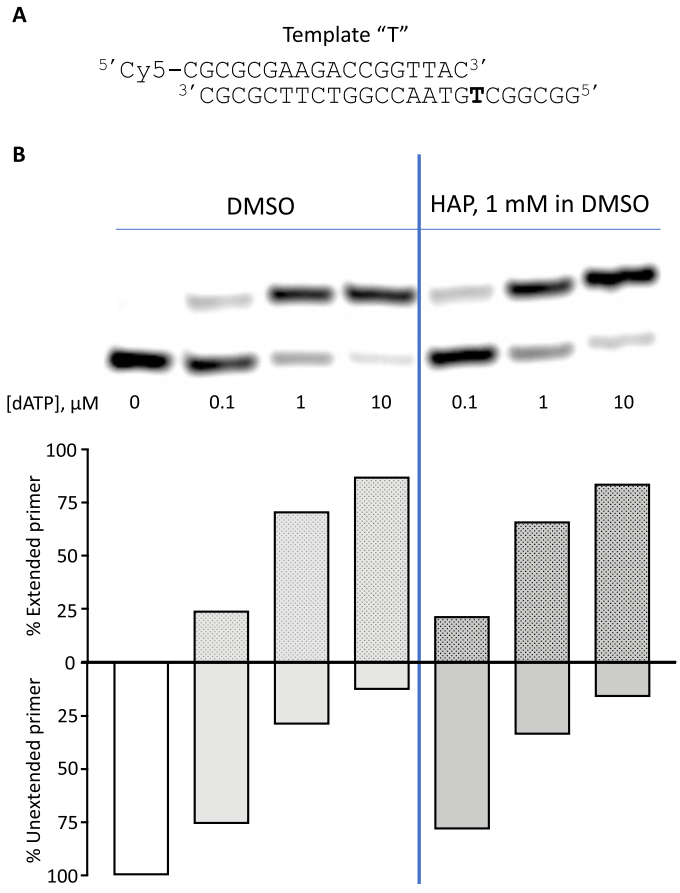

Fig. 2. 6-HAP does not inhibit DNA synthesis in vitro. (**A**) Primer-template design. (**B**) DNA polymerase primer extension reactions in the presence of 6-HAP or with control solvent, DMSO. 6-HAP (1 mM) did not prevent incorporation of dATP opposite template T, even when in 100- to 10,000-fold excess. The reaction was with the Exo⁻ Klenow fragment of *E. coli* Pol I, 0.25 U per reaction, for 10 min at 37°C. See Materials and Methods for a full description and fig. S1 for additional corroborating results.

the frequency of induced D-cycloserine–resistant (Cyc^r) mutants upon exposure in liquid medium (see Materials and Methods). As seen in Fig. 3A, overlapping dose-response curves were obtained using 250-fold less 6-HAP than the MO34 extract. We then determined the DNA changes in the *cycA* gene for 64 independent Cyc^r mutants induced by 6-HAP and 64 Cyc^r mutants induced by the MO34 extract at equimutagenic concentrations of 4 and 1000 ng/ml, respectively, in addition to 63 spontaneous Cyc^r mutants. A summary of the sequenced mutations is shown in Table 1, and the full spectrum is provided in the fig. S2. In contrast to the spontaneous mutation spectrum, which predominantly consists of deletions and duplications (indels) (78% of total mutations), exposure to both 6-HAP and MO34 extract caused a dramatic and specific increase in the proportion of transition base-pair substitutions (A·T→G·C and G·C→A·T) (Table 1 and Fig. 3, B and C), a result fully consistent with previously reported data on the mutagenic specificity of 6-HAP (*5*, *16*, *23*). Analysis of the distributions of the substitutions across the target sequence did not reveal any significant differences between 6-HAP– and MO34-induced mutations ($P = 0.59$), while both 6-HAP and MO34 extract produced spectra that were different from the spontaneous spectrum ($P = 0.02$

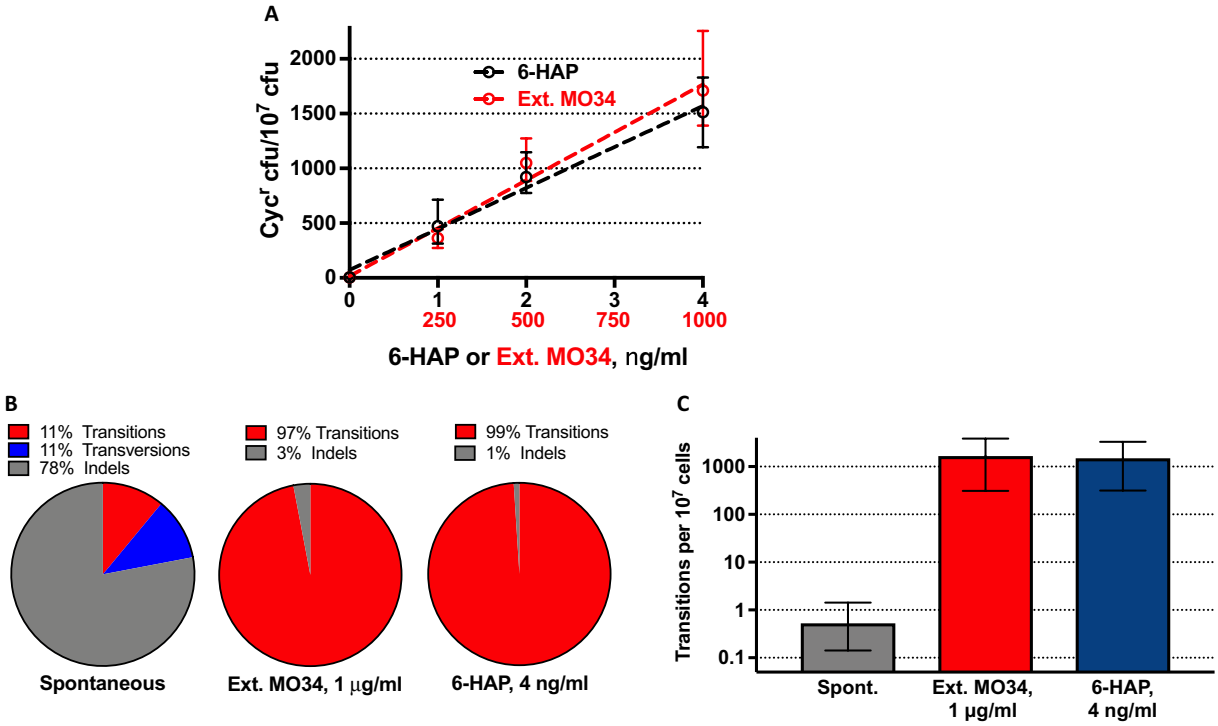

Fig. 3. Mutagenic specificity of MO34 is identical to that of 6-HAP. (**A**) An essentially identical linear dose-response curve can be obtained for the frequency of Cyc^r *E. coli* mutants as a function of 6-HAP or MO34 extract concentration. This requires 250-fold less of pure 6-HAP than of MO34 extract, likely representing the relative 6-HAP content of the extract. Presented are median mutant frequencies obtained from four separate experiments with 95% confidence intervals. (**B** and **C**) The extract from the *S. epidermidis* strain MO34 induces the same proportion of transition mutations as authentic 6-HAP (B) and with the same frequency (C).

Table 1. DNA sequence changes in spontaneous, MO34-, and 6-HAP–induced D-cycloserine–resistant *cycA* mutants.

Mutation type	Spontaneous No. (%)	MO34 (1 μg/ml) No. (%)	6-HAP (4 ng/ml) No. (%)
Transitions	**7 (11%)**	**64 (97%)***	**65 (99%)***
G·C→A·T	5 (7.9%)	19 (29%)	34 (52%)
A·T→G·C	2 (3.2%)	45 (68%)	31 (47%)
Transversions	**7 (11%)**	**0**	**0**
G·C→T·A	3 (4.8%)	0	0
G·C→C·G	1 (1.6%)	0	0
A·T→C·G	3 (4.8%)	0	0
Deletions 1 nt	**9 (14%)**	**0**	**1 (1%)**
Deletions >1 nt	**15 (24%)**	**2 (3%)**	**0**
Duplications	**9 (14%)**	**0**	**0**
IS elements	**16 (25%)**	**0**	**0**
Total mutations	63	66**	66**

*Significantly different from the spontaneous value (Fisher's exact test, $P < 10^{-4}$). **Two Cyc^r clones contained two mutations in the *cycA* gene.

and 0.01, respectively). Correlation analysis revealed a statistically significant similarity between the 6-HAP and MO34 spectra [linear correlation coefficient (CC), 0.343; $P < 0.01$], while both 6-HAP– and MO34-induced spectra did not show any significant correlation with the spontaneous spectrum (CC, 0.011 and −0.115). Therefore, our detailed mutational analysis strongly supports the contention that the 6-HAP present in the extract of strain MO34 has intrinsically the same mutagenic potential and specificity as chemically synthesized 6-HAP.

DISCUSSION

The results presented here confirm that the extract of the *S. epidermidis* strain MO34 contains 6-HAP as reported in (*3*), but in stark contrast with that report, we demonstrate that the 6-HAP produced by MO34 is highly mutagenic. Furthermore, we show that the active metabolite responsible for the biological effect of 6-HAP is a 6-HAP nucleotide, not the 6-HAP base itself (*3*). Our findings are in full agreement with previous data that have established a firm connection between 6-HAP–induced killing and the incorporation of 6-HAP nucleotide into DNA. These data include (i) the demonstrated hypersensitivity to 6-HAP–induced killing of yeast strains lacking the Ham1 d(6-HAP)TPase (*11*, *21*); (ii) suppression of this *ham1* hypersensitivity by mutations in replicative DNA polymerases that lower 6-HAP mutagenesis, such as *pol2*ts mutations affecting replicative DNA polymerase ε (*21*), or, alternatively, by increases in yeast ploidy (*11*, *24*); (iii) increased resistance to 6-HAP by *E. coli* strains containing the *dnaE915* antimutator DNA polymerase (*13*); (iv) demonstrated protection of 6-HAP–sensitive *E. coli* by expression of yeast Ham1p (*20*); (v) further sensitization of *E. coli* Δ*moa* strains by loss of the d(6-HAP)TPase encoded by the *rdgB* gene (*4*); and (vi) suppression of 6-HAP hypersensitivity of the *E. coli* Δ*moa* Δ*rdgB* double mutant by elimination of endonuclease V (*nfi* gene product)

(*4*). The repair enzyme endonuclease V is capable of recognizing HAP in DNA, producing endonucleolytic incisions at these sites, which may then lead to double-strand breaks, chromosomal fragmentation, and cell death (*25*–*27*).

We found no evidence in our study that the 6-HAP base itself can inhibit or interfere with a DNA polymerase reaction (*3*). In contrast, the active compound used by DNA polymerases is the deoxyribonucleoside triphosphate derivative of 6-HAP, d(6-HAP)TP, as amply corroborated by in vitro data on its incorporation by DNA polymerases (*12*–*14*) and in vivo data on the editing of 6-HAP–induced replication errors by the exonucleolytic proofreading function (*28*, *29*). 6-HAP, therefore, is a classical replicative mutagen, which, when activated to d(6-HAP)TP, can be incorporated into DNA by DNA polymerases primarily opposite template T or C residues, in this manner creating transition mutations during cycles of replication (*30*, *31*).

The putative anticancer action of 6-HAP does not contradict its high mutagenic activity, as many anticancer drugs are strong mutagens. When 6-HAP was first synthesized almost 50 years ago, it was tested for antiproliferative activity but appeared to have adverse effects (*32*). 6-HAP was shown to break chromosomes in epidermoid carcinoma (*33*). Newer studies have revealed the ability of 6-HAP to cause DNA breaks and apoptosis in human cancer cells (*15*, *34*). It is possible that a particular balance between proapoptotic and mutagenic activities of 6-HAP is required for anticancer activity. This balance might be concentration dependent and could be manifested differently depending on tissue type and the methods of 6-HAP administration.

The lack of 6-HAP–induced mutagenesis as reported in (*3*) was most likely due to usage of improperly chosen HAP concentrations. In the Ames test, the doses of HAP (0.5 and 1 μg/ml) that were applied to the Moco-deficient, HAP-hypersensitive strain TA100 (*35*, *36*) were too high and likely caused 100% killing during the incubation period in minimal medium in the presence of analog. Previous experimentation has shown that a fourfold lower concentration of 6-HAP (0.125 μg/ml) can kill approximately 96% of Moco-deficient bacterial cells during a 2-hour exposure in minimal medium (*8*, *37*). Therefore, only *his*$^+$ revertants of TA100 carrying a second suppressor mutation yielding HAP resistance (e.g., *yjcD*; (*7*)) can be selected under these conditions, which leads to a dramatic underestimation of HAP-induced *his*$^+$ reversion frequencies. Possibly, the 4-day incubation period applied to the TA100 cells (*3*) is also consistent with a requirement for production of suppressor variants. In the mouse lymphoma mutagenesis assay (*3*), on the other hand, the concentration of 6-HAP (0.25 μg/ml) was likely too low to induce mutations (assuming normal sensitivity of the tested cells to 6-HAP) (*15*). For example, the same concentration of 6-HAP does not induce mutations in wild-type *E. coli* grown in rich culture medium (*9*, *38*). We suggest that higher concentrations of HAP (at least 5 μg/ml) are required to induce mutations in the mouse lymphoma assay, as were used for the case of mutagenesis assays with Syrian hamster embryo fibroblasts (*39*, *40*) or in wild-type *E. coli* (*13*, *41*) and yeasts (*18*, *22*) grown in rich media.

MATERIALS AND METHODS

Chemicals

Extracts of *S. epidermidis* strains MO34 (6-HAP producing) and of control strain 1457, as well as the sample of chemically synthesized 6-HAP, were provided by T. Nakatsuji and R. L. Gallo (University of California, San Diego) (*3*). We also used 6-HAP (also called N^6-hydroxyadenine), purchased from MP Biomedicals (cat. # 101998, Santa Ana, CA) or Natland International Corporation [NIC; Research Triangle Park, NC; custom-synthesized for the National Institute of Environmental Health Sciences (NIEHS)]. The identity of the compounds was confirmed by mass spectrometry (MS) and nuclear magnetic resonance spectra by the companies and in-house analysis (fig. S3); they have the same biological properties (Fig. 1). Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was routinely used as a solvent.

Yeast strains

We used *S. cerevisiae* strains ES15 (*MAT*a *CAN1 his7-2 leu2Δ::kanMX ura3Δ trp1-289 lys2ΔGG2899-2900*) and ES18 (same, but *ham1::LEU2*). They are [ψ−] derivatives of Δl(−2)l-7B-YUNI300 (*42*), in which the *ade5-1* allele was corrected to Ade$^+$ by transformation with a polymerase chain reaction (PCR) fragment corresponding to the wild-type *ADE5,7* gene (*43*).

Bacterial strains

All experiments were performed with *E. coli* MG1655 (*fnr*$^+$ *rph-1*) (Coli Genetic Stock Center #6300) and its isogenic derivatives. MG1655-Δ*moaA753*::FRT-FRT was constructed by P1 transduction of the Δ*moaA753*::FRT-*kan*-FRT allele from the Keio collection (*44*) into MG1655, followed by elimination of the *kan* marker using plasmid pCP20, which expresses flippase (FLP) recombinase (*45*). MG1655 derivatives carrying the Δ*moaA753*::FRT-FRT, Δ*deoD780*::FRT-FRT, Δ*apt754*::FRT-FRT Δ*hpt743*::FRT-FRT, and Δ*gpt756*::FRT-*kan*-FRT alleles were likewise constructed by the addition of *kan*-containing gene deletions from the Keio collection followed by elimination of the *kan* marker using pCP20 on each iteration.

Genetic experiments for 6-HAP toxicity and mutagenicity

For spot tests with *E. coli*, saturated cultures grown in LB medium were diluted 30-fold in 1× M9 salts and transferred to M9 glucose plates (*46*) using a multiprong replicator device (approximately 0.1 ml of the culture per plate in total). After the spots had dried, a filter paper disc was placed on the center of each plate, and an appropriate volume of DMSO-dissolved extracts of *S. epidermidis* strains MO34 and 1457 (5 mg/ml) or 6-HAP, either synthesized in R. L. Gallo's lab or synthesized by NIC, was spotted onto the paper disc. A DMSO-only control was also included. The plates were incubated overnight at 37°C and inspected the next day for zones of inhibition around the discs. The plates were then replica plated on LB plates supplemented with rifampicin (100 mg/liter), incubated overnight at 37°C, and inspected for the appearance of Rif^r colonies around the discs. For quantitative mutagenesis tests with *E. coli*, at each concentration of MO34 extract or 6-HAP (NIC), 15 to 20 independent 1-ml M9 glucose cultures were started containing approximately 10^5 cells. The cultures were incubated with shaking for 24 hours at 37°C. The frequencies of D-cycloserine–resistant (Cyc^r) mutants occurring in the *cycA* gene were determined by plating 50 to 100 μl of a 10^{-1} dilution (for spontaneous samples) or a 10^{-2} dilution (for MO34- or 6-HAP–induced samples) on M9 glucose plates containing 50 μM D-cycloserine to obtain the number of Cyc^r colony-forming units (cfu) per milliliter, and by plating 100 μl of a 10^{-6} dilution on LB plates to obtain the total number of colony-forming units per milliliter. The experiments were repeated four times. To determine the exact nature of the Cyc^r mutations, the *cycA* gene was amplified from a large number of randomly chosen Cyc^r colonies from independent cultures in four experiments using primers cycAF1 (5′-CCCGTAAGCGTGTATTTT

TG-3′) and cycAR1 (5′-CCTGGAAAGCGATGTATAACG-3′), and the PCR products were sequenced with the same primers, as described (*47*).

For mutagenesis experiments with yeast, *S. cerevisiae* strains ES15 and ES18 (*ham1::LEU2*) were streaked out on YPDAU-rich medium [YPD (yeast extract, peptone, dextrose) plus adenine and uracil (*48*)]. For each strain, six single colonies were resuspended in individual tubes with YPD medium and grown until the cultures entered the early log phase of growth (3 to 6 hours). Each culture was then split and exposed to DMSO only, 6-HAP (MP Biomedicals), or MO34- or 1457-derived extracts. After overnight incubation with shaking, cells were plated to determine canavanine-resistant mutant frequencies using plates with canavanine or minimal complete plates, as described (*49*).

DNA polymerase reactions

We used oligonucleotides carrying a Cy5 fluorescent label at the 5′ end, which had the same exact sequences as used in (*3*). The DNAs were synthesized by Integrated DNA Technologies, Inc. (IDT) (USA), and we further purified them by polyacrylamide gel electrophoresis (PAAG) electrophoresis. Primer-template pairs (1 μM) were annealed in 50 μl of 50 mM KCl by heating the mixture to 90°C for 2 min floating in a beaker with 300 ml of water and allowing a slow cool down to room temperature. Polymerase reactions (total volume, 10 μl) were performed in 1× Klenow DNA polymerase buffer (NEB 2), 100 nM annealed substrate, 0.25 U (165 nM) of exonuclease-deficient DNA polymerase I Klenow fragment, and dNTPs at concentrations indicated in Fig. 2 or fig. S1 (ranging from 0.01 to 10 μM) in the presence or absence of 1 mM 6-HAP (MP Biomedicals). The reactions were incubated for 10 min at 37°C and terminated by formamide-EDTA dye stop solution. The products were denatured by brief heating at 90°C and resolved by electrophoresis on 16% PAAG-urea gels. The gels were scanned on a Typhoon imager using a red laser, and the bands were quantified using ImageJ software (National Institutes of Health). For each lane, the percentage of extended (or unextended) primer was calculated as the ratio of the intensity of the corresponding band over the total (extended plus unextended) intensity, multiplied by 100%.

Statistical methods

To assess differences among mutant frequencies, the Mann-Whitney *U* test was applied. Nonparametric confidence intervals for medians were calculated as described (*50*). A two-tailed Fisher's exact test was used to compare the relative proportions of specific types of mutation within sequenced *cycA* sample sets. In a different method, a Monte Carlo modification of the Pearson χ^2 test of spectra homogeneity (*51*) was used to compare mutation distributions. Calculations were done using the COLLAPSE program (*52*), and correlation analyses were performed using the STATISTICA package.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/9/eaaw3915/DC1

Fig. S1. HAP in a form of nucleobase does not interfere with DNA synthesis.

Fig. S2. Distribution of sequenced D-cycloserine–resistance mutations along the *cycA* gene.

Fig. S3. Confirmation of chemical identity of preparations of 6-HAP used in the study.

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Acknowledgments: We are grateful to T. Nakatsuji and R. L. Gallo (University of California, San Diego) for providing samples of the extract of MO34, control strains, and synthetic 6-HAP. We thank K. Bebenek and J. Williams of the NIEHS for the helpful comments on the manuscript for this paper. **Funding:** The study was funded by the UNMC Buffett Cancer Center Pilot grant 06_2018 (to Y.I.P.); the Intramural Research Program of the National Institutes of Health, National Library of Medicine (IBR); and project number Z01 ES065086 of the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (to R.M.S.). M.A.'s experimentation in Y.I.P.'s laboratory was supported by the National Cancer Institute Youth Enjoy Science (YES) Research Education Program (grant NCI R25 CA221777).
Author contributions: S.G.K., R.M.S., and Y.I.P. conceived the study. S.G.K. and R.M.S. performed experiments with bacteria, and M.A., E.A.M., and Y.I.P. performed experiments with yeast and DNA polymerase reactions in vitro. I.B.R. performed the statistical comparison of mutation spectra. S.G.K., R.M.S., and Y.I.P. wrote the manuscript, which was further edited by all authors. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors. The bacterial strains can be provided by SGK/RMS pending scientific review and a completed material transfer agreement. Requests for the yeast strains and oligonucleotides should be submitted to Y.I.P.

Submitted 15 December 2018
Accepted 15 August 2019
Published 11 September 2019
10.1126/sciadv.aaw3915

Citation: S. G. Kozmin, I. B. Rogozin, E. A. Moore, M. Abney, R. M. Schaaper, Y. I. Pavlov, Comment on "A commensal strain of *Staphylococcus epidermidis* protects against skin neoplasia" by Nakatsuji *et al*. *Sci. Adv.* **5**, eaaw3915 (2019).