



Light-induced mutagenicity in *Salmonella* TA102 and genotoxicity/cytotoxicity in human T-cells by 3,3'-dichlorobenzidine: a chemical used in the manufacture of dyes and pigments and in tattoo inks

Lei Wang, Jian Yan, William Hardy, Charity Mosley, Shuguang Wang, Hongtao Yu*

Department of Chemistry, Jackson State University, Jackson, MS 39217, USA

Received 4 August 2004; received in revised form 21 October 2004; accepted 24 October 2004

Available online 8 December 2004

Abstract

DCB, 3,3'-dichlorobenzidine, is used primarily as an intermediate in the manufacture of diarylide yellow or azo red pigments for printing ink, textile, paint, and plastics. It is also used in tattoo inks. In this article, we investigate light-induced toxicity of DCB in both bacteria and human Jurkat T-cells. DCB itself is not toxic or mutagenic to *Salmonella typhimurium* TA102, but is photomutagenic at concentrations as low as 2 μM and phototoxic at concentrations $>100 \mu\text{M}$ when bacteria are exposed to DCB and light at the same time (1.2 J/cm² of UVA and 2.1 J/cm² of visible light). Furthermore, DCB is both photocytotoxic and photogenotoxic to human Jurkat T-cells. Under a light irradiation dose of 2.3 J/cm² of UVA and 4.2 J/cm² of visible light, it causes the Jurkat T-cells to become nonviable in a DCB dose-dependent manner and the nonviable cells reaches 60% at DCB concentrations higher than 50 μM . At the same time, DNA fragmentation is observed for cells exposed to both DCB and light, determined by single cell gel electrophoresis (alkaline comet assay). As much as 5% (average) DNA fragmentation was observed when exposed to 200 μM DCB and light irradiation. This suggests that DCB can penetrate the cell membrane and enter the cell. Upon light activation, DCB in the cells can cause various cellular damages, leading to nonviable Jurkat T-cells. It appears, the nonviable cells are not caused solely by fragmentation of cellular DNA, but by other damages such as to proteins and cell membranes, or DNA alkylation. Therefore, persons exposed to DCB through environmental contamination or through tattoo piercing using DCB-containing inks must not only concern about its toxicity without exposing to light, but also its phototoxicity. © 2004 Elsevier Ireland Ltd. All rights reserved.

Keywords: 3,3'-Dichlorobenzidine; *Salmonella typhimurium* TA102; Phototoxicity; Photomutagenicity; Jurkat T-cell; Single cell gel electrophoresis (comet assay)

* Corresponding author. Tel.: +1 601 979 2174; fax: +1 601 979 3674.

E-mail address: yu@ccaix.jsums.edu (H. Yu).

1. Introduction

Tattoos, as a body art, have recently become more popular with 5–10% of the general population using tattoo/piercing in Europe, and high school students engaged in tattooing is increasing in the United States (Papameletiou et al., 2003). There are no strict laws and regulations in Europe that take into account the rapid increase of tattoos/piercing (Zenie et al., 2003). The European Commission has been trying to develop and implement regulations for the protection of human infections associated with tattoo/piercing (Walser, 2003).

Most colorants used for tattoos are pigments and dyes. Pigments are either metallic salts or organic compounds, but dyes are usually organic (Bäumler et al., 2003). Many synthetic organic colorants are in use in addition to the traditional inorganic pigments. One of the popular colorants is made of azo dyes (Bäumler et al., 2003). The azo colorants usually contain 3,3'-dichlorobenzidine (DCB), *o*-anisidine, or 4-chloro-toluidine. DCB has been assigned to a category 2B probable human carcinogen by the International Agency for Research on Cancer (IARC, 1987). Studies by Bäumler et al. pointed out that DCB can be found in the metabolites of azo pigments (Bäumler et al., 2003). In addition, industries had used large amounts of benzidine and benzidine congeners to produce commercial azo dyes for textiles, paper, leather and plastics in the past (Haley, 1975; Choudhary, 1996). DCB has also been found in commercial pigments such as pigment yellow 87 or pigment orange 16 since it is used as an intermediate in the manufacture of these pigments for printing ink, textile, paint, and plastics (Claxton et al., 2001; Bäumler et al., 2003). It is reported that DCB is also found in tattoo bands, hair dyes, folders of paper, toys, bed clothes, watch straps and airbrush inks (IARC, 1982; CFR, 1985; Zeilmaker et al., 2000a). Another source of DCB is from the reduction of azo dyes by intestinal microbes or environmental microorganisms (Claxton et al., 2001).

The concentrations of DCB and benzidine related products have been reported in many products. According to Zeilmaker et al. (2000a), DCB concentration is 340 µg/mg in inks including airbrush inks, benzidine concentrations are 337 µg/mg in the string of children's sweaters, 11–113 ppm in tattoo bands, and 80–340 ppm in 'airbrush' inks. It has been estimated that the daily dermal uptake for DCB is 0.80 ng/kg/day.

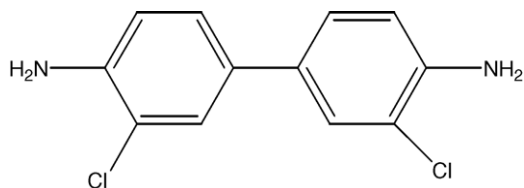


Fig. 1. Chemical structure of 3,3'-dichlorobenzidine.

It has been confirmed that DCB is mutagenic in *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and TA1538 with the presence of the S9 metabolic enzyme activation system (Garner, 1975; Prival et al., 1984; Iba, 1987; Chung et al., 2000). Among all dihalogenated benzidines, DCB is the most mutagenic (Savard and Josephy, 1986). DCB can be absorbed readily through skin (Lee and Shin, 2002). Although DCB has not been associated with cancer in man, there are sufficient evidences that it is carcinogenic in mice, rats, hamsters, and dogs (IARC, 1982). As with benzidine, the US Occupational Health and Safety Administration has strict regulations that workers should not get in contact with (CFR, 1985). The chemical structure of DCB is shown below in Fig. 1.

Photochemically, DCB is unstable and can dechlorinate under light irradiation (Banerjee et al., 1978; Nyman et al., 2002). It may be possible that during the photochemical transformation or dechlorination of DCB, reactive species may be formed that can cause phototoxicity including photomutagenicity. Therefore, the phototoxicity including photomutagenicity of DCB in *Salmonella typhimurium* bacteria strain TA102 and human Jurkat T-cells is examined in this article.

2. Material and methods

2.1. Material

DCB was purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. *Salmonella typhimurium* TA102 was kindly provided by Dr. Bruce Ames from the University of California (Berkeley, CA). Rat liver (S9, induced by Aroclor1254) was purchased from ICN Company (Aurora, OH). Human Jurkat T-cell (Jurkat TIB152) was from the American Type Culture Collection (Rockville,

MD). Most media and chemicals for cell culture were purchased from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Hyclone Laboratory (Logan, UT). Comet assay kits were purchased from Trevigen Company (Gaithersburg, MD). The Jurkat cells were cultured in a humidified atmosphere with 5% CO₂ at 37 °C for 24 h. RPMI 1640 medium was used for these non-adherent cells. Media was made complete by addition of 10% FBS and 1% of 100× antibiotic–antimycotic (containing both fungizone and pen/strep, GIBCO Cat. No. 15240-096).

2.2. Light source

A 300 W Xe/Hg(Xe) lamp from ORIEL Instruments (Stratford, CT) producing a full-spectrum light ranging from 300 to 800 nm was used. A Pyrex glass plate, which filtered out light with wavelengths less than 300 nm, was placed above the light beam at 40 cm from the light bulb. This distance is carefully maintained to obtain an UVA irradiance of 13.8 J/cm² h and visible light irradiance of 25.3 J/cm² h. Output energy was measured using a photo-radiometer (Cat. No. 2100) equipped with UVB (280–320 nm), UVA (320–400 nm), and visible light probes (400–750 nm) from Solar Light Co. (Philadelphia, PA).

2.3. Photomutagenicity assay

DCB photomutagenicity test was conducted based on the modified bacteria mutagenicity test developed by Maron and Ames (1983). A detailed procedure was described in two previous publications (Wang et al., 2003; Yan et al., 2004).

2.4. Bacteria viability

Bacteria viability test was conducted with a modified procedure (Ben-Hur et al., 1980). A 0.1 mL overnight cultured bacteria suspension (1×10^9 to 2×10^9 cells/mL, 10 h at 37 °C) was mixed with 0.1 mL of various concentrations of DCB solution in dimethyl sulfoxide (DMSO) and 2.5 mL PBS. Before irradiation, a 1.0 mL sample of the bacteria mixture was taken as the dark control. The rest of the sample was irradiated for 5 min (1.2 J/cm² of UVA and 2.1 J/cm² of visible light). After irradiation, another 1.0 mL sample was taken and both 1.0 mL samples were serially diluted with

sterilized, de-ionized water and poured onto the nutrient agar plates with the expected bacteria colonies of around 20–200 per plate. These plates were then put inversely in an incubator at 37 °C for 24 h. The colonies were counted with a colony counter (Bantex, Model 920A). All experiments were in triplicates.

2.5. Phototoxicity test of DCB in Jurkat T-cells

After the Jurkat T-cells grew to a concentration of no less than 1×10^5 cells/mL, the cells were harvested and centrifuged at 2000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with $1 \times$ PBS. Finally, the pellet was resuspended in $1 \times$ PBS to reach a cell concentration of approximately 5×10^5 cells/mL. Then the cell suspension was placed in a 96-well plate with 100 μL in each well. The concentrations of DCB were 2, 10, 50, 100, and 200 μM, obtained through a serial dilution of the freshly prepared 1 mM DCB stock solution in DMSO with $1 \times$ PBS. Then 100 μL of the desired DCB solution was added into each well containing cell suspensions. Two sets of 96-well plates were used with one covered with aluminum foil as the dark control, while the other was irradiated for 10 min with the 300 W Xe/Hg(Xe) (UVA 2.3 J/cm² and visible light 4.2 J/cm²). In each 96-well plate, nine-well sets were used for each DCB concentration. Among the nine wells at each DCB concentration, the cells in three wells were taken out for comet assay and the remaining six for cell viability assay.

2.6. Cell viability

The six wells for cell viability test received 100 μL of fluorescein diacetate (FDA, 10 ng/mL) in each well and incubated at 37 °C for 35 min. The plates were then read immediately using a Fluoroscan Ascent FL (Thermo Labsystems) with filters set at an excitation wavelength of 485 nm and emission wavelength of 538 nm. All experiments were in triplicates.

2.7. Alkaline comet assay

This assay has been used worldwide to detect cellular DNA fragmentations (Moneef et al., 2003). In this study, cell suspensions taken out from the three wells were centrifuged at 2000 rpm for 5 min. The pellets

were washed twice with cold $1 \times$ PBS at 4°C and re-suspended in media to obtain a cell concentration of 1×10^5 cells/mL. The following is a brief procedure for alkaline comet assay adopted from the manufacturer's instruction manual (Trevigen, 2001). In a 1 mL test tube, 20 μL of the cell suspension and 200 μL of melted agarose was mixed at 40°C , and 75 μL of the mixed suspension were pipetted onto a pre-warmed slide at 37°C . The slides were placed in a refrigerator at 4°C for 10 min before being placed in chilled lysis buffer at the same temperature for 45 min. Then the slides were tapped off extra buffer and immersed in freshly prepared alkaline solution (pH > 13, 0.25 M NaOH solution containing 0.1 μM EDTA) for 45 min at room temperature. Slides were then removed from the alkaline solution and washed twice by immersing in $1 \times$ TBE (tris-borate-EDTA) buffer for 5 min before being placed in a horizontal gel electrophoresis box (25 cm between the two electrodes in $1 \times$ TBE) and ran at 1 V per centimetre for 10 min. Afterwards, slides were placed in 70% ethanol for 5 min, removed, tapped off excess ethanol, and air-dried for 2.5 h at room temperature. Finally, 50 μL of diluted SYBR green solution was placed onto each circle of dried agarose and allowed to set for 4 h. The slides were then read with a fluorescence microscope equipped with DNA Damage Analysis Software (Loates Associates Inc.). Along with each set of experiment, a positive control, treated with 200 μM of potassium permanganate for 20 min at 4°C , was used. A DNA damage parameter, comet tail moment, was used for this study. The comet tail moment is the product of the distance and normalized intensity integrated over the tail length for a damage measure combining the amount of DNA in the tail with the distance of migration. A total of 70 cells per sample were scored and the tail moment was averaged. Statistical analyses were performed using the SAS system for windows (Release 8.00 TS level, SAS Institute Inc., Gary, NC).

3. Results

3.1. Phototoxicity and photomutagenicity of DCB in *Salmonella typhimurium* TA102

Salmonella typhimurium TA102, in the presence of DCB with concentrations of 0, 0.2, 0.5, 1.0, 2.0, 5.0,

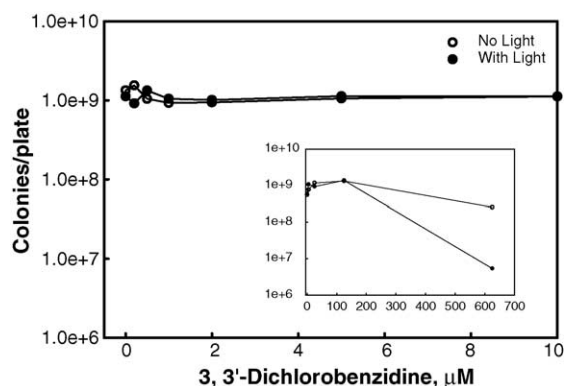


Fig. 2. Survival of *Salmonella typhimurium* TA102 in the presence of DCB with or without light irradiation (1.2 J/cm² of UVA and 2.1 J/cm² of visible light). Bacteria colonies were grown in Petri dishes with nutrient agar. Insert is the graph for higher DCB concentrations. All data points were the average reading from three Petri dishes.

10, 25, 50, and 100 μM , was irradiated for 5 min under a Xe/Hg(Xe) lamp (1.2 J/cm² of UVA and 2.1 J/cm² of visible light). The bacteria viability is shown in Fig. 2. There is no toxic effect observed whether with or without light irradiation in this DCB concentration range. However, at a higher concentration of 625 μM , bacteria colonies decrease >100 times under light irradiation and 10 times without light irradiation (insert in Fig. 2). These results indicate that DCB is toxic to TA102 at high concentrations (>100 μM) with or without light irradiation, but the toxicity is greater when the bacteria is exposed to DCB and concomitantly irradiated. However, the phototoxicity of DCB is negligible at concentrations <100 μM with a light dose of 1.2 J/cm² of UVA and 2.1 J/cm² of visible light.

Photomutagenicity of DCB in the concentration range from 0 to 100 μM was examined with the same light dose as the phototoxicity test and the results are shown in Fig. 3. Without light irradiation, DCB is not mutagenic in TA102 (lower line in Fig. 3). With light irradiation, DCB is photomutagenic in TA102, exhibiting four times higher the number of revertant colonies of the negative control and 2.4 times higher the number of revertant colonies of the light control (first point of the top line in Fig. 3). It also shows a 15% decrease of the number of revertant colonies at the DCB concentration of 100 μM comparing with the number of revertant colonies between 15 and 50 μM of DCB. A 43%

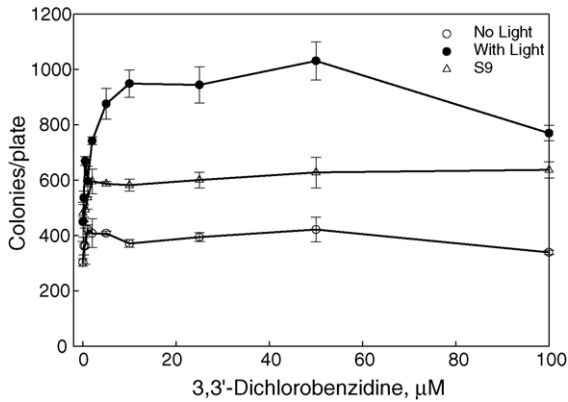


Fig. 3. Mutagenicity of DCB with *Salmonella typhimurium* TA102 with light irradiation or S9 metabolic activation. Samples were irradiated with 1.2 J/cm² of UVA and 2.1 J/cm² of visible light. All data are the average reading of three parallel Petri dishes. All experiments were repeated at least twice.

decrease at an even higher concentration of 625 µM was observed (data not shown). These results indicate that DCB is photomutagenic at concentrations of as low as 2 µM and phototoxic at higher concentrations of >100 µM. In comparison, the presence of the metabolic system, liver S9 homogenate (2 mg/plate), increases the number of revertant colonies compared with the negative control, but is more than 50% lower than using light as the activation system.

3.2. Photocytotoxicity and photogenotoxicity of DCB in human Jurkat T-cells

3.2.1. Photocytotoxicity

Human Jurkat T-cells were treated with DCB at concentrations of 0, 5, 10, 50, 100, and 200 µM. Fig. 4 shows the cell viability with or without light irradiation. Without light irradiation, a 20% cell death was observed due to exposure to DCB at concentrations >50 µM. There was no significant cell death under 10 µM of DCB. With 10 min of light irradiation (2.3 J/cm² of UVA and 4.2 J/cm² of visible light), the number of viable cells decreases in a DCB concentration dependent manner from 5 to 50 µM, reaching about 40% of viable cells remaining before leveling off at higher DCB concentrations. All data showed significant difference between with light and without light samples.

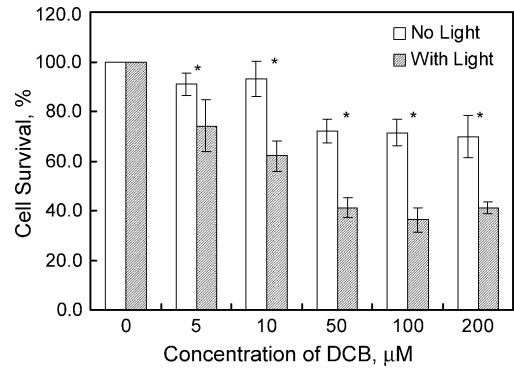


Fig. 4. Viability of human Jurkat T-cells after exposure to DCB with or without light irradiation (UVA, 2.3 J/cm² and visible light, 4.2 J/cm²). Total cell numbers were 10,000 cells/well. The results shown in this figure were the average from three independent experiments. Statistic analysis showed that the data with light vs. without light had significant differences.

3.2.2. Photogenotoxicity

Jurkat T-cells were treated with DCB and irradiated with light for 10 min. Cells were then treated with alkaline solution followed by electrophoresis. The cells on the slides were viewed with an EP-fluorescence microscope and photographed with a camera. A normal untreated cell is round in shape with little or no tail. Cells exposed to either DCB or light had very little changes in shape comparing with the untreated. Concomitant exposure to DCB and light caused fragmentation to the cellular DNA and long comet tail was observed.

Details of the DNA damage were analyzed by the comet tail moment (Fig. 5). There are very little damages to the cellular DNA if DCB is present, but without light irradiation. With light irradiation, the tail moment increases with the increase of DCB concentrations. An average of comet tail moment of five is observed due to exposure to 200 µM DCB and light. At this concentration, about 5% of the DNA was fragmented (data not shown). Based on 70 cells, all data have significant differences between with light and without light groups through statistical analysis with the SAS system. This implies that DCB can first penetrate the cell membrane into the cell nucleus and to be in the proximity of cellular DNA molecules to cause light-induced DNA damage. However, the DNA damage observed here is relatively small considering that up to 40%

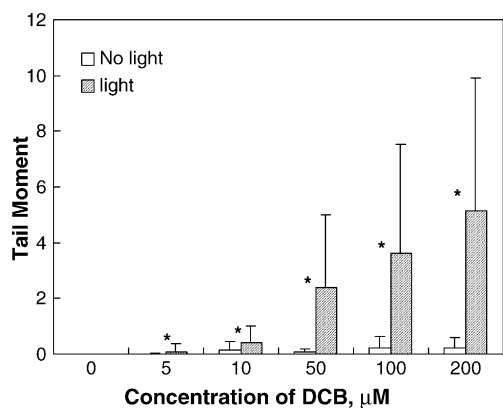


Fig. 5. DNA damage measured by the comet tail moment for human Jurkat T-cells in the presence of DCB upon light irradiation (2.3 J/cm² of UVA and 4.2 J/cm² of visible light). A total of 70 cells were scored for each point on this graph. There are significant differences between the light and without light groups through the calculation of SAS system analysis.

of the cells became nonviable under the same conditions. This indicates that DNA fragmentation may not be the main reason for nonviability of the Jurkat cells.

4. Discussion

DCB is a known animal carcinogen and a reasonably anticipated human carcinogen based on sufficient carcinogenicity data in experimental animals (IARC, 1982; Iba, 1989). There is no specific regulation on its use because DCB has physical superiority and economic advantage over other existing precursors for the preparation of pigments and toners (Iba, 1989; NTP, 2002). Human exposure to DCB can be through skin absorption, inhalation, and ingestion. Therefore, it is anticipated that DCB could be distributed in the inner organs as well as in the skin. In the inner organs, DCB toxicities, including carcinogenicity, is due to metabolic activation involving oxidation to highly reactive *N*-oxygenated intermediates by the microsomal cytochrome P-450 and flavin monooxygenase enzyme complexes in rodents (Iba, 1989; Canada, 1993). Although the 3,3'-dichlorobenzidine-derived *N*-oxygenated intermediates have not been unequivocally identified, they are believed to be responsible for the mutagenic and genotoxic effects (e.g. re-

lated to DNA binding) in bacterial and mammalian systems.

DCB molecules in skin cells are subject to light irradiation. The results presented here show that DCB is photomutagenic in *Salmonella* bacteria at a concentration of as low as 2 μM and photocytotoxic and photogenotoxic in human Jurkat T-cells at concentrations in the 2–100 μM range. According to Zeilmaker et al. (2000a), the concentration of DCB is 340 μg/mg in inks and the estimated daily dermal uptake is 0.80 ng/kg/day. Therefore, DCB concentrations shown phototoxicities are within the DCB concentrations exposed by humans.

DCB also showed stronger mutagenicity due to light activation than due to the metabolic activation with the S9 enzyme system. This indicates that DCB must undergo photochemical reactions to produce reactive, mutagenic species. As known, light irradiation of a DCB solution causes it to dechlorinate, thus forms reactive chlorine radicals and benzidine radicals (Banerjee et al., 1978; Nyman et al., 2002). As it is pointed out, DCB could bind covalently to macromolecules such as DNA in the presence of rat liver in vitro, these radicals induced by light can also react with biological molecules nearby such as lipid and cause cells to lose viability (Iba, 1989). Although nearly 60% of the Jurkat T-cells become nonviable due to exposure to light and DCB (>50 μM), only 5% of DNA fragmentation is observed by the alkaline comet assay. Compared to our previous study for the same test on azulene, up to 40% of cellular DNA is fragmented due to the exposure to azulene and light while nearly all the Jurkat T-cells remain viable (Wang et al., 2004). This indicates that these two compounds have different toxicity mechanisms and different photoreaction pathways with DNA. It is likely that photo-activated azulene can react with DNA, while photo-activated DCB react with mostly other biological molecules such as lipids in the cell. This is analogous to the reaction of enzyme-activated DCB with unsaturated fatty acids because both light irradiation and P-450 enzyme reaction produces DCB radicals (Iba, 1989). Therefore, DNA fragmentation caused by exposure to DCB and light should not be the main reason that causes the human Jurkat T-cells to become nonviable. Other damages such as to cell membrane elements and proteins in the cell, or other forms of DNA damage such as DNA alkylation, may be the sources of cytotoxicity for DCB plus light. Nonetheless, DCB is photomutagenic

in bacteria and photogenotoxic and photocytotoxic to human cells. Persons exposed to DCB through environmental contamination or through tattoo piercing using DCB-contaminated inks must not only concern about its toxicity without exposing to light, but also about its phototoxicity.

Acknowledgments

This research was in part supported by the National Institutes of Health: NIH SCORE S06 GM08047 and the US Army Research Office DAAD 1901-1-0733 to JSU. We thank NIH-RCMI for Core Molecular and Cellular Biology and Analytical Chemistry Facilities established at JSU.

References

- Banerjee, S., Sikka, H.C., Gray, R., Kelly, C.M., 1978. Photodegradation of 3,3'-dichlorobenzidine. *Environ. Sci. Technol.* 12, 1425–1427.
- Bäumler, W., Vasold, R., Lundsgaard, L., Talberg, H.J., 2003. Chemicals used in tattooing and permanent make up products. In: Papameletiou, D., Schwela, D., Zenie, A. (Eds.), *Workshop on Technical/Scientific and Regulatory Issues on the Safety of Tattoos, Body Piercing and of Related Practices*. Free available report in http://europa.eu.int/comm/consumers/cons_safe/news/eis_tattoo_proc_052003_en.pdf. European Commission, Ispra, VA, Italy, pp. 21–48.
- Ben-Hur, E., Prager, A., Green, A.P., Rosenthal, I., 1980. pH dependence of the phototoxic and photomutagenic effects of chlorpromazine. *Chem. Biol. Interact.* 29, 223–233.
- Canada, 1993. *Priority Substances List Assessment Report for 3,3'-Dichlorobenzidine* by Government of Canada. Health Canada and Environment Canada, Canada Communication Group Publishing, Ottawa, Ontario.
- Code of Federal Regulations (CFR), 1985. Office of the Federal Register, National Archives and Records Service, U.S. Government Printing Office, Washington, DC.
- Choudhary, G., 1996. Human health perspectives on environmental exposure to benzidine: a review. *Chemosphere* 32, 267–291.
- Chung, K.-T., Chen, S.C., Wong, T.Y., Li, Y.S., Wei, C.I., Chou, M.W., 2000. Mutagenicity studies of benzidine and its analogs: structure–activity relationships. *Toxicol. Sci.* 56, 351–356.
- Claxton, L.D., Hughes, T.J., Chung, K.-T., 2001. Using base-specific *Salmonella* tester strains to characterize the types of mutation induced by benzidine and benzidine congeners after reductive metabolism. *Food Chem. Toxicol.* 39, 1253–1261.
- Garner, R.C., 1975. Testing of some benzidine analogues for microsomal activation to bacterial mutagens. *Cancer Lett.* 1, 39–42.
- Haley, T.J., 1975. Benzidine revisited: a review of the literature and problems associated with the use of benzidine and its congeners. *Clin. Toxicol.* 8, 13–42.
- IARC, 1982. *IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, some industrial chemicals and dyestuffs*. International Agency for Research on Cancer, Lyon, pp. 239–256.
- IARC, 1987. Overall evaluations of carcinogenicity to humans. Free available in <http://monographs.iarc.fr/monoeval/crthgr02b.html>.
- Iba, M.M., 1987. Comparative activation of 3,3'-dichlorobenzidine and related benzidines to mutagens in *Salmonella typhimurium* assay by hepatic S9 and microsomes from rats pretreated with different inducers of cytochrome P-450. *Mutat. Res.* 182, 231–241.
- Iba, M.M., 1989. Activation of 3,3'-dichlorobenzidine: enzymic basis and toxicological consequences. *Drug Metabol. Rev.* 21, 377–400.
- Lee, J.H., Shin, H.-S., 2002. Determination of hemoglobin adducts formed in rats exposed orally with 3,3'-dichlorobenzidine by GC/MS-SIM. *Toxicol. Ind. Health* 18, 191–199.
- Maron, D.M., Ames, B.N., 1983. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* 113, 173–215.
- Moneef, M., Sherwood, B., Bowman, K., Kockelbergh, R., Symonds, R., Steward, W., Mellon, J., Jones, G., 2003. Measurements using the alkaline comet assay predict bladder cancer cell radiosensitivity. *Br. J. Cancer* 89, 2271–2276.
- NTP, 2002. *Report on Carcinogens*, 10th ed. National Toxicology Program. U.S. Department of Health and Human Services, Public Health Service.
- Nyman, M.C., Haber, K.S., Kenttamaa, H.I., Blatchley III, E.R., 2002. Photodechlorination of 3,3'-dichlorobenzidine in water. *Environ. Toxicol. Chem.* 21, 500–506.
- Papameletiou, D., Zenie, A., Schwela, D., 2003. Status report on the current situation, nature and size of the problem regarding the safety of tattoos, body piercing and of related practices in the EU. In: *Workshop on Technical/Scientific and Regulatory Issues on the Safety of Tattoos, Body Piercing and of Related Practices*. Free available report in http://europa.eu.int/comm/consumers/cons_safe/news/eis_tattoo_proc_052003_en.pdf, pp. 9–13.
- Prival, M.J., Bell, S.J., Mitchell, V.D., Peiperl, M.D., Vaughan, V.L., 1984. Mutagenicity of benzidine and benzidine-congener dyes and selected monoazo dyes in a modified *Salmonella* assay. *Mutat. Res.* 136, 33–47.
- Savard, S., Josephy, P.D., 1986. Synthesis and mutagenicity of 3,3'-dihalogenated benzidines. *Carcinogenesis* 7, 1239–1241.
- Trevigen, 2001. *Trevigen instruction manual: comet assay—reagent kit for single cell gel electrophoresis assay*. Free available in <http://www.trevigen.com/products/cometassay.html>.
- Walser, S., 2003. Opening address by the council of Europe. In: *Workshop on Technical/Scientific and Regulatory Issues on the Safety of Tattoos, Body Piercing and of Related Practices*. Free available report in http://europa.eu.int/comm/consumers/cons_safe/news/eis_tattoo_proc_052003_en.pdf. European Commission, Ispra, VA, Italy, pp. 6–8.
- Wang, L., Yan, J., Fu, P.P., Parekh, K.A., Yu, H., 2003. Photomutagenicity of cosmetic ingredient chemicals azulene and guaiazulene. *Mutat. Res.* 530, 19–26.

- Wang, L., Yan, J., Wang, S., Cohly, H., Fu, P.P., Hwang, H.-M., Yu, H., 2004. Phototoxicity and DNA damage induced by the cosmetic ingredient chemical azulene in human Jurkat T-cells. *Mutat. Res.* 562, 143–150.
- Yan, J., Wang, L., Fu, P.P., Yu, H., 2004. Photomutagenicity of 16 polycyclic aromatic hydrocarbons from the US EPA priority pollutant list. *Mutat. Res.* 557, 99–108.
- Zeilmaker, M.J., van Kranen, H.J., van Veen, M.P., Janus, J.A., 2000a. Cancer risk assessment of azo dyes and aromatic amines from tattoo bands, folders of paper, toys, bed clothes, watch straps and ink. RIVM Report 601503 019. Free available in <http://www.rivm.nl/bibliotheek/rapporten/601503019.pdf>.
- Zenie, A., Schwela, D., Papameletiou, D., 2003. Regulatory review in the EU. In: Papameletiou, D., Schwela, D., Zenie, A. (Eds.), *Workshop on Technical/Scientific and Regulatory Issues on the Safety of Tattoos, Body Piercing and of Related Practices*. Free available report in http://europa.eu.int/comm/consumers/cons_safe/news/eis_tattoo_proc_052003.en.pdf. European Commission, Ispra, VA, Italy, pp. 74–77.