

Light-Induced Cytotoxicity and Genotoxicity of a Sunscreen Agent, 2-Phenylbenzimidazole in *Salmonella typhimurium* TA 102 and HaCaT Keratinocytes

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Abstract: 2-Phenylbenzimidazole (PBI) is an ingredient found in sunscreen agents. PBI can absorb the UV portion of the solar light and undergo a series of light-induced reactions to cause adverse effects in humans. Therefore, chemical and photochemical toxicity of PBI were investigated in the bacteria *Salmonella typhimurium* TA 102 and human skin keratinocyte cells. There is no appreciable bacteria death due to the exposure to PBI alone, indicating that PBI is not chemically toxic to the bacteria at a dose as high as 625 μ M. However, exposure to PBI and a solar simulator light (300-W Xe/Hg lamp, 30 min, 18.6 J/cm², equivalent to 30 min outdoor sunlight) causes significant bacteria death: 35% at 25 μ M and 55% at 625 μ M PBI. Exposure of the bacteria to light and PBI at doses 5-25 μ M causes the bacteria to revert, an indication of mutation. In the presence of PBI but without light irradiation, the number of revertant bacteria colonies is around 200 due to spontaneous mutation. Combination of light irradiation and PBI causes the number of revertant TA 102 colonies to increase in a dose dependent manner, reaching a maximum of around 1700 revertant colonies at 25 μ M PBI. At higher PBI concentrations, the number of revertant colonies remains constant. This result clearly indicates that PBI is photomutagenic in TA 102. Exposure of the human skin HaCaT keratinocytes in aqueous solution in the presence of PBI causes the cell to lose its viability with or without light irradiation. There is no significant difference in cell viability for the light irradiated or non-irradiated groups, indicating PBI is not photocytotoxic. However, exposure of the cells to both PBI and light irradiation causes cellular DNA damage, while exposure to PBI alone does not cause DNA damage.

Keywords: 2-Phenylbenzimidazole, Sunscreen Agent, Photo-Ames Test, *Salmonella* TA 102, HaCaT Keratinocytes

Introduction

There has been a shift in the paradigm of the American lifestyle. In today's society, the development of leisure activities, holiday habits, along with tanning through sunbathing or artificial tanning devices for cosmetic purposes, has caused an increase in ultraviolet radiation (UVR) exposure [1, 2]. Unnecessary sunlight exposure is known to induce acute and chronic modifications in the skin such as erythema, immune suppression, premature skin aging, and skin cancer [1, 3-5]. Natural sunlight includes UVA (320-400 nm), UVB (280-320 nm), UVC (200-280 nm), and visible (400-700 nm) radiation. UVA irradiation can penetrate into the dermal layer of the skin. UVB radiation, commonly referred to as the erythema band, is largely absorbed in the

epidermis with a small portion reaching the upper dermal layer. The UVC radiation or the "germicidal radiation" does not reach the earth's surface [4-6].

Wearing sunscreen, in conjunction with protective clothing, avoiding sun exposure, and refraining from tanning salons helps mitigate the aforementioned harmful effects [1, 3]. Sunscreens are topical preparations that reduce the deleterious effects of UVR by absorption, reflection, or scattering [3]. Sunscreens can be divided into two categories: chemical and physical [7]. Chemical sunscreens provide protection by absorbing UV radiation while physical sunscreens prevent UV radiation from reaching the skin [1, 3]. The difference between physical and chemical sunscreens is that the physical sunscreens are usually dense formulations with ingredients that do not selectively absorb UVR but rather reflect and scatter all

UVR and visible radiation. These sunscreens tend to be non-photosensitizing and broad spectrum. Chemical sunscreens are usually non-opaque and contain an absorbing chemical. These sunscreens are usually colorless because they lack visible light-absorbing chemicals which have proven to be more cosmetically acceptable to most individuals. However, to be effective a sunscreen should have a wide range of absorbance with excellent UVB absorbance. The sun protective factor (SPF) value of sunscreens benefits consumers because this rating indicates the effectiveness of the sunscreen to protect against sunburn or erythema [7]. However, SPF is limited in that it is unable to indicate how efficient sunscreens are at protecting the skin from UVA-induced damage or how long-term use will lower the risk of developing skin cancer.

para-Aminobenzoic acid (PABA) used to be a UV filter in sunscreen formulations. PABA has recently been discovered to increase the formation of a particular DNA defect in human cells, therefore increasing the risk of skin cancer in individuals who lack the repair mechanisms of these cellular defects. For this reason, PABA has been banned as an ingredient used in the manufacture of sunscreen agents because of the associated health risks which included its failure to provide sunburn protection for normal human skin [3].

2-Phenylbenzimidazole (PBI, Figure 1), is an ingredient in sunscreen agents that has been reported to cause DNA photodamage [8, 9]. This damage occurs through both Type I and Type II mechanistic pathways upon exposure to UVB irradiation. It also exhibits oxidizing and reducing properties [8]. The aim of this study is to investigate the phototoxic and photomutagenic effect of PBI in the bacteria, *Salmonella typhimurium* strain TA 102 and human skin HaCaT keratinocytes upon concomitant exposure to PBI and light radiation. The photocytotoxicity and genotoxicity of PBI in human skin keratinocytes will provide direct link to human health.

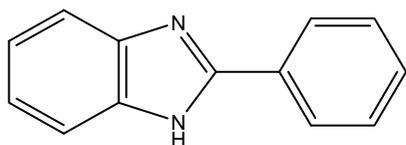


Figure 1: Chemical structure of 2-phenylbenzimidazole

Materials and Methods

Materials

Dimethyl sulfoxide (DMSO), 8-methoxypsoralen (8-MOP), and PBI were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI). Dr. Bruce Ames from the University of California (Berkeley, CA) nicely provided *Salmonella typhimurium* strain TA 102. Dr. Norbert Fusenig of the German Cancer Research Centre (Heidelberg, Germany) kindly donated the HaCaT keratinocytes, the predominant cell type in the epidermis. The Comet assay kit was from Trevigen Company (Gaithersburg, MD). The following materials were

purchased from American Type Cell Culture (Manassas, VA): Trypsin EDTA, Fetal Bovine Serum (FBS), and Dulbecco's minimum essential medium (DMEM). Penicillin/streptomycin and phosphate buffered saline (PBS) were from Fisher Scientific (Houston, TX).

Light Source

The irradiation source used was a 300 W Hg/Xe(Xe) solar simulator lamp from ORIEL Instruments (Stratford, CT). It encompasses the UVA, UVB, and visible light regions of the solar radiation. A Pyrex glass filter was placed atop the platform aligned with the pathway of the light beam. This arrangement allowed the sample contained within its respective Petri dish to be placed atop the platform and irradiated by the light beam positioned beneath it. The Pyrex glass also served as a filter to remove light of wavelengths <300 nm.

Phototoxicity Test in *Salmonella typhimurium* TA 102

The following was prepared a day prior to the experiment: the bacterium was cultured, the nutrient agar was prepared and autoclaved and the remaining materials were sterilized. The PBI stock solution (1 mM) was prepared in DMSO and 5-fold serial diluted with DMSO to obtain the desired concentrations.

Petri dishes containing 6.0 mL physiological water (0.5% NaCl), 1.5 mL of 0.2 M phosphate buffer, 300 μ L of the respective PBI sample dilution, and 300 μ L of TA 102 culture solution were prepared. The negative control used 300 μ L of DMSO to substitute the 300 μ L PBI solution. Under sterile conditions, seven test tubes were each filled with 9.0 mL of physiological water and 1.0 mL of the above mixture with various PBI concentrations. A 1.0 mL aliquot was pipetted into a petri dish and the labeled petri dish was irradiated for the designated time interval (0, 15, 30 min). After irradiation (or kept in the dark for the controls), nutrient agar (10 mL at 40°C) was poured into each of the Petri dishes, the Petri dishes were gently rotated to ensure even distribution and allowed 30 min to solidify followed by incubation at 37°C in an inverted position for 48 h to minimize condensation on the agar surface. The viable bacteria colonies were counted after incubation using the Bantex Colony Counter Model 920A.

Bacteria Mutagenicity Test in *Salmonella typhimurium* TA 102

The photomutagenicity assay was carried out with TA 102 as previously described with modifications [10-12]. The bacteria strain TA 102 was selected because it was more resistant to light irradiation than strains TA 98 and TA 100. This histidine auxotrophic strain contained an ochre mutation in the *hisG* gene, which increased its sensitivity to reversion and enabled TA 102 to readily detect numerous types of mutagens such as X-rays and UV light as well as cross-linking agents such as psoralens and mitomycin C [13]. Because TA 102 has the R-factor resistance plasmid, this

increased its ability to undergo chemical and spontaneous mutations by enhancing an error-prone DNA repair system. Therefore, because TA 102 has an error-prone DNA repair system, this allows for the detection of DNA damage due to light irradiation or exposure to chemical.

Preparation of Petri dishes containing the minimal agar

In a 2L flask, 30g of agar and 1860mL of distilled water was added and covered with aluminum foil. The flask was stirred on a hot plate for 30 min until the mixture was completely dissolved. The mixture was transferred to three 1 L glass containers, autoclaved for 15 min while covered with aluminum foil, and combined into two 1 L containers. The combined solutions were stirred on the hot plate and received 20mL of 50× Vogel Bonner and 50mL of 40% glucose solution. The minimal agar solution (30mL) was poured into each petri dish under the Environmental/Biohazardous hood and the Petri dishes were irradiated with UV light for 15 min while the minimal agar solidified. The petri dishes were then covered with lids and stored in a large plastic container covered with Aluminum foil until needed.

Top agar

In two 250 mL flasks, 0.6g agar, 0.5g NaCl, 100mL distilled water were mixed under stirring on a hot plate until complete dissolution. The flasks with the top agar were autoclaved for 15 min and 10 mL of histidine/biotin stock solution was added in the presence of an alcohol burner.

Bacteria culture

The stored bacteria TA 102 (50μL), Nutrient Broth No. 2 (12mL), and tetracycline (50μL) were mixed in a 50mL conical tube under sterile conditions. Thereafter, the loosely sealed cap was secured with masking tape and placed into the gyrorotary incubator for 10 h at 210 rpm, which promoted the growth of the bacterium.

Test tubes containing the mixture of 3.5mL of 0.02M sodium phosphate buffer, 700μL of the PBI in DMSO, and 700μL of TA 102 were vortexed and placed into the gyrorotary incubator for 20 min at 210 rpm to homogenize. Then, the 0.7 mL of this bacteria-PBI mixture was pipetted into the test tubes containing 2.0 mL of the top agar prepared the previous day in the Dri-bath at 45°C. The mixture was vortexed and poured onto the minimal agar petri dishes. The control petri dishes were kept in the dark and covered with aluminum foil while the other petri dishes were irradiated in an inverted position on the light source platform for 15 min rotated at 7.5 min. Two negative controls containing 4% DMSO in culture medium were used with one placed in the gyrorotary incubator for 20 min and the other not. The positive control chemical used in this study was 8-MOP (10μg/plate) irradiated for 2 min. 8-MOP is a

photomutagenic chemical used to ensure the correct setup of the experiment. After irradiation, the control and irradiated petri dishes were incubated for 48 hrs at 37°C and the number of revertant colonies was counted with a colony counter (Bantex, Model 920A). A repeat experiment was carried out to ensure accuracy of the data.

Cytotoxicity and Genotoxicity in Skin HaCaT Keratinocytes

Cell culture

The culture and treatment of the human skin HaCaT keratinocytes followed the procedure published previously with modifications [14]. The cells were grown in the media containing 10% FBS in DMEM and 1% penicillin/streptomycin in CO₂ incubator (5% CO₂) at 37°C. After reaching the desired cell concentration, the skin was washed twice with 1× PBS, treated with 0.25% trypsin/EDTA, and incubated for an additional 5 min at 37°C to ensure cell detachment. The detached cell in suspension was centrifuged for 5 min at 2000 rpm, supernatant discarded, cell pellet washed twice with 1× PBS, and finally resuspended with 1× PBS to adjust the concentration to ~ 1 × 10⁵ cells/mL.

Cytotoxicity test

Two 96-well plates (one as control without light irradiation) were divided into six nine-well regions. Equal volumes (100μL) of the cell suspension and PBI in 1×PBS were added to the designated regions of the nine wells of the 96-well plates. Each region of the plate represented a different PBI concentration. The desired concentrations of PBI in 4% DMSO were achieved via serial dilutions of the DMSO stock solution with PBS (0-50μM). The control plates were in the absence of light for a total of 135 min. The treated plates were irradiated region-by-region for 15 min each. After irradiation, six wells of the nine well were added with fluorescein diacetate (FDA, 100μL of 10 ng/mL) and the other three were taken out for Comet assay described later. The plates with added FDA were placed in a 5% CO₂ incubator for 35 min at 37°C. After incubation, the fluorescence intensity of FDA of each well was read using a Biosystem Fluoroskan II Microplate Reader (Helsinki, Finland) with the excitation and emission wavelength filters set at 485 nm and 538 nm, respectively.

Comet Assay

Immediately following irradiation, the first three wells were harvested and combined with molten LMAgarose at 42°C at a ratio of 1:10 (V/V). After mixing, 75μL of it was immediately pipetted onto the each of the two Comet slide wells. The slides were positioned flat in a tray at 4°C in the refrigerator for 10 min before immersed in a prechilled lysis solution at 4°C for 45 min to break the cell membrane. The excess buffer on the slide was tapped off before they were immersed in freshly prepared alkaline solution for 45 min

in the dark at room temperature. The slides were washed twice for 5 min in $1\times$ TBE buffer and placed onto the horizontal electrophoresis apparatus immersed in $1\times$ TBE buffer. A voltage of 1 Volt/cm was applied for 10 min for the DNA in the cell to migrate out of the cell nuclei. The slides were washed twice with 70% ethanol, covered with metal foil, and allowed to air dry overnight. Each of the slide wells was added with 50 μ L of diluted SYBR Green staining solution and air-dried overnight. The slides were viewed by an epifluorescence microscope to determine the extent of DNA damage to the HaCaT cells. The Comet tail moment, the product of the distance and the normalized intensity integrated over the tail length, measures the damage combining DNA contained in the tail with the distance of migration. A total of 75 cells per slide were scored to calculate the tail moment.

Statistical Analysis

Two-way analysis of variance (ANOVA) was performed to determine the statistical difference for the data. The P-value ($P < 0.05$) indicated there was a significant difference in the data. There was no correlation between the data of the irradiated HaCaT keratinocytes and those skin cells not irradiated with simulated solar light.

Results

Cytotoxicity of PBI in *Salmonella typhimurium* strain TA102

Figure 2 illustrates the effect of PBI on TA 102 viability at various concentrations. As the concentration of PBI increases, the number of colonies per plate decreases. The number of viable bacteria colonies remained is about 65% and 45% due to the exposure to 125 μ M and 625 μ M PBI and light, respectively, compared to the control without PBI. This indicates that PBI is phototoxic to TA 102 at concentrations $> 125 \mu$ M. In addition, the amount of TA 102 viable colonies for the 15 and 30 min irradiation time period is not significantly different. In other words, at $< 125 \mu$ M PBI, more than 65% of the bacteria are viable. Therefore, the following photomutagenicity tests were carried out at PBI concentrations below 125 μ M.

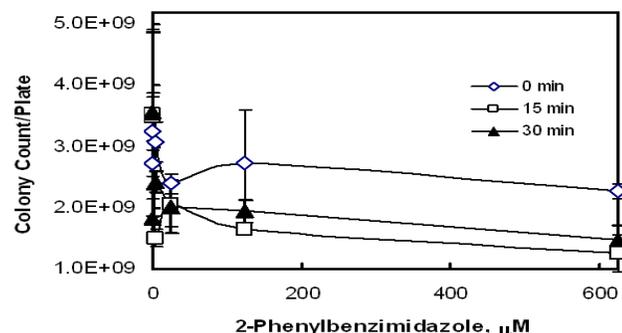


Figure 2: Cytotoxicity of 2-phenylbenzimidazole in TA102

Photomutagenicity of PBI in *Salmonella typhimurium* TA 102

Figure 3 depicts the photomutagenic activity of PBI under. The control plates containing 0-125 μ M PBI without light irradiation produced 220-252 revertant colonies per plate. However, the plates containing PBI with light irradiation demonstrated that with the increase of PBI concentration, the number of revertant colonies increases. At 0 μ M PBI, the number of revertant colonies is 287, but it gradually increases to 1700 at 25 μ M PBI and thereafter levels off. This clearly indicates that the combination of PBI and light irradiation causes mutation in *Salmonella* TA 102.

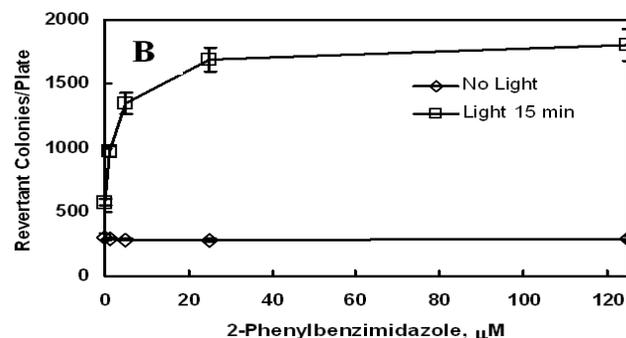


Figure 3: Photomutagenicity of 2-phenylbenzimidazole in TA 102

Cell Viability of HaCaT Keratinocytes Exposed to PBI and Light

Figure 4 illustrates the effect on HaCaT keratinocytes by PBI in the absence and presence of light irradiation. The overall cell viability decreases due to the exposure to PBI with or without light, but there are no differences between the light exposure group and dark control group. The amount of skin cells that survived after exposure to 5 μ M PBI was 80%, the same percentage that survived upon concomitant exposure to PBI and solar simulator irradiation. Similarly, treatment with 25 μ M PBI with no light irradiation resulted in the death of approximately 32% of the skin cells; whereas 35% of the cells died due to the exposure to 25 μ M PBI and light irradiation.

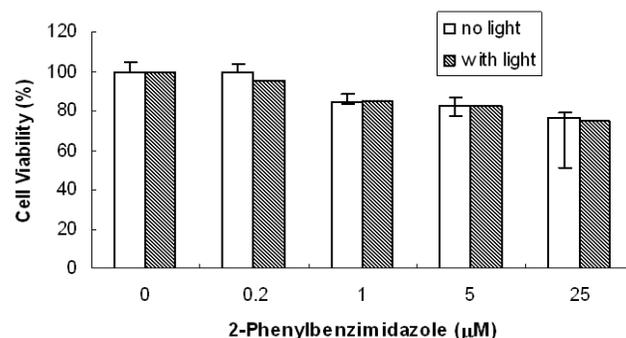


Figure 4: Cell viability of 2-phenylbenzimidazole in HaCaT keratinocytes. Statistical analyses indicate that the DNA tail moments are significantly different for all the light irradiated from the non-irradiated.

Genotoxicity and Photo-Genotoxicity of PBI in HaCaT Keratinocytes

As can be visualized in Figure 5, no appreciable DNA damage is seen for the skin cells treated with PBI up to 25 μM and kept in the dark. However, DNA damage is seen as the DNA tail moment for skin cells receiving concomitant exposure to simulated solar light and PBI. Significant DNA damage is seen within the 0.2–25 μM of PBI, indicating that the combination of light and PBI is genotoxic to the skin HaCaT keratinocytes. Although the standard deviation of the DNA damaged tail moment is large, the average of the tail moment is significantly larger in those skin cells exposed to solar simulator irradiation compared to those non-irradiated skin cells ($P < 0.001$).

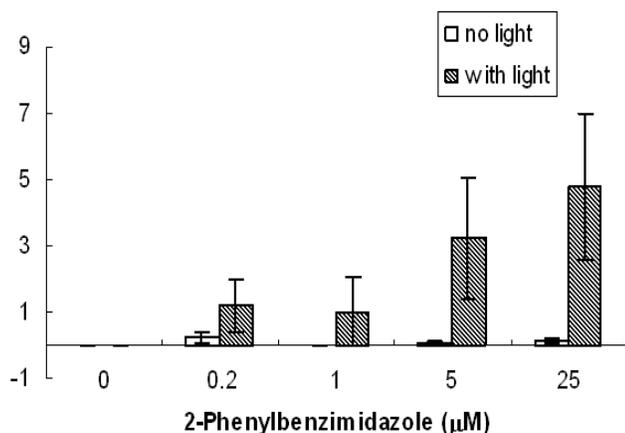


Figure 5: Cell genotoxicity of 2-phenylbenzimidazole in HaCaT keratinocytes. Statistical analyses indicate that the DNA tail moments are significantly different for all the light irradiated from the non-irradiated.

Discussions

When developing cosmetic products, it is essential that the UV absorbers are chemically and photochemically inert [15, 16]. Failure to ensure this factor may compromise this beneficial shielding effect if the photo-excited sunscreen agents decompose readily or react chemically with cellular components, such as DNA and protein [9]. It may also result in the rearranging of chemical bonds causing the formation of new molecules. This may inadvertently reduce the absorbance and, ultimately, alter the toxicological properties [1].

Benzimidazoles are used as fungicides and anti-helminthic agents [17]. The nature of the side groups on the benzimidazole rings greatly influence the action of the compound [18]. PBI is more potent in inducing chromosome damage in onion than 2-methylbenzimidazole following 24 h exposure in a dose-dependent response [17]. However, PBI does not induce chromosome loss in *Saccharomyces cerevisiae* strain D61.M and also lacks anti-mitotic activity [18, 19]. The commercially available UVB blocker, 2-phenylbenzimidazole-5-sulfonic acid (PBSA) also

sensitizes the production of singlet oxygen, resulting in the generation of 4,8-dihydro-4-hydroxy-8-oxo-2'-deoxyguanosine when irradiated with UVB in oxygenated solution in the presence of 2'-deoxyguanosine. Both compounds were also found to photo-induce the formation of alkali-labile cleavage sites in both single- and double-stranded DNA [1, 8, 9].

From the results presented above, PBI is photomutagenic in *Salmonella* TA 102 at a concentration as low as 1 μM . At 25 μM , the number of revertant colonies caused exposure to light irradiation and PBI at the same time is 1700 per plate compared to 220 revertant colonies per plate generated by the light-deprived control at the same concentration. This is a clear indication that PBI is strongly photomutagenic in TA 102 and is not mutagenic without light irradiation. The studies on the HaCaT keratinocytes demonstrated PBI did not affect the cell viability despite the increasing PBI concentrations or concomitant exposure to simulated solar irradiation. A slight decrease in cell viability was observed at 5 μM and 25 μM , respectively, with or without light irradiation. Therefore, it can be concluded that PBI in the 1–25 μM concentration range alone or in combination with light irradiation does not affect the skin cell viability. However, the combination of PBI in the 1–25 μM concentration range and light irradiation caused significant DNA damage in the HaCaT keratinocytes while no damage is observed without light irradiation.

Exposure to solar UV radiation (UVA and UVB) is believed to cause photoageing, photoimmunosuppression, and skin cancer and the precise mechanisms have yet to be elucidated [20]. Sunscreen chemicals play an important role in photo-protection. However, it is vital to ensure that a sunscreen formulation retains photostability over the entire solar UV spectrum. The photoprotection of sunscreens is lost if they are used as an acceptable preventive mechanism for longer sun exposure [5]. The accumulation of DNA damage during intense or repeated exposures should be considered as a relevant risk factor [20]. Photodamage is predicted to become a major threat to public health in the coming decades [1]. In general, the kind of sun protection best used by any person without a history of skin cancer will depend on frequency, duration, and intensity of sun exposure; as well as an individual's desire to avoid the acute and long-term consequences of exposure to sunlight [3].

Inbaraj et al. reported that the photochemical reactions of the sunscreen agent PBSA and its parent analog, PBI, may include the production of reactive oxygen species (ROS) [8]. Furthermore, the phosphorescence of PBI illustrated that, upon irradiation, its triplet states become populated. Its lifetime in this triplet states was measured to be 2 ms, long enough for PBI to react with oxygen under optimum temperature conditions [8]. The generation of singlet oxygen has demonstrated to be sensitized by many sunscreen agents. Hence, it was concluded that PBSA had the photosensitizer potential to interact with DNA, while serving as a barrier between skin cells and the harmful

effects of direct sunlight. Our study clearly indicates that PBI can cause mutation in bacteria and damages to the cell nuclear DNA in human skin cells when in combination with simulated solar light. This means that when a person applies a sunscreen containing PBI and goes outdoors under the sunlight, he or she might be at risk of skin cancer, photoageing, or photo-immunosuppression.

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