HEALTH AND MEDICINE

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

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Kozmin *et al.* contend that observations previously reported regarding the antimicrobial and antitumor activities of 6-*N*-hydroxy aminopurine (6-HAP) were incorrect. Their conclusions rely on poorly characterized reagents and focus strictly on in vitro techniques without validation in relevant mammalian model systems. We are pleased to be able to illuminate the weaknesses in their technical comment. The totality of current results continues to support our original conclusion that a strain of the common human commensal skin bacterium, *Staphylococcus epidermidis*, produces 6-HAP that can inhibit tumor growth.

THE REAGENT USED BY KOZMIN *ET AL.* WAS NOT 6-HAP

In their technical comment (*1*), the authors report that they used a commercially available form of 6-*N*-hydroxy aminopurine (6-HAP) purchased from MP Biomedicals (Chemical Abstracts Service no. 5667-20-9). We will refer to this chemical as 6-HAP-MPB. The authors used this reagent to test in vitro mutagenic activity. To investigate their discrepancies with our findings, we purchased 6-HAP-MPB from this supplier and observed that the reagent upon which they based their conclusions is not 6-HAP. That 6-HAP-MPB is not 6-*N*-hydroxy aminopurine is obvious from the package label that states this commercial product has a molecular weight of 1151.13 (Fig. 1). This does not correspond to the mass of 6-HAP (theoretical mass, 151.0489). To determine whether this was only a typographical error, we next applied high-resolution electrospray ionization time of flight mass spectrometry (HR-ESI-TOF-MS) analysis to the commercial lot of 6-HAP-MPB (Fig. 2). Three major peaks [mass/charge ratio (m/z) = 257.2476, 285.2789, and 445.3736] were detected by positive mode that correspond to $C_{16}H_{33}O_2$, $C_{18}H_{37}O_2$, and $C_{21}H_{46}N_7O_3$, respectively. No mass corresponding to 6-HAP was detected in this lot of 6-HAP-MPB. Furthermore, since authentic 6-HAP has potent antimicrobial activity against group A *Streptococcus*, we also tested the antimicrobial function of the commercial compound used by Kozmin *et al.* 6-HAP-MPB did not have antimicrobial activity (Fig. 3). We therefore suggest that their conclusions are based on an unknown substance and not 6-HAP. Furthermore, the identification error made by Kozmin *et al.* is not without precedent. Synthetic products assumed to be 6-HAP have been studied in previous papers (*2*, *3*). Mutagenic activity was reported for a compound assumed to be 6-HAP without characterization of the chemical structure of the reagent used for testing (*4*, *5*). Kozmin *et al.* repeated this error and also based their conclusions on an uncharacterized commercial product.

Validation of reagents is essential for all experimentation, but particularly necessary in this case. In our report, we identified 6-HAP from an unbiased screen of bacterial products without prejudice. The chemical structure of the active molecule produced from *Staphylococcus epidermidis* MO34 was solved by high-performance liquid chromatography, high-resolution ESI-MS, isotope incorporation assay, ^{1}H nuclear magnetic resonance, 2D-gHMBC (two-dimensional gradient-selected heteronuclear multiple-bond correlation) spectral data, and fragmentation profile on ESI-MS. This analysis was followed by repeat chemical synthesis and validation of the synthetic molecule [see original figures from our manuscript (*6*): Fig. 1D and figs. S1 to S5]. Given the results from these multiple independent approaches, we confirmed that we were working with 6-HAP. No such characterization was done by Kozmin *et al.*, and therefore, the conclusions they reached are not valid.

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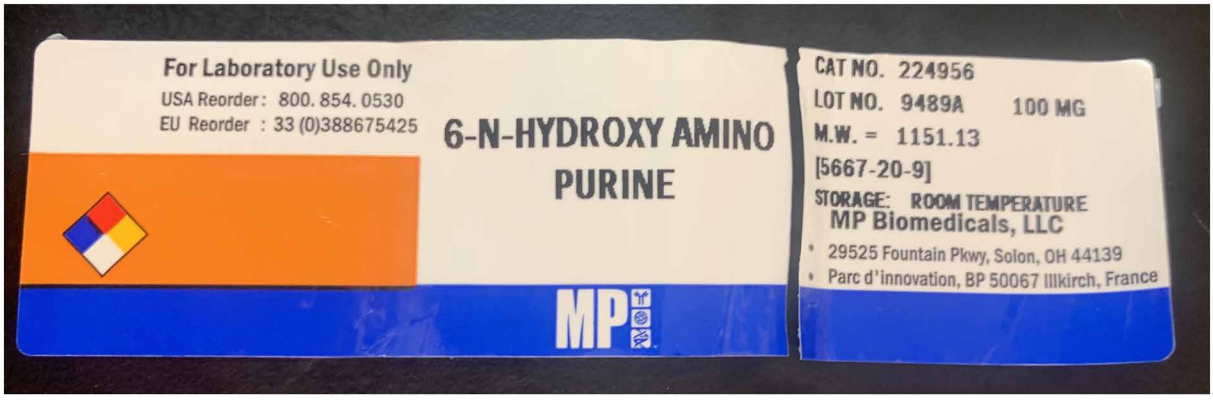
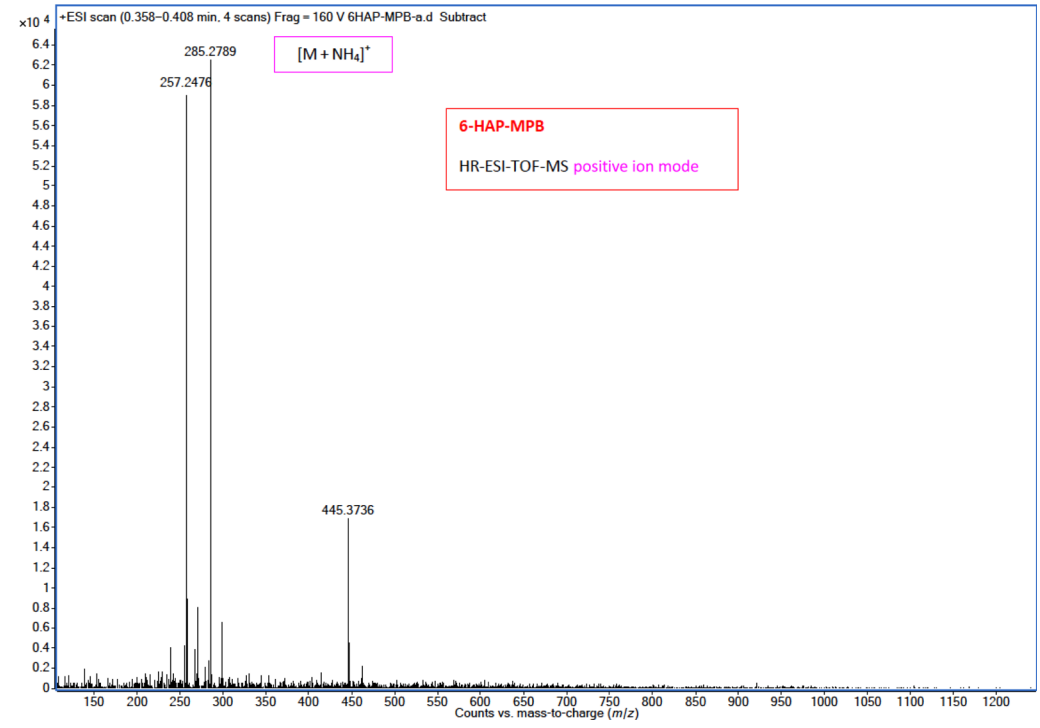


Fig. 1. Molecular weight of 6-HAP-MPB on the package label does not correspond to that of 6-HAP. (Photo credit: Teru Nakatsuji, University of California, San Diego.)

A



B

Mass measured	Theoretical mass	Delta (ppm)	Estimated composition
257.2476	257.2475	0.4	$[C_{16}H_{33}O_2]^+$
285.2789	285.2788	0.4	$[C_{18}H_{37}O_2]^+$
445.3736	444.3662	0.25	$C_{21}H_{46}N_7O_3$

Fig. 2. 6-HAP-MPB does not contain a chemical that is identical to 6-HAP. (**A**) 6-HAP-MPB was dissolved in 99% acetonitrile/1% TFA (trifluoroacetic acid) and subjected to high-resolution ESI-TOF-MS. Peaks were scanned between *m/z* 100 and 1250 in positive ion mode. (**B**) Estimated chemical composition of three major peaks detected in 6-HAP-MPB. None of them correspond to that of 6-HAP (theoretical mass, 151.0489).

MUTAGENESIS ASSAYS USED BY THE AUTHORS WERE NOT PHYSIOLOGICALLY RELEVANT

To support their conclusions with the unknown chemical in 6-HAP-MPB, they also performed some experiments using a crude synthetic product and an extract of the culture supernatant of *S. epidermidis* MO34. These were given to them with information that these reagents required further characterization and purification. These crude products are unstable. 6-HAP is spontaneously deoxidized within 24 hours in aqueous solutions at normal atmosphere and room temperature. This results in a molecule with a chemical formula of $C_5H_5N_5$, which does not have biological activity (Fig. 4). Without validation of their working reagents, their conclusions must be considered unreliable.

Even if Kozmin *et al.* had used authentic 6-HAP, the assays they used were not physiologically relevant. To measure mutagenic activity by Ames test or a mouse lymphoma assay, target cells must be incubated with a mutagen for 4 and 7 days, respectively. This is much longer than the term of stability of 6-HAP in solution. In addition, as shown in our original manuscript, mitochondorial amidoxyme–reducing components in normal human cells rescue cells from the antimetabolite activity of 6-HAP. As a consequence of this rapid inactivation reaction,

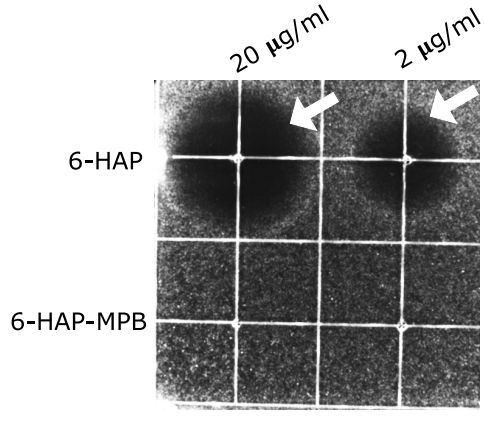
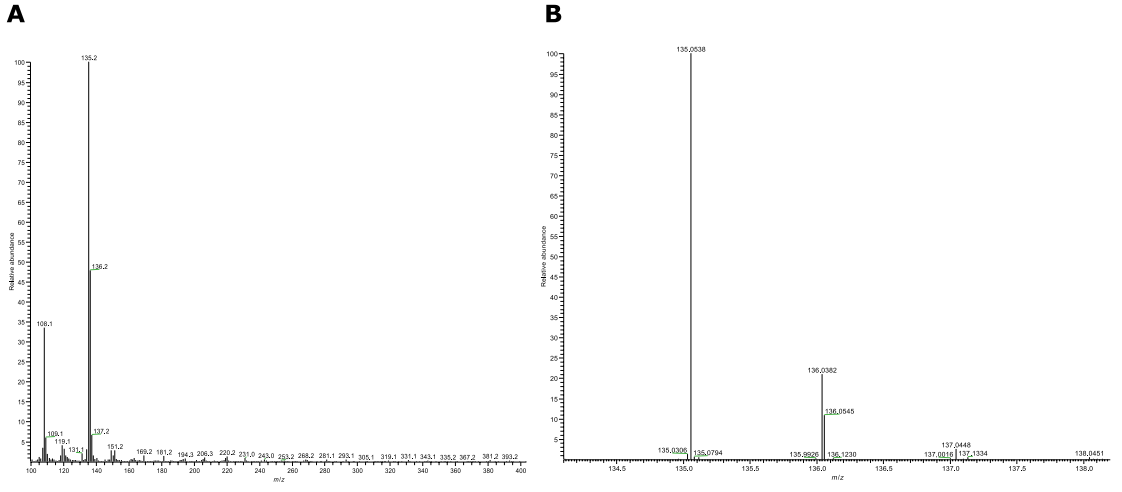


Fig. 3. 6-HAP has antimicrobial activity, but 6-HAP-MPB does not. Antimicrobial activity of indicated concentration of 6-HAP or 6-HAP-MPB was measured against group A *Streptococcus* (NZ131) with a radial diffusion assay. Black circle of zone of growth inhibition represents antimicrobial activity of 6-HAP (arrows). The method of this assay has been provided in our original manuscript.



C

Mass measured	Theoretical mass	Delta (ppm)	Estimated composition
135.0538	135.0539	−0.7	$C_5H_5N_5$
136.0382	136.0380	1.5	$C_5H_4N_4O$

Fig. 4. 6-HAP can be spontaneously deoxidized in solution within 24 hours. (**A** and **B**) Native 6-HAP was dissolved in heated H_2O (60°C) at 0.1 mg/ml and incubated at room temperature for 24 hours. Degradation of 6-HAP was characterized by low-resolution ESI-TOF-MS (A), followed by the high-resolution mode (B). All 6-HAP was degraded after a 24-hour incubation. (**C**) Estimated chemical composition of two major peaks detected in (B).

6-HAP does not show systemic toxicity in mice and does not inhibit the growth of normal human keratinocytes.

To avoid the natural instability of 6-HAP, Kozmin *et al.* conducted their mutagenic assays using a Δ*moaA* bacterial mutant, which cannot detoxify 6-HAP to the canonical nucleobase. They detected mutagenic activity only in the Δ*moaA* mutant but not in wild-type bacteria. This illuminates another example of why the conclusions in their commentary are not physiologically relevant. The authors also argue that 0.5 and 1 μg/ml at which we tested for mutagenic activity by Ames test was too high; however, *Escherichia coli* TA100 used for the Ames test is not killed at these concentrations by 6-HAP. We have also shown that this concentration is relevant since the final yield of purified 6-HAP is 7 mg from 6.4 liters of conditioned media of *S. epidermidis* MO34. Therefore, the concentration we tested was comparable to the concentrations which bacteria can produce in vivo. The authors' claim of a false-negative Ames test is without data to support it.

The authors also invert their argument to claim that the concentration of 6-HAP we used in our mouse lymphoma assay (0.25 μg/ml) was too low. They suggest use of at least 5 μg/ml of 6-HAP to test mutagenic activity in this assay. However, they ignored data showing that native 6-HAP at >0.2 μg/ml inhibits BrdU (bromo deoxyuridine) incorporation in the mouse lymphoma cell line F5178 (parental cell line of T5178Y$tk^{+/-}$ used for mouse lymphoma assay). Thus, we tested the mutagenic activity on mouse lymphoma at the highest concentration that did not inhibit cell proliferation. These toxic effects of 6-HAP on cells that lack mitochondorial amidoxyme–reducing components explain the antitumor effects of 6-HAP. It cannot be a mutagen in transformed cells because of its toxicity and cannot be a mutagen in normal cells because of its instability.

MOUSE ASSAYS SHOW 6-HAP INHIBITS TUMOR GROWTH

Last, but perhaps most importantly, the authors do not mention data from our publication that validated conclusions by using two different mouse models. We demonstrated that repeated injections with validated and purified 6-HAP inhibited growth of a melanoma cell line growing in mice. We also showed that applying *S. epidermidis* MO34 that produces 6-HAP, but not a control *S. epidermidis* strain, suppressed new tumor formation in a two-stage carcinogenesis model. Mutagenesis was not observed in mice injected with 6-HAP or colonized by an MO34 strain for 12 weeks. These observations in mice were also supported by metagenomic evidence that *S. epidermidis* strains with the capacity to produce 6-HAP are common in healthy human populations. Together, these data in mice and observations in humans make it highly improbable that 6-HAP promotes mutagenesis in physiological settings.

CONCLUSIONS

We disagree with the statements by Kozmin *et al.* that 6-HAP is mutagenic. In direct contrast to the comments of these authors, our results were based on an unbiased approach followed by careful chemical characterization, physiologically relevant bioassays, mouse experiments, and human observations. These results all support the prior conclusion that 6-HAP is protective against tumor development. We encourage further careful work to address this issue. Although either conclusion is highly relevant and highlights the importance of the microbiome to human health, a careful evaluation of currently available data suggests that 6-HAP is a beneficial product of the skin microbiome and not a mutagenic chemical.

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