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Photomutagenicity of cosmetic ingredient chemicals azulene and guaiazulene

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Abstract

The photomutagenicity of the popular skin conditioning agents azulene and guaiazulene were tested in *Salmonella typhimurium* TA98, TA100 and TA102. Following irradiation with UVA and/or visible light, both azulene and guaiazulene exhibited mutagenicity 4–5-fold higher than the spontaneous background mutation. In contrary, naphthalene, a structural isomer of azulene, was not photomutagenic under the same conditions. Azulene was photomutagenic when irradiated with UVA light alone, visible light alone, or a combination of UVA and visible light. Azulene and guaiazulene are not mutagenic when the experiment is conducted with the exclusion of light. Therefore, extreme care must be taken when using cosmetic products with azulene/guaiazulene as ingredients since after applying these products on the skin, exposure to sunlight is inevitable.

Keywords

Azulene; Guaiazulene; Photomutagenicity; Light irradiation; Ames test; TA102

1. Introduction

Azulene is a non-alternate hydrocarbon, consisted of an unsaturated seven member ring fused with an unsaturated five member ring (Fig. 1). In contrast to its non-polar structural isomer naphthalene, azulene has a highly polar asymmetric structure, with a large permanent dipole moment of 0.80 ± 0.02 D [1]. As such, azulene absorbs the red light in the visible region (600 nm band) for the first excited-state transition and in the UVA light (330 nm band) for the second excited-state transition to initiate possible excited-state photochemical reactions [2]. Due to its light absorption, azulene aqueous solutions produces a beautiful blue color. This is drastically different from naphthalene which absorbs light only in the UVB region (<315 nm).

Azulene and its alkyl derivative guaiazulene, 1,4-dimethyl-7-isopropylazulene (Fig. 1), are popular gradients in beauty, cosmetic, skin, and body care products [3,4]. Azulene is an essential oil derived from German chamomile plant *Matricaria chamomile*. In addition to its wonderful aroma, this oil is an anti-inflammatory, anti-spasmodic, and anti-microbial agent [5,6]. Reports from various sources including the United States Food and Drug Administration showed that azulene is a popular ingredient for various products for human uses [3–5]. Its toxicology to experimental rodents, including mutagenicity and the clinical assessment of its safety, has been reported [5,6]. When dosed orally, azulene exhibited acute toxicity of LD₅₀ of 3 g/kg in mice and 4 g/kg in rats, respectively. When azulene was

injected into the brains of rats, it caused non-space-occupying lesions [7]. Azulene was found not mutagenic in *Salmonella typhimurium* bacteria strains TA98, TA100, TA1535, and TA1537 with and without the presence of an S9 activation enzyme system [5]. In clinical studies, some patients showed allergic reactions to azulene [8].

We previously reported that under UVA light irradiation, azulene can cause DNA single strand cleavage [9] and DNA damage can lead to mutation. Therefore, we report in this paper that both azulene and guaiazulene are photomutagenic in *Salmonella* TA102 with concomitant irradiation by UVA and/or visible light.

2. Materials and methods

2.1. Materials

S. typhimurium TA98, TA100, and TA102 strains were kindly provided by Dr. Ames from the University of California (Berkeley, CA). Azulene, guaiazulene, naphthalene, and 8-methoxypsoralen (8-MOP) were purchased from Sigma–Aldrich Chemical Co. (Milwaukee, WI). Other chemicals and solvents were used in their highest purity grade.

2.2. Light sources

Two light sources were used in this study: a fixed-intensity UV transilluminator light with a emission band at 365 nm from six 15 W UVA tubes from Spectronics Corporation (Westbury, NY), and a 300 W Xe/Hg(Xe) lamp from ORIEL Instruments (Stratford, CT) producing a full-spectrum light ranging from 300 to 800 nm. A Pyrex glass plate (1 mm in thickness) was placed on top of the light beam at 14.2 cm for the UV transilluminator light and at 40 cm for the Xe/Hg(Xe) lamp. This distance is carefully maintained to obtain the same UVA irradiation energy for both light sources. The Pyrex glass efficiently filtered off lights at wavelengths below 300 nm (0% transmittance) and only allowed <20% to pass through for lights between 300 and 320 nm as tested by a CARY 3 UV-Vis absorption spectrometer from Varian Inc. (Houston, TX). The output energies for the Xe/Hg(Xe) lamp were 7.0 mW/cm² for visible light (400–700 nm), 3.8 mW/cm² for UVA light (315–400 nm), and 0.012 mW/cm² for UVB light (280–315 nm). Output energy was measured using a photo-radiometer equipped with UVA, UVB, and visible light probes from SOLAR Light CO. (Philadelphia, PA) after the Pyrex filter glass. The output energies for UVB and visible light for the transilluminator lamp were zero and for UVA was 3.8 mW/cm², the same as the Xe/Hg(Xe) lamp. Although UVB is known to be much more mutagenic [10], an control experiment showed that irradiation with the Xe/Hg(Xe) lamp (>10 J/cm² of UVA light) only increased the mutation frequencies by 1.5–1.9 times comparing with spontaneous background as will be shown later.

2.3. Photomutagenicity assay

The assays were conducted in *S. typhimurium* histidine auxotrophic strains TA98, TA100, and TA102 concomitant with irradiation by light in the presence of azulene, guaiazulene, naphthalene, or azulene photo-products, following the method of Maron and Ames [11], a standardized procedure for the assay in the absence of light irradiation, and the experimental conditions by Utesch and Splittgerber [12] with modifications. Utesch and Splittgerber developed a protocol for bacterial photomutagenicity testing restricting the use of light irradiation doses that induced mutations not higher than two-fold of the control, or maintained bacterial viability higher than 50%, both in the absence of any test compound. As such, to fulfill the criteria, the total medium and UVA light doses used by Utesch and Splittgerber are: 3 and 6 mJ/cm² for TA98, 1 and 2 mJ/cm² for TA100, and 18 and 36 mJ/cm² for TA102, respectively. In our case, irradiation with the 300 W Xe/Hg lamp itself causes the number of revertant bacteria colonies of TA102 to increase 1.5–1.9 times

comparing to the number of revertant colonies due to spontaneous mutation with a UVA dose of 1.1–10 J/cm² or the combined UVA and visible light dose of 3.5 J/cm², whereas it needs 2.5 J/cm² UVA light from the transilluminator light to reach the same number. A higher light dose (>10 J/cm² of UVA) would not produce more revertant colonies, rather bacteria death is observed. Therefore, a UVA dose of 6.5 J/cm² is used for all experiments involving TA102. For TA98 and TA100, since these two strains are more light sensitive, a smaller light dose of 0.24 J/cm² is used. This is much higher than the dose used by Utesch and Splittgerber [12]. Under these light doses, both TA98 and TA100 were able to survive the light and produce the number of revertant colonies from the spontaneous mutation. For the positive control, in the presence of 10 µg/plate of 8-MOP, the number of revertant bacteria colonies for TA102 increases steadily as the light dose increases before reaching a maximum number of about six times the revertant colonies of the spontaneous mutation. This result is comparable to the literature values [12–15].

2.3.1. Procedure—One milliliter of a freshly prepared solution of the test chemical (azulene, guaiazulene, naphthalene, or azulene photoproducts) in dimethylsulfoxide was placed in a 13 mm × 100 mm capped culture tube, to which was added with 1 ml of overnight culture of the tester strain and 5 ml of 0.2 M sodium phosphate buffer (pH 7.4). The azulene photoproducts were obtained by irradiating the azulene solution containing 1 ml of 13.5 mM azulene plus 6 ml of the phosphate buffer for 30 min before mixing with the 1 ml overnight culture of TA102. After mixing, the test tube was incubated at 37°C for 20 min at 210 rpm with a loose fitting cap. In a beaker, melted top agar (100 ml) was added with 10 ml histidine/biotin solution (both 0.5 mM) and mixed thoroughly. Two milliliters (2 ml) of the top agar/histidine/biotin mixture was transferred into each of the 13 mm × 100 mm capped culture tubes at 45 °C in a heating block, followed by addition of 0.7 ml pre-incubated azulene-TA102 culture solution (the pre-incubation was for 20 min for complete interaction of the bacteria with azulene. A later experiment showed that pre-incubation is actually not necessary). The combined 2.7 ml mixture was poured onto the minimal glucose agar plates with the cover on and was left on a leveled surface to allow the agar to harden before they were placed on top of a Pyrex glass (1 mm in thickness) support upside down. Light was allowed to irradiate from the bottom up through the Pyrex glass and the culture plate cover (<0.5 mm) for a desired time period. In a separate test with a Varian CARY 3 UV-Vis absorption spectrometer, we found that the Pyrex glass efficiently filtered off light below 300 nm (0% transmittance) and only allow small portions of light between 300 and 320 nm to pass through (<20% transmittance). The culture plate cover allows light >280 nm to pass through. After irradiation, plates were incubated for 48 h at 37 °C and the revertant colonies were counted with a colony counter (Bantex, Model 920A).

The variability in assays conducted in triplicate was generally <±20%. All test chemicals were assayed on at least two separate occasions with similar results. A negative solvent control and a positive (8-MOP for TA102) control were used throughout all the experiments.

3. Results

3.1. Photomutagenicity of azulene and guaiazulene in *Salmonella typhimurium* tester strain TA102

Photomutagenicity of azulene, guaiazulene, and naphthalene were conducted with the Xe/Hg(Xe) lamp. The concentrations of the chemicals were 0, 62.5, 125, 250, 500 µM (or 0, 22, 43, 86, and 172 µg/plate for azulene). Upon mixing the bacteria with the testing chemical, the agar mixture was irradiated for 30 min to achieve a light dose of 6.5 J/cm² of UVA and 12.6 J/cm² of visible light. After 48 h of incubation, the numbers of revertant bacteria colonies per plate for TA102 were counted. They are listed in Table 1 and plotted versus the concentration of the testing chemicals in Fig. 2.

While the control experiments show that azulene or guaiazulene without light irradiation is not mutagenic in TA102 in this concentration range, combination of light irradiation causes the number of revertant bacteria colonies to increase with the increase of azulene or guaiazulene concentrations. As shown in Fig. 2, this number of increase of revertant colonies for both azulene and guaiazulene exhibits a dose–response relationship. At the highest azulene concentration, the number of revertant bacteria colonies is about five times of the control without light irradiation for azulene and four times of the control for guaiazulene. The number of revertant colonies reaches a plateau at a dose of 125 μM for azulene and 250 μM for guaiazulene, respectively. Therefore, the five or four-fold increase in revertant bacteria colonies of TA102 indicates that the combination of azulene or guaiazulene with light irradiation is mutagenic. In other words, both azulene and guaiazulene are photomutagenic. In contrast, naphthalene is not mutagenic or photomutagenic at 100, 500, and 2500 μM under the same experimental conditions (Table 1). However, exposure to naphthalene and irradiation did increase the number of revertant bacteria colonies 1.5–1.9 times of the media control, similar to the number obtained for the light control. Therefore, naphthalene is not photomutagenic under these conditions. The reason that naphthalene is not photomutagenic is possibly because it does not absorb UVA and visible light used in this study as will be discussed in the discussion section. Testing with UVB light on the photomutagenicity of naphthalene is difficult because UVB itself is strongly mutagenic [10].

3.2. Mutagenicity and photomutagenicity of azulene photoproducts

During the tests for azulene phototoxicity, it was found that there was no photomutagenicity response if older azulene solutions were used. Therefore, all the tests involving azulene must be prepared fresh. To understand this observation, a freshly prepared azulene solution in the phosphate buffer containing 17% dimethylsulfoxide was irradiated with the Xe/Hg(Xe) lamp for 30 min. After the irradiation, the azulene solution was mixed with TA102 and subjected to test with or without light irradiation as described in the experimental section. Both showed that the number of revertant bacteria was similar to that of the media control (Table 1). This indicates that light irradiation may have transformed azulene into one or more photoproducts that are neither mutagenic nor photomutagenic. Further study is needed to identify the photo-product(s) of azulene.

3.3. Both UVA and visible light can activate azulene and thus become mutagenic

Further evaluation of the effect of light wavelengths on azulene photomutagenicity was conducted with four different wavelengths of light: (i) full spectrum light, 300–800 nm from the Xe/Hg(Xe) lamp without optical filter (but with a Pyrex glass filter). This light has about 30% of UVA comparing with 8.7% UVA for the outdoor sunlight [16]; (ii) UVA light, 365 $\pm$ 15 nm light using the UV-transilluminator; (iii) >400 nm visible light from the Xe/Hg(Xe) lamp after a >400 nm optical filter from Andover Corporation (Salem, NH). This light lacks the UVA component comparing with the outdoor light; and (iv) >450 nm visible light from the Xe/Hg(Xe) lamp after a >450 nm optical filter from Andover Corporation. All samples had the same amount of azulene at 500 μM and irradiated with 6.5 J/cm^2 UVA and/or 12.6 J/cm^2 visible light. Fig. 3 shows that 6.5 J/cm^2 UVA light (365 nm, bar #3) or 12.6 J/cm^2 visible light (>400 nm, bar #4) are able to increase the number of revertant colonies more than twice of the light control (neighboring bar on the right) and the azulene dark control (bar control). With 12.6 J/cm^2 visible light of >450 nm, the number of revertant colonies only increases by 1.6 time of the control (bar #5). With 6.5 J/cm^2 UVA and 12.6 J/cm^2 visible light, the number of revertant bacteria colonies increases more than five times of the control, signifying a strong mutagenic response. Since the light doses are different, the relative photomutagenicity of azulene with these wavelength of light cannot be compared.

However, a point is clear that UVA alone, visible light alone, or combination of UVA and visible light can all activate azulene and thus make it mutagenic.

3.4. Photomutagenicity of azulene, guaiazulene, and naphthalene in *Salmonella typhimurium* tester strains TA98 and TA100

When TA98 and TA100 are exposed to light (Xe/Hg, visible/UVA = 2:1) doses of 0, 0.12, 0.24, 0.48, 1.5, 2.4 J/cm², the number of revertant colonies caused by light exposure are listed in Table 2. This number for TA98 remains the same below the light dose of 0.24 J/cm², while higher doses of light would kill some of the bacteria and cause the number of revertant colonies to decrease. For TA100, exposure to light at all doses listed here caused the number of revertant colonies to increase to about double the number of colonies as a result of spontaneous mutation. Therefore, a light dose of 0.24 J/cm² is used for determining the mutagenicity of azulene, guaiazulene and naphthalene at a concentration of 500 µM for both strains. This light dose is more than 40 times greater than the light dose used by Utesch and Splittgerber [12] for photomutagenicity tests. Under these experimental conditions, all three compounds were found not photomutagenic in TA98 and TA100.

4. Discussion

4.1. Azulene photomutagenicity

The results presented here show for the first time that the widely used ingredient chemical, azulene or guaiazulene commercially, is photomutagenic in *S. typhimurium* TA102. Since the presence of 250 µM azulene/guaiazulene increases the number of revertant bacteria colonies of TA102 about five times, this compound can be classified as a moderate to strong photomutagen. At concentrations of as low as 25 µM, the photomutagenic effect is still evident. The highest concentration used for these experiments is about two orders of magnitude lower than the concentration of azulene in some of the cosmetic formulations of 1% azulene, or 78 mM [3]. Even with dilutions due to application on the skin, a concentration of higher than the experimental concentration of 25 µM can still be expected. After topical application of azulene/guaiazulene beauty products, customers are likely to be exposed to sunlight irradiation, which is composed of approximately 91% visible (>400 nm), 8.7% UVA (315–400 nm), and 0.3% UVB (280–315 nm) light [16]. Exposure to solar irradiation alone can cause skin cancer [10,16–19]. Among all wavelengths, UVB radiation is responsible for most of the photocarcinogenic effect of sunlight [10]. The results presented here suggest that irradiation with relatively harmless UVA and visible sunlight can turn the normally not mutagenic azulene/guaiazulene into mutagenic compounds. Since azulene is so widely used in our daily lives as cosmetic products [3–5], its safety needs to be carefully evaluated further. So far, its use has been considered safe because its mutagenicity and acute toxicity are negligible [5,8] and it is even beneficial as a weak anti-inflammatory agent [6].

Mechanistically, azulene absorbs visible or UVA light and is promoted to the first or second excited-states. We recorded the absorption spectra for both azulene and guaiazulene and found that they both have broad absorption bands at 340 nm in the UVA region and 600 nm in the visible region (data not shown, also see reference by Tetreault et al. [2] for azulene). It is known that molecules in the excited-states can undergo a variety of excited-state reactions, producing free radicals, reactive oxygen species, or more toxic stable photoproducts, thus causing phototoxicity [20,21]. It is likely the short-lived reactive species produced during the photolysis of azulene that are damaging the bacteria and causing mutation. However, it seems that azulene can quickly turn into unidentified forms or photoproducts that are not mutagenic or photomutagenic since prephotolysis of azulene solution diminished its photomutagenicity. In our preliminary study, when azulene is

irradiated together with pure DNA or human skin cells, azulene can cause DNA strand cleavage (Parekh et al. [9] and unpublished results). However, the reactive species that are causing the mutagenicity and the biological targets that are damaged due to the excited-state reactions need further investigation. As reported to date, the photochemistry of azulene in terms of photoproduct(s) identification and studies of light generated reactive intermediates have not been reported. There is merely one report on the photochemical reaction of substituted azulene, not azulene itself [22]. In comparison, naphthalene does not absorb UVA or visible light and is found to be not photomutagenic under these experimental conditions.

4.2. Photomutagenicity assay

Although the *Salmonella* bacterial mutagenicity bioassay developed by Ames has been a convenient and most commonly used mutagenicity bioassay, conduct of this bioassay concomitant with photo-irradiation for photomutagenicity determination is relatively new. Several European communities have recently published guidelines for testing chemicals with regard to photomutagenicity [12–15]. Although ideal photomutagenicity assay is to use the level of light only to photo-activate the chemicals that results in mutation, not to cause death of the bacteria. The major concern is that light radiation, particularly from UVB light, can damage DNA and results in cell death if the damage is not timely and efficiently repaired. *Salmonella* TA98 and TA100 strains lack the DNA excision repair enzymes and thus are very sensitive to light. Other factors also complicate the study, including absorption of the light by the medium, the plate, and the agar. Thus, optimal experimental conditions for the radiation of bacteria with and without the presence of chemical are yet to be developed. In this study, we followed some of the criteria of Utesch et al. [12] to determine photomutagenicity of azulene and guaiazulene in *S. typhimurium* tester strains TA98, TA100, and TA102. TA102 fits all the criteria and is suitable for use in photomutagenicity testing. However, TA98 and TA100 are too sensitive to light to be efficiently used for photomutagenicity test. Under our experimental conditions, it was found that both azulene and guaiazulene are photomutagenic in TA102, but not in TA98 and TA100.

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Fig. 1.
Chemical structure of azulene, guaiazulene and naphthalene.

Fig. 2. Photomutagenicity test of azulene and guaiazulene with *S. typhimurium* TA102. Samples were irradiated with 6.5 J/cm² of UVA and 12.6 J/cm² of visible light using the 300 W Xe/Hg(Xe) lamp.

Fig. 3.

Effect of light wavelengths on azulene (172 µg/plate) photomutagenicity in *S. typhimurium* TA102. The light doses for the full spectrum light #1: 6.5 J/cm² of UVA and 12.6 J/cm² of visible light; #2: 6.5 J/cm² of UVA at 365 nm; #3: 12.6 J/cm² of visible light of >400 nm; #4: 12.6 J/cm² of visible light of >450 nm. The dark bars on the right side are the controls with light but without azulene.

Table 1

Number of revertant colonies of TA102 upon irradiation with the 300 W Xe/Hg lamp in the presence of testing chemicals^a

	0 μ M	62.5 μ M	125 μ M	250 μ M	500 μ M
Azulene, no light	312 $\pm$ 21	426 $\pm$ 104	477 $\pm$ 158	390 $\pm$ 111	380 $\pm$ 104
Azulene + light ^b	477 $\pm$ 69	1439 $\pm$ 172	1985 $\pm$ 337	1975 $\pm$ 282	2099 $\pm$ 235
Guaiazulene, no light	431 $\pm$ 62	472 $\pm$ 21	422 $\pm$ 76	408 $\pm$ 81	321 $\pm$ 52
Guaiazulene + light ^b	541 $\pm$ 42	820 $\pm$ 84	1018 $\pm$ 135	1389 $\pm$ 131	1549 $\pm$ 71
Azulene photoproduct, no light	302 $\pm$ 42				334 $\pm$ 41
Azulene photoproduct + light ^b	376 $\pm$ 60				357 $\pm$ 82
Naphthalene, no light	408 $\pm$ 76		422 $\pm$ 35 ^c		422 $\pm$ 29
Naphthalene + light ^b	490 $\pm$ 159		752 $\pm$ 69 ^c		798 $\pm$ 41

^aThe negative control was with media and no light. The number of revertant colonies as a result of the spontaneous mutation of TA102 ranges from 230 to 370 in all the experiments. Positive control was with 10 μ g/plate 8-methoxypsoralene irradiated with the Xe/Hg lamp for 2 min, producing the number of revertant colonies in the range of 2200–3100 per plate.

^bThe light dose was 6.5 J/cm² for UVA light and 12.6 J/cm² for visible light.

^cThe concentration for naphthalene in this column was 100 μ M. At 2500 μ M, the number of revertant colonies were 390 $\pm$ 65 and 623 $\pm$ 143 for without and with irradiation, respectively.

Table 2

Photomutagenicity test of azulene, guaiazulene, and naphthalene using TA98 and TA100 and Xe/Hg(Xe) lamp

	<u>Azulene (500 μM)</u>		<u>Guaiazulene (500 μM)</u>		<u>Naphthalene (500 μM)</u>	
	No light	+0.24 J/cm ²	No light	+0.24 J/cm ²	No light	+0.24 J/cm ²
TA98	64	14	37	28	37	18
TA100	193	202	96	165	83	92