Regular article

Analysis of Photomutagenicity of Thiabendazole with UVA Irradiation: Absence of 8-Hydroxyguanosine Formation

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We recently reported the photomutagenic property of thiabendazole [2-(4-thiazolyl)-1*H*-benzimidazole, TBZ], a post harvest fungicide, in bacterial and mammalian cells. In the present study we investigated its photomutagenic potential along with several of its structurally-related pesticides including benomyl, mefenacet, tricyclazole, and imazalil. None of the compounds except TBZ showed photomutagenicity with UVA-irradiation in *S. typhimurium* TA100, TA98, or *E. coli* WP2uvrA/pKM101. Since TBZ consists of thiazole and benzimidazole rings, we then investigated photomutagenic potential of thiazole, benzimidazole, and 2-phenylbenzimidazole. Only 2-phenylbenzimidazole showed very weak photomutagenicity with UVA-irradiation. These results indicate both benzimidazole ring and thiazole ring are necessary for UVA-activation of TBZ. We measured 8-hydroxydeoxyguanosine (8-OHdG) in salmon sperm DNA exposed to TBZ and UVA by HPLC-ECD, and found that 8-OHdG was not responsible for the photomutagenesis of TBZ.

Key words: photomutagenicity, UVA, TBZ, pesticide, 8-OHdG

Introduction

Thiabendazole [2-(4-thiazolyl)-1*H*-benzimidazole, TBZ] is approved in Japan as a post-harvest fungicide for imported citrus fruits during transport and storage. We previously reported that TBZ shows potent photomutagenicity following UVA irradiation (320–400 nm) for 10 min at a dose rate of 2.5 J/m²/sec in *Escherichia coli* strain WP2uvrA/pKM101 and *S. typhimurium* strains TA97, TA104, and TA100 (1–3). Structurally related compounds containing imidazole or thiazole, such as benomyl, mefenacet, tricyclazole, and imazalil are widely used in agriculture as pesticides. The first purpose of the present study was to investigate whether those compounds are also photomutagenic.

TBZ photomutagenicity is observed when bacterial cells are irradiated with UVA in its presence, but neither pre-irradiation of TBZ just before adding the cells, nor pre-treatment of cells with TBZ before UVA-irradiation generates mutagenicity (1,3). It seems likely, therefore,

that the photomutagenic TBZ products are unstable and/or rapidly react with other molecules before being incorporated into the cells. One likely mutagenic species could be oxygen radicals. The contribution of reactive oxygen species to photomutagenicity has been reported for many chemicals, including methylene blue (4), chlorpromazine (5,6), benzo[a]pyrene (7), and several *N*-nitroso compounds (8–11). The second purpose of the present study was to investigate the possibility that 8-hydroxydeoxyguanosine (8-OHdG) is formed in DNA exposed to TBZ and UVA *in vitro*.

Materials and Methods

Chemicals, bacterial strains and media: Thiabendazole [2-(4-thiazolyl)-1*H*-benzimidazole, TBZ, CAS No. 148-79-8], benomyl [methyl 1-(butylcarbamoyle)-2-benzimidazolecarbamate, CAS No. 17804-35-2], mefenacet (2-benzothiazol-2-yloxy-*N*-methylacetanilide, CAS No. 73250-68-7), tricyclazole (5-methyl-1,2,4-triazolo[3,4-*b*]benzothiazole, CAS No. 41814-78-2), imazalil {1-[2-(2,4-dichlorophenyl)-2-allyloxyethyl]-1*H*-imidazole, CAS No. 35554-44-0}, benzimidazole (CAS No. 51-17-2), 2-phenylbenzimidazole (CAS No. 716-79-0), and thiazole (CAS No. 288-47-1) were purchased from Wako Pure Chemical Industries Ltd., (Tokyo). Figure 1 shows the structures of these compounds. Angelicin (CAS No. 523-50-2) was obtained from Sigma-Aldrich Co., (MO, USA). Compounds were dissolved in dimethylsulfoxide (DMSO) just before the experiments. The final concentration of DMSO in cell suspension during UVA irradiation was 4% or less. We used *S. typhimurium* strains TA100 (*hisG46*, *uvrB*, *rfa*/pKM101), TA98 (*hisD3052*, *uvrB*, *rfa*/pKM101), and *E. coli* strain WP2uvrA/pKM101 [*trpE65*, *uvrA*] for the reverse mutation assays and cultured them in Nutrient medium (Oxoid, No.2) at

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Fig. 1. Chemical structure of TBZ and its structurally-related compounds investigated.

37°C with shaking. Minimal glucose agar plates consisted of Vogel-Bonner E medium supplemented with 2% glucose and 1.5% agar (12). Top agar (0.6% agar and 0.5% NaCl) contained 0.05 mM D-biotin and 0.05 mM L-histidine for (*S. typhimurium*) or 0.05 mM L-tryptophan (for *E. coli*).

UVA irradiation and reverse mutation assay: We used a black-light fluorescent lamp (National Black Light Blue, FL15BL-B, 15W, Matsushita Electric Industrial Co., Japan) that emitted wavelengths of 300–400 nm as the UVA source. To filter out UVB wavelengths below 320 nm, we inserted a 5-mm thick soft glass plate between the culture dishes and the lamp. UVA was irradiated from a distance of 20 cm at a dose rate of 2.5 J/m²/sec (UVX Radiometer, Model UVX-36, Ultra-Violet Products, Upland, CA, USA) on a 24-well multiplate with lid. We placed 0.1 mL of overnight culture ($1-3 \times 10^9$ cells/mL) in each well of a 24-well multiplate containing 0.5 mL 100 mM sodium phosphate buffer (pH7.4). We added the test compound solution (5–20 μ L) dissolved in DMSO, mixed it in well, and irradiated the mixture with UVA for 10 min at room temperature. We then added the mixture in each well to 2 mL molten top agar in a test tube kept at 46°C, immediately poured the mixture onto a minimal glucose agar plate, incubate the plates for 2 days at 37°C, and then counted the number of His⁺ or Trp⁺ revertant colonies. All experiments were conducted in duplicate, the controls in triplicate. We judged as positive a doubling or greater increase over the control value together

with a dose-related response. Angelicin was used as a positive control for photomutagenicity (13).

8-OHdG assay: We dialyzed salmon sperm DNA (Wako Pure Chemical) dissolved in TE buffer at a concentration of 5 mg/mL against 10 mM Na-phosphate buffer (pH7.4). TBZ was dissolved in methanol at a concentration of 10 mg/mL. We mixed DNA (0.5 mg) and TBZ (0.05 and 0.1 mg) into 0.25 mL sodium phosphate buffer in each well of a 24-well multiplate (final concentrations of TBZ were 2 and 4 mg/mL). We used *N*-nitrosopyrrolidine (Sigma-Aldrich, CAS No. 930-55-2) dissolved in buffer as the positive control (10). We irradiated the mixture with UVA for 15, 30, and 60 min and then dialyzed the reaction mixture against water. We treated the dialyzed DNA with Nuclease P1 (5 unit, Seikagaku Corp., Tokyo) in sodium acetate buffer (20 mM, pH4.8) at 37°C for 2 h, and then by bacterial alkaline phosphatase (1 unit, Takara Bio Inc., Shiga, Japan) in Tris-HCl buffer (100 mM, pH8.0) at 37°C for an additional 2 h. We then removed the enzymes by ultrafiltration (14,000 g, 10 min) with Microcon YM-30 (Millipore Corp., USA), added 1 μ L 500 mM EDTA (pH8.0) to the filtered samples, and kept the mixture at –20°C. We measured the amount of deoxyguanosine (dG) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) using a high-performance liquid chromatography (HPLC) system (Tosoh, Japan). The system was composed of two pumps (CCPM-II), a degasser (SD-8020), an autosampler (AS-8020), a column oven (CO-8020), a reverse-phase column (Hydrosphere C18, 5 μ m, 150 $\times$ 4.6 mm ID, YMC, Japan), a UV detector (UV-8020), and an electrochemical detector (ECD, EC-8020). We used Tosoh HPLC Multi Station software (LC-8020) for data acquisition. A 25 μ L of sample was injected into the HPLC system. 8-OHdG and dG were eluted at a flow rate of 1 mL/min. We used two solvents for gradient elution-solvent A (2% acetonitrile in 10 mM phosphate buffer and 1 mM EDTA, pH 7.0) and solvent B (8% acetonitrile in the same buffer). The elution conditions were as follows: 0–5 min 0% solvent B; 5–20 min, 0–100% solvent B; 20–25 min 100% solvent B isocratic; 25–30 min 100–0% solvent B. The column temperature was maintained at 35°C. 8-OHdG was detected with the ECD equipped with glassy carbon working electrode operated at +500 mV versus an Ag/AgCl reference electrode. dG was measured by UV absorption at 254 nm. Data were expressed as 8-OHdG/10⁵ dG (nM).

Results and Discussion

Table 1 shows the results of photomutagenicity test on pesticides with *E. coli* WP2uvrA/pKM101 and *S. typhimurium* TA100 and TA98. No mutagenicity was observed with benomyl, mefenacet, tricyclazole, or im-

Table 1. Photomutagenicity of test pesticides

Compound	Dose $\mu\text{g}/\text{plate}$	Revertants/plate [†]		
		WP2uvrA/pKM101	TA100	TA98
Solvent control		83	113	17
UVA control		94	107	19
TBZ	50	377	165	18
	100	591	257	23
	500	623	246	21
Benomyl	10	90	105	14
	50	84	94	18
	100	64	71	11
	500	28	33	4
Mefenacet	50	98	99	14
	100	90	97	16
	500	102	92	12
	1000	98	102	14
Tricyclazole	50	85	109	17
	100	84	114	15
	500	51	100	16
	1000	28	67	13
Imazalil	5	72	111	14
	10	67	113	10
	50	79	114	11
	100	57	111	9
	200	*	*	*
Angelicin	2	948	472	18

*Growth inhibition, [†]Mean of duplicate plates.

Cells were irradiated with UVA for 10 min in the presence of the chemical.

azalil, while UVA-preirradiated TBZ induced reverse mutations in WP2uvrA/pKM101 and TA100. Since the photomutagenic property was dependent on the structure of TBZ, we then investigated photomutagenic potential of thiazole, benzimidazole, and 2-phenylbenzimidazole with WP2uvrA/pKM101. 2-Phenylbenzimidazole precipitated at doses of 100 μg or more, which limited effective UVA-irradiation. Thiazole and benzimidazole could be tested up to a dose of 500 μg without precipitation. Neither thiazole nor benzimidazole showed photomutagenicity with UVA-irradiation. On the other hand, the photomutagenicity of UVA-irradiated 2-phenylbenzimidazole was marginal at a dose of 50 μg (Fig. 2). The weak increase was reproducible in the four independent experiments (the maximum increase in each experiment was 2.4, 2.2, 1.9, and 2.0 times the control; data not shown). The limited activity of the compound might be due to its low solubility. On the other hand, 2-phenylbenzimidazole-5-sulfonic acid, a derivative of 2-phenylbenzimidazole, is widely used in sun-screen and cosmetics because of its strong absorption in the UVB region (14–17). Stevenson and Davies (17) reported that 2-phenylbenzimidazole and 2-phenylbenzimidazole-5-sulfonic acid caused guanine base damage in DNA by sensitizing photooxidation by singlet oxygen *via* both type I and type II mechanisms

Fig. 2. Photomutagenic activity of TBZ and 2-phenylbenzimidazole with UVA-irradiation in *E. coli* WP2uvrA/pKM101. The mixture of bacterial cells and test compound in 0.5 mL of phosphate buffer was irradiated with UVA for 10 min. Data are the means of 2 experiments. Benzimidazole (open circle); Thiazole (open square); 2-Phenylbenzimidazole (open triangle); TBZ (closed circle).

when they are exposed to narrow-band UVB radiation or natural sunlight. We, therefore, investigated photomutagenic potential of 2-phenylbenzimidazole-5-sulfonic acid with UVA-irradiation using WP2uvrA/pKM101 and TA100 at doses up to 200 $\mu\text{g}/\text{plate}$. No photomutagenicity was detected with UVA-irradiated 2-phenylbenzimidazole-5-sulfonic acid (data not shown). These results indicate that both the benzimidazole ring and the thiazole ring are necessary for UVA-activation of TBZ.

In order to investigate the possible contribution of 8-OHdG in photomutagenesis of TBZ, we assayed 8-OHdG formation in DNA exposed to TBZ and UVA *in vitro*. Salmon sperm DNA solution was irradiated with UVA for 15, 30, and 60 min in the presence of TBZ at 200 and 400 $\mu\text{g}/\text{mL}$. The results of analysis are shown in Fig. 3. No increase of 8-OHdG in DNA was detected with any irradiation time at any dose of TBZ, while *N*-nitrosopyrrolidine at 1 mg/mL used as a positive control caused 8-OHdG formation. The data suggest that UVA-treatment of TBZ does not generate oxidative damage in DNA, and 8-OHdG is not likely the mechanism responsible for the photomutagenesis of TBZ. We previously investigated inhibitory effects of several scavengers for reactive oxygen species such as ethanol, dimethyl sulfoxide, mannitol, histidine, ascorbic acid, and epigallocatechin on photomutagenic activation of TBZ and found that these scavengers did not show any apparent inhibitory effects. In addition, neither catalase nor SOD inhibited the photomutagenicity of TBZ in reverse mutation assays using *E. coli* WP2

Fig. 3. 8-OHdG in DNA exposed to TBZ and UVA. UVA was irradiated for 15, 30, and 60 min on the mixture of DNA and TBZ (200 and 400 μg/mL) in phosphate buffer. Without UVA (white bar); UVA 15 min (light gray bar); UVA 30 min (dark gray bar); UVA 60 min (black bar). NPR (*N*-nitrosopyrrolidine, 1000 μg/mL) was used as the positive control.

uvrA/pKM101 (2). We also reported that TBZ with UVA irradiation caused G:C→A:T transitions most frequently, followed by A:T→T:A transversions and A:T→G:C transitions. However, induction of G:C→T:A transversions was very low (1). G:C→T:A transversions are known to be typical mutations caused by oxidative DNA damage (18). Therefore, a different mechanism, perhaps direct interaction with DNA, must be involved in the photomutagenicity of UVA-irradiated TBZ. We suspect that the production of radicals followed by DNA bulky adduct formation is involved, but that awaits future investigation.

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