Acetyl zingerone: An efficacious multifunctional ingredient for continued protection against ongoing DNA damage in melanocytes after sun exposure ends

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Received 20 June 2019, Revised 6 September 2019, Accepted 16 September 2019

Keywords: Acetyl zingerone, ultraviolet radiation (UVR), dark cyclobutane pyrimidine dimers (dark-CPDs), quencher, antioxidant, scavenger

Abstract
OBJECTIVE: Recent research has shown that significant levels of cyclobutane pyrimidine dimers (CPDs) in DNA continue to form in melanocytes for several hours in the dark after exposure to ultraviolet light (UVR). We document the utility of a new multifunctional ingredient, 3-(4-hydroxy, 3-methoxybenzyl)-pentane-2,4-dione (INCI acetyl zingerone (AZ)), to protect melanocytes against CPD formation after UVR exposure ends.

METHODS: The use of AZ as an intervention to reduce CPD formation after irradiation was assessed in vitro by comparing kinetic profiles of CPD formation for several hours after irradiation in cells that were untreated or treated with AZ immediately after irradiation. Multifunctional performance of AZ as an antioxidant, quencher and scavenger was established using industry-standard in vitro chemical assays, and then, its efficacy in a more biological assay was confirmed by its in vitro ability to reduce intracellular levels of reactive oxygen species (ROS) in keratinocytes exposed to UVA radiation. Molecular photostability was assessed in solution during exposure to solar-simulated UVR and compared with the conventional antioxidant α-tocopherol.

RESULTS: Even when added immediately after irradiation, AZ significantly inhibited ongoing formation of CPDs in melanocytes after exposure to UVA. Incubation with AZ before irradiation decreased intracellular levels of UVA-induced ROS formation in keratinocytes. Compared with α-tocopherol, the molecular structure of AZ endows it with significantly better photostability and efficacy to neutralize free radicals (•OH, •OOH), physically quench singlet oxygen (1O2) and scavenge peroxynitrite (ONOO−).

CONCLUSION: These results designate AZ as a new type of multifunctional ingredient with strong potential to extend photoprotection of traditional sunscreens and daily skincare products over the first few hours after sun exposure ends.

Résumé
OBJECTIF: Une étude récente a montré que des taux significatifs de dimères cyclobutyliques de pyrimidine (Cyclobutane Pyrimidine Dimers, CPD) dans l’ADN continuaient à se former dans les mélanocytes pendant plusieurs heures, dans l’obscurité, après que leur exposition aux radiations ultraviolettes (UAV) ait pris fin. Nous documentons l’utilité d’un nouvel ingrédient multifonctionnel, le 3-(4-hydroxy, 3-méthoxybenzyle)-pentane-2,4-dione (INCI acétyle zingéron (AZ)), pour protéger les mélanocytes contre la formation de CPD une fois l’exposition aux rayonnements UV terminée.

MÉTHODES: L’utilisation d’AZ en tant qu’intervention pour réduire la formation de CPD après exposition aux ultraviolets a été évaluée in vitro en comparant les profils cinétiques de la formation de CPD pendant plusieurs heures après irradiation dans des cellules non traitées et dans des cellules traitées à l’AZ immédiatement après exposition. La performance multifonctionnelle de l’AZ comme agent antioxydant, absorbant et éliminateur a été établie à l’aide de dosages chimiques in vitro standard pour l’industrie, après quoi son efficacité à un dosage plus biologique a été confirmée par sa capacité in vitro à réduire les taux intracellulaires d’espèces réactives de l’oxygène (ROS) dans les kératinocytes exposés au rayonnement UV. La photostabilité moléculaire a été évaluée en solution pendant l’exposition UV simulée de rayonnements solaires et par rapport au traitement antioxydant conventionnel α-tocophérol.

RÉSULTATS: Même lorsqu’il a été ajouté immédiatement après exposition, l’AZ a inhibé la formation continue de CPD dans les mélanocytes après l’exposition aux UV et ce de façon significative. Une incubation avec de l’AZ avant exposition a entraîné une diminution des taux intracellulaires de formation des ROS, induits par le rayonnement UV, dans les kératinocytes. Par rapport au α-tocophérol, la structure moléculaire de l’AZ lui confère une photostabilité significativement meilleure ainsi qu’une plus grande efficacité pour neutraliser les radicaux libres (•OH, •OOH), absorber physiquement l’oxygène singulet (1O2) et éliminer le peroxynitrite (ONOO−).

CONCLUSION: Ces résultats montrent que l’AZ, considéré comme un ingrédient multifonctionnel d’un type nouveau, jouit d’un fort potentiel de prolongation de l’effet photoprotecteur des écrans solaires traditionnels et des produits de soins de la peau pendant quelques heures après la fin de l’exposition au soleil.

Introduction
Damage to DNA is arguably the most important type of photodamage initiated by sun exposures. Research has identified that...
cyclobutane pyrimidine dimers (CPDs) represent the main class of DNA lesions responsible for the induction of melanoma and non-melanoma skin cancers [1]. Other research also suggests strong links between CPD formation and photoaging [2,3] as well as being a key event that triggers the production of the immunomodulatory cytokine TNF-α [4] and onset of erythema in skin during acute exposures to the sun [5].

Until recently, it was widely held that CPD formation occurred solely during irradiation itself, when DNA directly absorbs UVR to form electronically excited state species that subsequently undergo photochemical transformations to yield CPDs (referred to here as light-CPDs) [1]. However, recent research challenges this notion that CPDs in skin only arise during periods of UV exposures. A recent study in melanocytes [6] documented that CPDs continued to increase by 50% or more after irradiation ended, in the dark and independent of additional UVR photons. More recently, CPD formation in the dark (now referred to as dark-CPDs) was also reported to occur in keratinocytes not only in vitro but also in vivo in human skin at measurable levels within the first 2 h after exposure to UVA/visible radiation [7,8].

These results clearly signal that the formation of dark-CPDs in the absence of UVR follows a different mechanism of formation from the one yielding light-CPDs under direct irradiation. To explain these surprising results in melanocytes [6], a chemiexcitation pathway of dark-CPD formation was invoked, involving generation of unstable dioxetanes as intermediates from a reaction between peroxyxynitrite and melanin fragments; the dioxetanes then thermally decay to yield excited state triplet carbonyl species. Once formed, excited triplet carbonyls then transfer their energy of excitation to nearby DNA bases to form the same type of excited states that form when DNA directly absorbs UVR. Generation of excited state species from chemical reactions as opposed to direct absorption of UVR constitutes chemiexcitation and is referred to as ‘photochemistry without light’. It is well known that 1,2 dioxetanes represent efficient sources of excited triplet state carbonyls [9] and that they can play significant roles in biological systems [10,11].

These recent discoveries that dark-CPDs continue to form in skin long after the end of UV exposures prompt thinking of new intervention strategies for additional photoprotection that work in concert with traditional sunscreens. Sunscreens provide excellent protection from UVR that stimulates onset of erythema (i.e., 290–360 nm) [12], but they exhibit diminishing absorption of longer UVA rays (380–400 nm) and virtually no absorption of short-wavelength visible (400–450 nm) radiation. This latter feature is all the more important since as much as 50% of the reactive oxygen species (ROS) formed in human skin during sun exposures is caused by higher energy visible radiation [13], which is beyond the protective capacity of sunscreens. Hence, sunscreens possess important gaps in photoprotective properties and would benefit from the use of supplemental ingredients to bolster their photoprotective properties.

In this report, we showcase the molecule 3-(4-hydroxy, 3-methoxybenzyl)-pentane-2,4-dione (INCI: acetyl zingerone; AZ) as a new type of multifunctional skincare ingredient. The molecular structure of AZ was inspired by those of curcumin and zingerone (a key component in ginger) found in nature (Fig. 1) and then intentionally constructed to possess high efficacy to neutralize key UVR-induced ROS. Herein, we document the ability of AZ in vitro to prevent dark-CPD formation in melanocytes within the first few hours after UVA exposure and then rationalize AZ’s efficacy to inhibit dark-CPD formation in terms of its multifunctional properties to perform as an antioxidant, physical quencher of singlet oxygen and scavenger of peroxynitrite with high efficiencies compared with α-tocopherol, one of skin’s chief non-enzymatic antioxidants [14] and a common ingredient added to sunscreen products.

**Materials and methods**

**Acetyl Zingerone**

The target compound, 3-(4-hydroxy, 3-methoxybenzyl)-pentane-2,4-dione (INCI: Acetyl Zingerone; AZ), was prepared in two steps: (1) by condensation of vanillin (4-hydroxy-3-methoxybenzaldehyde) with acetylacetone to obtain 3-(4-hydroxy, 3-methoxybenzyldiene)-pentane-2,4-dione (INCI: Acetyl Zingerone; AZ) as the target compound, 3-(4-hydroxy, 3-methoxybenzyl)-pentane-2,4-dione (INCI: acetyl zingerone; AZ) as a new type of multifunctional skincare ingredient. The molecular structure of AZ was inspired by those of curcumin and zingerone (a key component in ginger) found in nature (Fig. 1) and then intentionally constructed to possess high efficacy to neutralize key UVR-induced ROS. Herein, we document the ability of AZ in vitro to prevent dark-CPD formation in melanocytes within the first few hours after UVA exposure and then rationalize AZ’s efficacy to inhibit dark-CPD formation in terms of its multifunctional properties to perform as an antioxidant, physical quencher of singlet oxygen and scavenger of peroxynitrite with high efficiencies compared with α-tocopherol, one of skin’s chief non-enzymatic antioxidants [14] and a common ingredient added to sunscreen products.

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![Figure 1](image-url)  
Figure 1 The design of acetyl zingerone (AZ, Fig. 1A) was inspired by curcumin (Fig. 1D) and zingerone (Fig. 1E) structures. Zingerone is a key chemical component found in ginger. In solution, AZ exists in keto–enol tautomers [1A (keto) and 1B & 1C (enol)]
spectral analysis. Acetyl zingerone (trade name: Synoxyl® AZ) is commercially available from Sytheon, Boonton, New Jersey, USA. AZ is REACH-registered (EC® 820-605-0) and is safe for topical applications as documented by the studies available for review on ECHA website.

Method of analysis by HPLC

Acetyl zingerone was dissolved in methanol and analysed by HPLC. Retention time: about 27.3 min for keto and 34.5 min for enol forms. Standard and sample concentration: 1 mg mL⁻¹ (standard and sample should be injected immediately (fresh) after preparation). Column: XTerra RP18, (250 × 4.6) mm, 5 µm; flow rate: 1 mL min⁻¹; detection by UV at 280 nm; column oven temperature: 30°C, sample temperature: 4°C; injection volume: 10 µL; run time: 60 min; elution mode: gradient programme: time/% of Mobile phase B: 0/10, 5/10, 20/20, 40/80, 50/80, 55/10, 60/10; Mobile phase A: 0.1% trifluoroacetic acid (TFA) in water; and Mobile phase B: 0.1% TFA in acetonitrile (80:20 v/v).

Measurement of dark-CPD formation in melanocytes

Experiments were performed with C57BL/6 mouse melanocytes. AZ was solubilized at 20 mg mL⁻¹ in DMSO and then further diluted with water for the final test concentration of 25 µg mL⁻¹. Cells were plated and grown to 70–80% confluence after which they were washed with PBS (Gibco 10010023, calcium and magnesium free) and irradiated in PBS with UVA (75 kJ m⁻²) using F20T12BL lamps (Spectra Mini, Daavlin, Bryan, OH) passed through a 3-mm thick glass plate filter and a BD Falcon polystyrene Petri dish lid to remove UVC (100–280 nm) and most UVB (280–315 nm). UVA output was measured prior to irradiation using a 500C radiometer (National Biological Corp., Twinsburg OH). Immediately after irradiation, AZ was added and cells were then incubated further for 6 h. DNA was harvested immediately after irradiation for the 0h time point (in the absence of AZ) and then at 1, 2, 4 and 6 h. DNA was purified using a QIAamp Blood Kit (Qiagen, Cat. No. 51104 or 51106) and CPD was quantified using an ELISA (Cat. CAC-NM-DND-001, Clone TDM-2, Cosmo Bio, Inc., Carlsbad, CA) following manufacturer’s protocol (Cat. NMDND001), with experimental details provided elsewhere [6].

Measurement of absorbance spectrum

The UV/visible spectrum of AZ was recorded on a Jasco V-530 UV/Vis spectrophotometer in a 10-mm path length quartz cuvette.

Measurement of intracellular ROS in keratinocytes

Experiments were performed with C57BL/6 mouse melanocytes. AZ was added and cells were then incubated further for 6 h. DNA was harvested immediately after irradiation using Applied Biosystem Fluorimeter Cytofluor 4000. This part of the study was done by following the procedure developed by Valencia & Kochevar [15]. DCFH-DA is a non-polar compound that freely diffuses into cells, where it is hydrolysed (oxidized) to the non-fluorescent polar derivative DCFH and thereby trapped within the cells. In the presence of free radicals, DCFH is oxidized to the highly fluorescent DCF. This hydrolysis is proportional to the concentration of the intracellular ROS and can be detected in living cells by fluorometry at ex/em 488/530 nm [16]. Cell numbers were quantified using the sulfurhodamine B colorimetric method [17]. Each experimental condition was tested in at least 4 repeats. Water was the negative control. P values representing statistical significance were calculated using the paired t-test, and the threshold of statistical significance adopted was P = 0.05.

In vitro determination of efficiencies to neutralize key ROS

Acetyl zingerone and δ-tocopherol were solubilized separately in ethanol (70% v/v) and water (30% v/v), respectively, and then, profiles of these two compounds for neutralizing key ROS were determined essentially by following the method of Mullen et al. [18] and represented as μmole trolox equivalent per gram in Table 1.

Physical quenching of singlet oxygen (¹O₂)

Five millilitre of 1 mg mL⁻¹ AZ or δ-tocopherol was added to the reaction mixture used to generate singlet oxygen consisting of

Table 1 Comparison of effectiveness of AZ to δ-tocopherol to neutralize different types of reactive oxygen species (ROS)*

| Compounds                  | Peroxyl | Hydroxyl | Superoxide Anion | Peroxynitrile | Singlet Oxygen |
|----------------------------|---------|----------|------------------|---------------|---------------|
| Acetyl Zingerone           | 25,325  | 9723     | ND               | 1139          | 7180          |
| δ-Tocopherol               | 1432    | 94       | ND               | 9             | 2636          |
| Effectiveness Ratio (AZ/δ-tocopherol) | 17.7    | 39.6     | Not applicable   | 126.6         | 2.7           |

ND = not detected.

*Results expressed as μM Trolox equivalent/g ± 5%.

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5 mL 0.16 mg mL\(^{-1}\) lithium molybdate, 4 mL 0.01 M NaOH and 5 mL of 0.015% H\(_2\)O\(_2\) [19] with solution pH 5. Aliquots (500 µL) from the reaction mixture were removed every 2 min and added to 1 mL of 1 mg mL\(^{-1}\) curcumin to stop the reaction before HPLC analysis to monitor the degradation of AZ or \(\alpha\)-tocopherol.

Photochemical stability

Acetyl zingerone and \(\alpha\)-tocopherol were solubilized separately in 50% aqueous ethanol and ethanol, respectively, and put in 5-mL Opticlear Vials (Kimble Glass, Chicago Heights, IL). Test vials were then placed in a Rayonet RPR-100 photochemical reactor equipped with four RMR-3000 (UVB) and four RMR-3500 (UVA) lamps (Southern New England Ultraviolet Company, Brandford, CT). This arrangement reflects a simulated daylight condition. 100 µg L\(^{-1}\) of the sample was irradiated with 13 J cm\(^{-2}\) at 31°C. The degradation of AZ and \(\alpha\)-tocopherol was determined by the HPLC method described earlier.

Results

Acetyl zingerone (AZ) significantly reduces dark-CPD formation within 1 h after UVA exposure in melanocytes

The potential of melanocytes to continue the formation of CPDs in the dark (i.e. dark-CPDs) for at least 2 h post-UVA irradiation appears in the kinetic profile in Fig. 2. The data for each time point were normalized with respect to the level of CPD formed at the initial irradiation. Thus, the ‘zero’ time point on the graphs reference the extent to which CPDs formed during irradiation, presumably following direct absorption of UVA by DNA, whereas the dark-CPDs created after irradiation clearly arise by a different mechanism of formation in the dark in the absence of exposure to UVA radiation.

In untreated controls, after irradiation was stopped, dark-CPDs continued to increase significantly in melanocytes by about 40% (Fig. 2) before they levelled off and eventually declined as nucleotide excision repair became apparent, in agreement with the kinetic profiles reported previously for this cell type [6].

The ability of AZ to prevent dark-CPD formation in melanocytes was determined by incubating cells with AZ (25 µg mL\(^{-1}\)) immediately after exposure to UVA radiation and then measuring CPD formation over the same time course as the controls for 6 h post-irradiation. Inspection of the results in Fig. 2 reveals that when added after irradiation, treatment with AZ significantly reduced dark-CPD formation within the first 2 h post-irradiation. Notably, in both experiments, the levels of CPDs present in controls after 6 h were also comparable to those for AZ-treated cells, suggesting that AZ did not interfere with normal cellular abilities to repair...
Acetyl zingerone protects against on-going DNA damage

Acetyl zingerone (AZ) significantly reduces intracellular ROS formation in keratinocytes after UVA exposure

The ability of AZ to reduce the burden of UVA-induced ROS formation in biological systems was determined using keratinocytes to avoid any effect of melanin. Intracellular ROS was quantified by measuring the fluorescence from the oxidation product of dichlorofluorescin diacetate (DCF-DA) using an established protocol wherein cells were pre-treated with either 25 µg/mL or 50 µg/mL of AZ prior to irradiation. DCFA-DA is advantageous in that it is oxidized by several of the ROS induced in cells by UV exposure (including peroxyl and hydroxyl radicals, singlet oxygen, peroxynitrite and hydrogen peroxide) [20,21]. Results appear in Fig. 3 and confirm that relative to the untreated controls, incubation with AZ significantly reduced intracellular ROS formation induced by UVA exposure in a dose-dependent manner by −35% and by −46% for the low and high doses, respectively.

Acetyl zingerone (AZ) possesses negligible absorbance in the UV spectral region

The absorbance spectrum shown in Fig. 4 confirms that AZ provides only weak tailing absorbance in the UVB region (290–320 nm) that arises from its maximum absorbance peak at 280 nm, with virtually no absorbance in the UVA (320–400 nm) region. This effectively rules out that AZ exerts its protective effects during UVR exposure through a sunscreen-type effect. This observation, coupled with the finding that AZ prevents dark-CPD formation in melanocytes after irradiation, led us to investigate other performance attributes of AZ to help explain how it mediates its photoprotective benefits.

Acetyl zingerone (AZ) is an effective antioxidant, scavenger of peroxynitrite and physical quencher of \(^{1}\)O\(_{2}\)

Effectiveness of AZ to neutralize key ROS, including peroxyl radicals, hydroxyl radicals, superoxide anion radicals, peroxynitrite and singlet oxygen, was assessed using industry-standard chemical methods. Results appear in Table I along with a direct comparison against \(\alpha\)-tocopherol as a positive control. Effectiveness ratios show that AZ possesses superior efficacy compared with \(\alpha\)-tocopherol by factors ranging from 2.7 for singlet oxygen to 127 for peroxynitrite. AZ’s capacity to neutralize singlet oxygen compared with \(\alpha\)-tocopherol was also of interest. As \(^{1}\)O\(_{2}\) represents an electronically excited state, it can be neutralized by either chemical or physical quenching. Physical quenchers relax \(^{1}\)O\(_{2}\) back to its \(^{3}\)O\(_{2}\) ground state through an energy transfer process without being chemically degraded, whereas chemical quenchers react chemically with \(^{1}\)O\(_{2}\) and in the process become sacrificed. To determine the extent to which AZ and \(\alpha\)-tocopherol function as chemical versus physical quenchers, we quantified via HPLC the stability of the molecules over the time course of the method used to assess their effectiveness against \(^{1}\)O\(_{2}\). The results in Table II reveal that at the
end of the method, only 35% of α-tocopherol remains compared with 82% for AZ. Thus, these results support that AZ is a more effective physical quencher of $^1\text{O}_2$, which helps explain AZ’s enhanced capacity to neutralize $^1\text{O}_2$ compared with α-tocopherol.

Acetyl zingerone (AZ) is more photochemically stable than α-tocopherol

Molecular photostability was determined by quantifying levels of AZ and α-tocopherol remaining while individual solutions were exposed to 130 kJ m$^{-2}$ solar-simulated UV radiation over a time course of 30 min. Surprisingly, the results in Fig. 5 show that over the irradiation period α-tocopherol was completely degraded chemically while 87% of AZ remained molecularly intact.

Discussion

In this study, we confirm previously published results [6] that significant levels of dark-CPDs continue to form in melanocytes for hours after exposure to UVA radiation ends and show that the use of a unique ingredient, acetyl zingerone (AZ), can intervene effectively in the chemiexcitation pathway of dark-CPD formation to reduce them by ca 82% (Fig. 2) after the first hour of post-irradiation compared with untreated control. The fact that AZ was added to melanocytes after UVA exposure further proves that AZ exerts its protective benefits by means other than a sunscreen-type effect. Our hypothesis is that the effectiveness of AZ originates from its unique molecular structure that enhances its ability to function as an antioxidant, scavenger and physical quencher. By definition, physical quenchers represent molecules that can accept the energy of excitation from other excited states to restore them to their original ground states while at the same time having the ability to dissipate the excess energy they acquire in a harmless manner (typically in the form of heat). In this way, the molecular integrity of physical quenchers remains intact. This differs from the normal action of antioxidants or scavengers that function by reacting chemically with ROS and as a result become consumed or sacrificed in the process.

The different functional groups that equip AZ with antioxidant, scavenger and physical quencher properties are highlighted in Fig. 6. These properties were probed by comparing AZ directly to α-tocopherol in a battery of standard chemical assays. Effectiveness ratios displayed in Table I substantiate AZ’s superior performance compared with α-tocopherol against major forms of ROS known to form in skin during periods of sun exposure, including peroxyl and hydroxyl radicals, as well as the non-radical species singlet oxygen and peroxynitrite [22,23].

Of particular interest was the finding that AZ is better than α-tocopherol in neutralizing $^1\text{O}_2$ by a factor of 2.7-fold. Analysis to examine the chemical stability of AZ and α-tocopherol over the exposure period to $^1\text{O}_2$ further revealed that AZ was significantly more stable than α-tocopherol by 82% versus 35% (Table II). As $^1\text{O}_2$ is an electronically excited state species of molecular oxygen ($^1\text{O}_2$), it is possible that it could be deactivated through physical quenching or chemical reaction. These results provide direct evidence that AZ scavenges $^1\text{O}_2$ mainly through physical quenching as opposed to chemical reaction. A great many organic compounds have been studied for their ability to scavenge $^1\text{O}_2$ with many compounds functioning by a combination of physical quenching and chemical reaction. Physical quenching capabilities appear to be more favourable for electron-rich compounds, such as amines, phenols and methoxybenzenes [24,25]. The generally accepted mechanism for deactivation of $^1\text{O}_2$ through physical quenching involves

![Figure 5](image-url)

**Figure 5** Comparison of the photostability profiles of AZ and α-tocopherol when solutions of each molecule were exposed to 130 kJ m$^{-2}$ solar-simulated UV radiation over 30 min and sampled at regular intervals to quantify the amount of each molecule remaining by HPLC. Data are representative of two independent experiments.

![Figure 6](image-url)

**Figure 6** Acetyl zingerone (AZ) contains different functional groups that equip it with multifunctional properties including as an antioxidant via hydrogen atom donation from the phenolic group, a physical quencher of $^1\text{O}_2$ from methoxy-substituted aromatic ring and a scavenger of ONOO$^-$ through the two enolizable carbonyls of the 3-methyl-2,4-pentanedione group.

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the formation of an exciplex between the organic compound and \(^1\)O\(_2\) with varying degrees of charge transfer character with eventual decay by intersystem crossing to yield oxygen (\(^1\)O\(_2\)) and the organic compound in their ground states [26].

The other noteworthy result from the effectiveness profile is the superior ability of AZ to scavenge peroxynitrite (ONOO\(^-\)). Peroxynitrite reacts both as an oxidant and nucleophile [27] and has been shown to react with enolizable carbonyls, such as 3-methyl-2,4-pentanediene, through either one-electron oxidation or nucleophilic addition to the carbonyl group [28]. This functional group on AZ most likely imbues it with exceptional scavenging capabilities towards ONOO\(^-\) and further accounts for its superior scavenging properties compared with \(\alpha\)-tocopherol. Indeed, the most striking feature of AZ’s performance profile in Table I overall is the versatility of AZ to neutralize conventional free radicals (ROO\(^•\) and \(\mathbf{Ho}\)•) as an antioxidant, physically quench excited state species (\(^1\)O\(_2\)) and scavenge strong nucleophiles (ONOO\(^-\)) through carbonyl addition, with high efficiencies.

Although the antioxidant, scavenging and physical quenching properties of AZ were substantiated using non-biological chemical...
assays, the results in Fig. 3 confirm that AZ also significantly reduces ROS intracellularly in keratinocytes exposed to UVA radiation. The fact that AZ displays minimal absorption of UVB rays, with no absorption of UVA radiation (Fig. 4), further reinforces the molecule’s unique properties to attenuate ROS. These results also verify that AZ not only functions efficiently in biological systems, as predicted by the chemical assays, but that AZ does not function as a photosensitizer during UVA exposure. This latter point is important since it has been documented that many compounds, while effective in chemical assays, can become pro-oxidants under UVR that increase rather than decrease ROS levels [29].

The multifunctional properties that enable it to neutralize ROS so efficiently also help explain how AZ might intervene to decrease dark-CPD formation in melanocytes. Potential modes of intervention by AZ are illustrated in Fig. 7, which outlines the mechanism proposed by Premi et al. [6] for dark-CPD formation in melanocytes. Briefly, dark-CPD formation shows dependency on the presence of ONOO−, which continues to form after irradiation stops following UVA activation of the enzymes NADPH oxidase and iNOS to generate O2•− and NO• as precursors that react at the diffusion-controlled limit to form ONOO− (Stage 1). Peroxynitrite is the key ROS oxidant that reacts chemically with melanin fragments to form putative high-energy dioxetanes as intermediates that degrade thermally to produce excited triplet states of carbonyl compounds (Stage 2). These triplet carbonyls then undergo energy transfer to DNA with subsequent formation of dark-CPDs (Stage 3). AZ’s strong scavenging and antioxidant properties presented here support intervention in Stage 1 by scavenging ONOO− before it can undergo reaction to form dioxetane intermediates. The hydrogen atom donor properties of its antioxidant function support its potential to act in Stage 2 by reducing dioxetane intermediates to vicinal diols, especially since this has been reported for α-tocopherol [30] and since the antioxidant properties of AZ (ability to donate a hydrogen atom) presented here are clearly superior to those of α-tocopherol. In either case, AZ would essentially prevent the formation of excited state triplet carbonyls needed for energy transfer to DNA in Stage 3 and thus would prevent dark-CPD formation, as observed experimentally (Fig. 2).

Also shown in Fig. 7 is the potential use of triplet quenchers in Stage 3 to prevent dark-CPD formation by physically deactivating excited triplet carbonyls before they can transfer their energy to DNA. In order for this mode of intervention to be energetically viable, however, the energy level of the excited triplet carbonyls must be higher than or comparable to that of AZ. We recently calculated the triplet energy of AZ as 82 kcal mol−1 (unpublished data), which approximates the value of acetone of 78 kcal mol−1 [31]. Additional support that the excited triplet carbonyls formed within melanocytes fall within this ballpark comes from the finding that the use of DBAS (sodium 9,10-dibromoanthracene-2-sulpho-nate) as a diagnostic probe for excited triplet carbonyls not only amplified their weak chemiluminescence but also prevented dark-CPD formation in cultured melanocytes. This is significant since DBAS only monitors excited triplet carbonyls whose energy levels are greater than 70–74 kcal mol−1 [32]. While this makes it conceivable that AZ could function as a triplet quencher, this possibility still requires further experimental verification. Interestingly, physical quenching of photoexcited states formed in skin following UVA absorption by endogenous chromophores has been suggested as a novel approach to reduce photodamage from photooxidative stress and to enhance the photoprotective properties of sunscreens [33].

While previous research for dark-CPD formation in melanocytes after irradiation with UVA found a strong dependence upon ONOO−, we think it is also important to highlight that singlet oxygen (1O2) represents another UVB-induced ROS that forms within cells and is capable of producing high-energy dioxetane intermediates. Abundant scientific literature supports that 1O2 can react smoothly with a variety of functional moieties (including indoles, enamines, olefins, vinyl ethers and enols) in organic molecules to form dioxetane intermediates [34–37]. In addition, the side chains of certain amino acids (tryptophan, tyrosine and histidine) are postulated as major targets of 1O2 attack in biological systems [38] forming dioxetanes (or other endoperoxides) as initial intermediates upon reaction with 1O2. Owing to its short lifetime, 1O2 is thought to only exist during irradiation; however, it has also been reported that endoperoxides can serve as carriers of 1O2 in biological systems with capacity to release 1O2 upon thermal decomposition [39,40]. Initial formation of endoperoxides during irradiation would be a way to transport and extend the availability of 1O2 within skin cells even after irradiation ends. Although not observed here with melanocytes irradiated with UVA, the use of full-spectrum radiation, including UVB, UVA and visible radiation, might have ramifications for the involvement of 1O2 in CPD formation by chemiexcitation in melanocytes or keratinocytes either during or after irradiation. If so, our results predict that AZ would have the ability to trap out 1O2 efficiently by physical quenching to prevent dioxetane formation and hence prevent CPD formation.

Inherent photostability is an essential molecular property for ingredients recommended for use in topical formulations in order to deliver sustained protective benefits to skin during periods of sun exposure. The photostability profiles in Fig. 5 illustrate that AZ is relatively stable compared with α-tocopherol. Whereas α-tocopherol completely degraded, AZ sustained 87% of its molecular structure intact over the course of irradiation. This suggests that AZ could be an effective ingredient when added to traditional sunscreens over entire periods of sun exposure and daily skincare products.

In order to be effective on human skin in vivo, AZ must partition into epidermal layers to access melanocytes and keratinocytes, as suggested here by the in vitro results. The extent to which molecules partition into the epidermis is influenced by the nature of the vehicle in which they reside. This will be an important factor for consideration to optimize the efficacy of AZ in topical formulations. Adding AZ to optimized sunscreen formulations would be advantageous since it could allow AZ to partition into skin during sun exposures so that it would be at sites within the upper layers of skin to intervene optimally in the formation of dark-CPDs immediately after sun exposures.

In conclusion, these studies support that AZ as a single ingredient with multifunctional properties and enduring photostability would be useful as a supplement in traditional sunscreens to shore up their protection against dark-CPD formation after sun exposure as well as to help skin cope with the excess ROS formed in skin during sun exposures. While sunscreens form a protective film on top of skin’s surface to intercept UVR before it reaches the underlying skin, AZ could add additional photoprotective benefits beneath the sunscreen film within the upper layers of skin and is suitable for inclusion into both sunscreen and daily skincare products.

Acknowledgement

The authors would like to thank Sytheon for funding this research.
Acetyl Zingerone protects against on-going DNA damage

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