UVA-Induced Genetic Effects of Thioridazine, Mesoridazine and Sulforidazine: an *in vitro* Study

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This *in vitro* study focuses on the UVA-induced reactions with DNA of thioridazine (TRZ), and two of its major metabolites (TRZ-2-sulfoxide or mesoridazine, MRZ; and TRZ-2-sulfone or sulforidazine, SRZ). TRZ binds covalently to DNA upon UVA-irradiation. Under comparable irradiation conditions, MRZ binds to a lesser extent and almost no binding was observed with SRZ. Besides, photo-induced genetic effects were investigated by means of a differential DNA repair test in *E. coli*. The photo-induced genetic effects in *E. coli* decreased from TRZ, MRZ to SRZ, which corresponds with their capacity for UVA-induced binding to DNA. TRZ, MRZ and SRZ differed in their rate of photodecomposition rather than in the *intrinsic* reactivity towards DNA of the instable intermediates formed.

Irreversible binding to DNA was also observed upon treatment with peroxidase, which is known to oxidize phenothiazines *via* the formation of reactive radical cation species. As both the colour of the intermediate and its reactivity towards DNA were comparable for peroxidase treatment and exposure to UVA, we assume that the radical cation is the reactive species in the latter case as well.

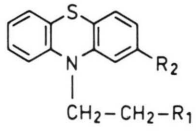
Introduction

Like many other compounds, for instance polycyclic aromatic hydrocarbons, acridine derivatives or methylene blue, phenothiazine drugs are able to form molecular complexes with nucleic acids. The phenothiazine nucleus can only become partially intercalated between two adjacent base pairs because its non-planar structure makes total insertion unlikely [1].

Although in principle complexes with DNA are reversible, they may cause serious biological effects because of interference with essential processes controlled by this biomacromolecule. Besides, irreversible binding to DNA, which increases this risk, is more probable when a molecule has already formed an (intercalation) complex before it is (non-)enzymatically activated.

The foregoing justifies the interest in the (ir)reversible interactions between phenothiazine drugs and DNA.

Most studies concern chlorpromazine (CPZ; Fig. 1), a prototype of this class of antipsychotic drugs. For instance De Mol and Maanders [1] described reversible binding of, amongst other compounds, CPZ to calf thymus DNA. Intercalation of the phenothiazine nucleus between two adjacent DNA base pairs appeared to play a role. Irreversi-



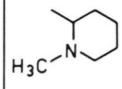
R_1	R_2	compound
$-CH_2-N\begin{matrix}CH_3\\CH_3\end{matrix}$	$-Cl$	chlorpromazine CPZ
	$-S-CH_3$	thioridazine TRZ
	$-\overset{\overset{O}{\|}}{S}-CH_3$	mesoridazine MRZ
	$-\overset{\overset{O}{\|\|}}{\underset{\underset{O}{\|\|}}{S}}-CH_3$	sulforidazine SRZ

Fig. 1. Structural formulae of chlorpromazine (CPZ), thioridazine (TRZ), TRZ-2-sulfoxide (mesoridazine, MRZ) and TRZ-2-sulfone (sulforidazine, SRZ).

Abbreviations: CPZ, chlorpromazine; DCM, dichloromethane; MRZ, mesoridazine; SRZ, sulforidazine; TRZ, thioridazine.

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ble binding of CPZ to DNA *in vitro*, as a result of enzymatic activation, has also been studied [2]. Less is known with regard to biological effects which may be ascribed to interaction of CPZ with DNA. When rats were treated with dimethylbenzanthracene, CPZ increased the incidence and size of tumors [3]. Prenatal administration of CPZ to chick embryos caused a "curled toe" anomaly, indicating that this agent is a potential physiological, instead of anatomical, teratogen for chickens [4].

Thioridazine (TRZ; Fig. 1) is another anxiolytic and antipsychotic member of the phenothiazine group. The evaluation of the DNA reactivity of TRZ has been the subject of some papers. Chromosome damage was not observed in lymphocytes of individuals exposed to therapeutic doses of TRZ [5, 6]. In the micronucleus test, TRZ showed at most minimal activity. On the other hand TRZ-induced sex-linked recessive lethal mutations and translocations in Oregon-S males of *Drosophila melanogaster* and dominant lethal mutations in male mice [7]. In addition some effects on meiotic chromosomes of male mice were found by Murthy and Subramanyam [8].

However, in 1983 Matter *et al.* [9] re-evaluated the genotoxic potential of TRZ using the mouse and Chinese hamster micronucleus test, chromosome analysis of Chinese hamster bone marrow cells, the mouse dominant lethal test, a Salmonella/microsome test and, sex-linked recessive lethal tests in *Drosophila melanogaster*. In all of these tests TRZ was found to be non-mutagenic and non-genotoxic.

Not only enzymatic activation but also UV-radiation as a cause of phenothiazine-induced DNA damage has received considerable attention (see, for example [10]), because several phenothiazines can provoke phototoxic/photoallergic reactions in man [11].

CPZ has become most notorious due to its adverse (sun)light induced effects. Near-UV photoexcited CPZ induced single strand breaks in DNA [12] and photoinduced irreversible binding of CPZ to DNA was observed [7, 13]. This resulted in genetic effects in *S. typhimurium* [14] and in *E. coli* [13].

TRZ also causes adverse photobiological effects [11] similar to those of CPZ. Satanove and McIntosh [15] described a gray to purple skin pigmentation and ocular opacity in humans exposed to TRZ or CPZ and UV (or visible radiation). TRZ was found to be almost as phototoxic as CPZ in *Candida albicans* and in mice [16], and its phototoxic effects were further shown with the use of tissue culture techniques [17]. Marko *et al.* [18] found that phenothiazines, including TRZ, photochemically induced dynamic nuclear polarization in nucleic acid bases and nucleotides. This polarization was explained by electron transfer from photoexcited phenothiazine to the bases.

Whether UVA-induced reactions of TRZ with DNA can result in genetic effects is the subject of this paper. It was investigated by means of a bacterial test system. Besides, attention was paid to the UVA-induced formation of covalent bonds between TRZ and DNA *in vitro*.

It is known from studies with chlorpromazine that metabolism of this drug yields products differing from the parent drug with regard to their photosensitizing potency [19, 20]. Photo-induced binding to DNA and photo-induced genetic effects were therefore also studied for two important metabolites of TRZ [21, 22]; TRZ-2-sulfoxide (mesoridazine; MRZ; Fig. 1) which is used as antipsychotic drug, and TRZ-2-sulfone (sulforidazine; SRZ; Fig. 1).

The photo-induced genetic effects of TRZ, MRZ and SRZ were studied with the use of a differential DNA repair test in *E. coli*.

Materials and Methods

Thioridazine-HCl, mesoridazine-mesylate and sulforidazine were obtained from Sandoz Pharmaceuticals (East Hanover, N.J.). The latter two compounds were converted to the corresponding HCl salt before use. [Benzene ring ^{3}H] CPZ, specific activity 22.4 Ci mmol^{-1} (New England Nuclear, Dreieich, W. Germany) and unlabeled CPZ (Specia, Paris, France) were used.

Irradiation procedure

A Rayonet Photochemical Reactor (Southern New England UV Co.) equipped with RUL 3500 A lamps was used. These lamps emit UVA radiation from 320–380 nm, with a maximum at 350 nm. The light intensity was measured with an UVX radiometer equipped with an UVX-36 sensor (UV-Products, San Gabriel Cal.). The samples, in pyrex test tubes with an inner diameter of

1.3 cm, were irradiated in a carrousel. The temperature remained below 25 °C during the experiments.

Photobinding to DNA

A solution of 0.1% (w/v) highly polymerized calf thymus DNA (Sigma No D-1501, Sigma Chemical Company, MI) in 0.15 M saline was prepared (DNA-solution). Of the phenothiazines 3 mM solutions in 0.15 M saline were made and stored at 4 °C in vials shielded from light. Of each of these solutions 0, 0.15, 0.30 and 0.45 ml were pipetted into pyrex test tubes and the total volume was brought to 4 ml with 0.15 M saline. While shaking the tubes, 1 ml of the DNA-solution was added per tube. The tubes were placed in the dark for 1 h (20 °C). Next the tubes were irradiated for 15 min (I = 14 W/m^2) and kept in the dark for 16 h (20 °C).

After this period 1 ml 2% Na_2CO_3 (aq) and 5 ml dichloromethane (DCM) were added. After shaking and centrifugation, the DCM-layer, containing unbound phenothiazine-derived molecules, was removed. Extraction was completed by repeating this procedure twice.

The UV-spectrum of the aqueous layer was recorded (Jasco 7850 UV/VIS spectrophotometer, B& L systems, Zoetermeer, The Netherlands) and the phosphate concentration, a measure of the amount of DNA, was determined according to Nakamura [23].

To test if binding also occurred in the dark, the procedure was repeated without 15min irradiation.

Binding to DNA induced by peroxidase-hydrogen peroxide

Peroxidase- and H_2O_2 solutions were made by dissolving peroxidase (Sigma No P-8375, Sigma Chemical Company, MI) and H_2O_2 (30%) in 0.15 M NaCl, at final concentrations 0.5 mg/ml and 0.7% (v/v), respectively. Two ml DNA-solution, 0–2.8 ml 3 mM phenothiazine-solution, 3.6–0.8 ml 0.15 M NaCl, 0.2 ml peroxidase-solution, and 0.2 ml H_2O_2-solution were pipetted into pyrex test tubes (total volume 6 ml). The tubes were kept in the dark for 17 h (20 °C). Thereafter unbound molecules were removed by DCM extractions as mentioned above. This experiment was also done with ^{3}H-labeled CPZ.

Photodecomposition of phenothiazines

The percentage of phenothiazine photodecomposed after 15 min irradiation was determined for the highest concentration. Before unbound phenothiazine-derived molecules were removed by DCM extractions, samples were taken and analyzed by reverse phase HPLC (column Chrompack, RP-8, 7 μm, l = 10 cm, i.d. = 0.4 cm; eluent acetonitrile: ammoniumcarbonate 0.4% (aq) = 9:1; flow = 1 ml/min) equipped with a Packard 1040 A photodiode array detector and a Schimadzu C-R 3 A integrator (Shimadzu corporations, Kyoto, Japan).

Differential DNA-repair test with E. coli K 12 strains

The colony forming ability (survival) of repair-deficient *E. coli* K 12/343/753 (lac+, uvr B/ rec A), and of *E. coli* K 12/343/765 (lac–, uvr+. rec+) with an intact repair system was determined. The test was performed according to Mohn *et al.* [24]; more details, *e.g.* preparation of PEPS, NRS-Agar and PBS, are found in Ref. [24]. Overnight cultures (O.C.) of the bacterial strains were prepared by inoculating 24 ml PEPS-bouillon with # 753 and 10 ml with # 765 and incubating overnight at 37 °C while shaking to reach the stationary growth phase. After centrifugation (Runne Zentrifugen, Heidelberg, West Germany) at 5000 rpm of a mixture of 24 ml of # 753 O.C. and 0.75 ml of # 765 the pellet was resuspended in 6 ml PBS (*E. coli* suspension). Of each of the phenothiazines a 5 mM solution was made in sterile water. Of these solutions 0.2 ml were pipetted into a pyrex test tube together with 6.8 ml PBS and 3.0 ml of the *E. coli* suspension. This mixture was preincubated for 1 h in the dark. The tube was irradiated for 30 min (I = 7.5 W/m^2), 0.5 ml samples were taken during this period. These samples were post-incubated for 1 h in the dark and after dilution plated on NRS-Agar media in triplicate. The *E. coli* strains used differ in their auxotrophic requirements, # 765 is lac– and # 753 lac+. Therefore their individual survival can be determined in mixtures in which both strains are present. This is done by plating on the same agar medium, with neutral red as a pH indicator,

on which the lac+ colonies appear red and lac− colonies white [24]. Plates were kept at 37 °C and the red (# 753) and white (# 765) colonies were counted after 24 h.

As a control *E. coli* suspensions *without* phenothiazines were processed in the same way. After irradiation some of the samples taken were incubated for 1 h and processed further as mentioned above. To the remaining samples, phenothiazine solutions were added to reach concentrations comparable with those mentioned above. These samples were kept in the dark for 2.5 h before plating.

Results and Discussion

Binding to DNA

Both UVA irradiation (λ_{max} = 350 nm) and peroxidase treatment of solutions containing TRZ produced a transient blue colour, probably due to one or more intermediates formed from TRZ. Gubitz *et al.* [25] have shown that both peroxidase treatment and UV irradiation (254 nm) of TRZ produced a reactive, blue coloured radical cation. In the present study, irradiation of TRZ with longer wavelength UV (UVA, λ_{max} = 350 nm) apparently also resulted in photo-induced ionization. The same observation was made for MRZ where both peroxidase treatment and UVA irradiation yielded a purple coloured intermediate. UVA irradiation of SRZ did not produce a coloured product whereas a slightly yellow colour was observed upon peroxidase treatment.

Peroxidase-treatment of CPZ produced a red coloured radical cation [2], however, in contrast to TRZ and MRZ, the same UVA irradiation of CPZ did not yield this species. Photo-induced ionization of CPZ to this coloured radical cation occurred upon irradiation with short wavelength UV (254 nm, [24]) but at longer wavelengths (> 270 nm) photo-induced dechlorination was observed rather than ionization. The latter, photohomolysis into promazinyl radicals, has been demonstrated in an *in vitro* study by Motten *et al.* [26], and in a recent *in vivo* study by Schoonderwoerd *et al.* [27].

Ionization of the phenothiazines either by peroxidase treatment (CPZ, TRZ, MRZ, SRZ) or by UVA irradiation (TRZ, MRZ) resulted in binding to DNA accompanied by a change in DNA absorption spectra, with an extra shoulder at 340 nm. As a representative example, this is illustrated in Fig. 2 for UVA-induced binding of TRZ. The shapes of the absorption curves were the same for all mentioned phenothiazines, only the height of the shoulder differed.

The quantitative evaluation of DNA binding is not straightforward as the molar extinction coefficients of the DNA-phenothiazine adducts are not known. Besides, radiochemically labeled TRZ, MRZ and SRZ were not available.

However, since ^{3}H-labeled CPZ was available the binding of this compound to DNA after peroxidase treatment could easily be determined by liquid scintillation counting of the aqueous phase after the DCM extractions. The amount of CPZ-binding to DNA was also calculated by taking for the extinction coefficient of the CPZ-DNA adduct at 340 nm the molar extinction coefficient of CPZ itself at 310 nm. The values obtained in this way were 15% lower than the correct ones determined by liquid scintillation counting. Apparently the extinction coefficient of CPZ at 310 nm is 15% higher than that of its DNA-adduct at 340 nm.

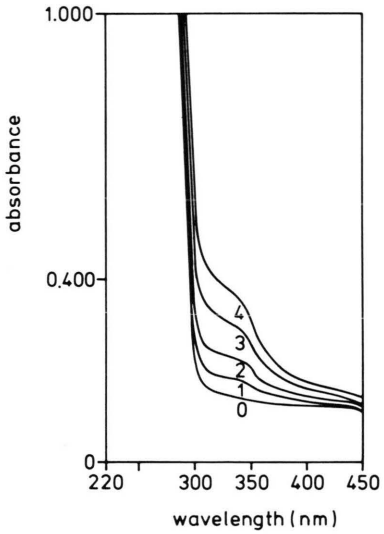


Fig. 2. UVA-induced binding of thioridazine to DNA causes the introduction of a shoulder at 340 nm in the absorption spectrum of DNA. 0 = absorption spectrum of DNA, 1→4 increase of starting concentration of thioridazine (see Materials and Methods). Incubation of DNA with (high concentrations of) thioridazine in the dark did not cause any difference compared to the spectrum of DNA alone (0).

The chromophoric group in TRZ, MRZ and SRZ does not differ much from that of CPZ (maximum absorption for all these phenothiazines is at 310 nm). This appeared to be the case for the DNA-adducts as well (shoulder at 340 nm). This is comprehensible if it is taken into account that both the peroxidase-induced reaction of CPZ and the UVA-induced reactions of TRZ, MRZ (and SRZ) with DNA occur *via* phenothiazine radical cations. It was, therefore, assumed that the extinction coefficient of the DNA-adducts of TRZ, MRZ and SRZ was also 15% lower than that of their corresponding parent compounds.

With this assumption, and using data from the Nakamura phosphate determination, the UVA- and peroxidase-induced binding of TRZ, MRZ, and SRZ, per nucleotide were calculated and are presented in Fig. 3 and 4, respectively.

From these figures it becomes clear that sulfoxidation of the side chain results in reduced binding of the phenothiazine to DNA (binding TRZ > MRZ > SRZ). Without UVA irradiation, no binding was detected for all three phenothiazines.

Irradiation or peroxidase treatment of the phenothiazines in the absence of DNA gave products which could all be extracted with DCM.

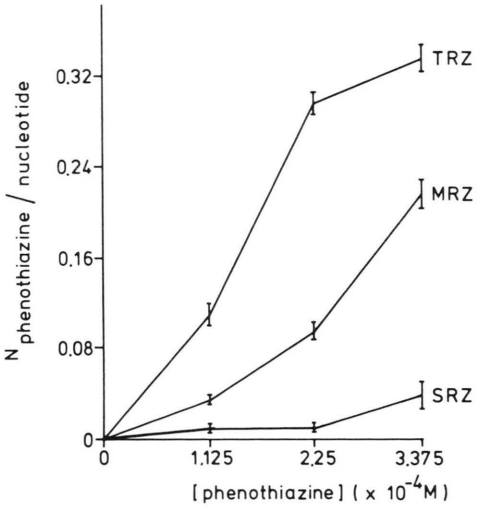


Fig. 3. UVA-induced DNA binding (expressed as the number of phenothiazine molecules bound per nucleotide) of thioridazine, mesoridazine and sulforidazine at increasing molar concentrations of phenothiazine ([phenothiazine]). Without UVA-irradiation no binding was observed. The bars represent the standard deviations of 3 independent experiments.

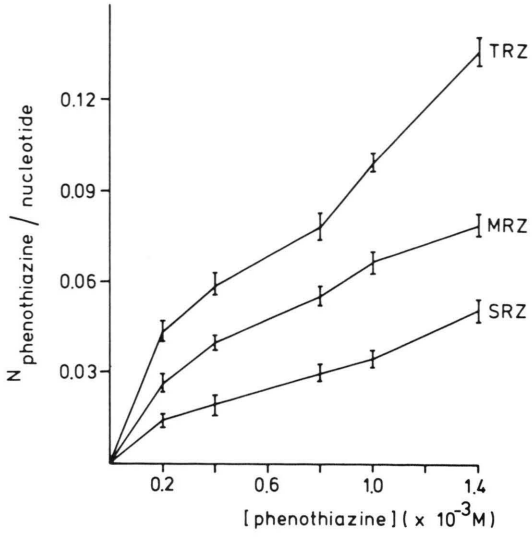


Fig. 4. Peroxidase-induced DNA binding (expressed as the number of phenothiazine molecules bound per nucleotide) of thioridazine, mesoridazine and sulforidazine at increasing molar concentrations of phenothiazine ([phenothiazine]). The bars represent the standard deviations of 3 independent experiments.

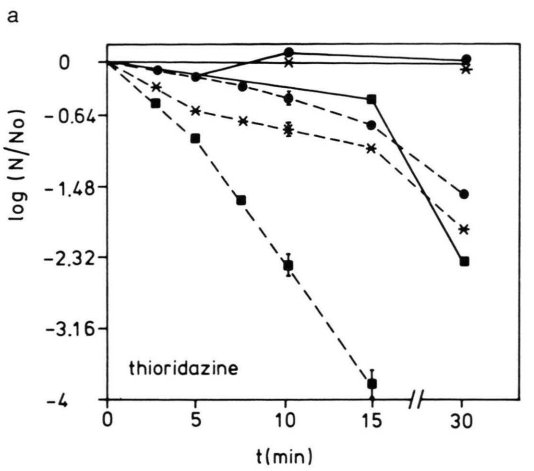
HPLC-analysis of the irradiated DNA samples with the highest concentration of phenothiazines showed that the percentage of photodecomposed phenothiazine was 85, 65, and 10%, respectively for TRZ, MRZ, and SRZ. It is not within the scope of this article to present details of the photodecomposition. No decomposition was observed in non-irradiated solutions and in those not exposed to peroxidase.

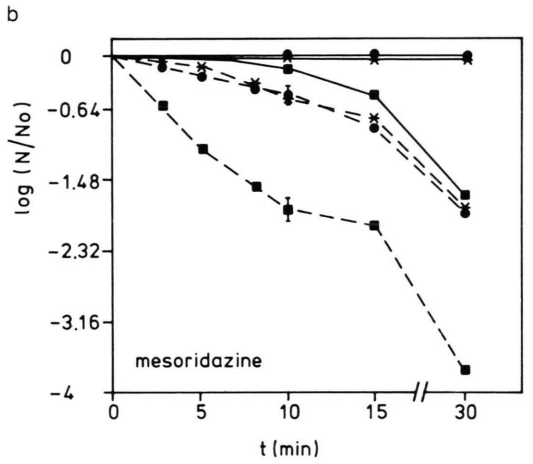
DNA-repair test

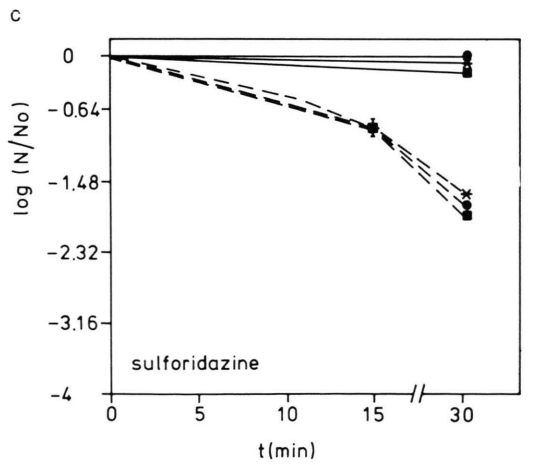
In Fig. 5a, b and c the survival of colonies (log N/N_0) for TRZ, MRZ and SRZ, respectively, is plotted against irradiation time. For each phenothiazine three situations were studied.

- Survival of both strains upon UVA irradiation only;
- Survival of strains first irradiated followed by dark incubation in the presence of TRZ, MRZ and SRZ; and
- Survival after UVA irradiation in the presence of these compounds.

As can be seen in Fig. 5c the addition of SRZ to irradiated strains, or irradiation of the bacteria in







the presence of SRZ did not cause a decrease in colony-forming ability compared to the controls without SRZ. Apparently SRZ is not phototoxic in this test system.

For MRZ (Fig. 5b) dark incubation of the irradiated strains with MRZ also had no effect compared to irradiation only. However, irradiation in the presence of MRZ caused a decrease in survival for both the # 765 and the # 753 *E. coli* strains. It should be noted that, especially until 10 min there is no decrease in colony forming ability of the # 765 strain indicating that, at least partly, the decrease of the number of colonies of the # 753 strain is caused by DNA damage which is repairable by the # 765 strain. After 10 min the colony forming ability of the # 765 also decreased, possibly as a result of damage to other biomolecules (proteins, lipids [28] or by non-repairable DNA damage.

Addition of TRZ to irradiated bacterial suspensions resulted in a decrease of the number of colonies of the # 753 strain but not for the # 765 strain. Apparently even non-irradiated TRZ has genetic effects, at least on irradiated *E. coli*. Irradiation of both strains in the presence of TRZ gave results comparable to those obtained with MRZ, but the effects were even more pronounced.

It has been speculated that radical intermediates of CPZ can be generated *in vivo* during the oxidative metabolism of the drug [29]. Indeed oxidation occurs upon incubation with peroxidase [24]. De Mol *et al.* [13] have shown that this results in covalent binding of CPZ to DNA (and albumin) and in repairable DNA damage in *E. coli*. In the present study peroxidase-induced binding was also found for TRZ, MRZ and SRZ. In this respect it is noteworthy that non-irradiated TRZ caused genetic effects in irradiated *E. coli*, possibly by enzymatic oxidation or by UVA-induced damage of membranes (the latter may facilitate the penetration of TRZ).

Fig. 5. Survival (log N/N_0) of *E. coli* after UVA-irradiation alone (●), UVA-irradiation followed by incubation with a phenothiazine drug in the dark (★) or UVA-irradiation in the presence of a phenothiazine drug (■). Survival was studied for two strains of *E. coli:* repair-deficient *E. coli* K 12/343/753 (lac+, uvr B/rec A), (– – –), and repair-competent *E. coli* K 12/343/765 (lac–, uvr+, rec+) (——). The bars represent the standard deviations of 3 independent experiments.

Several studies [25, 30, 31] have shown that irradiation of TRZ with short wave-length UV (254 nm) results in photo-ionization. Gubitz *et al.* [25] have observed that ionization also occurred after treatment with peroxidase. We found that irradiation with UVA with longer wave-length (*i.e.*, λ_{max} = 350 nm) also resulted in photo-ionization as the same coloured intermediates were found as after treatment with peroxidase. The rate of photodecomposition of the phenothiazines decreased from TRZ, MRZ to SRZ. Oxidation of the sulfur which is substituted at the 2-position of the phenothiazine molecule thus makes this compound less susceptible for (photo-)ionization.

Also the photo-induced covalent binding to DNA and the photo-induced genetic effects in *E. coli* decreased from TRZ, MRZ to SRZ.

There is a good correlation between the amount of phenothiazine decomposed after irradiation and the amount bound to DNA. If, for the highest concentrations of TRZ, MRZ and SRZ, the degree of photobinding to DNA (Fig. 3) is divided by the percentage of photodecomposed phenothiazine, the figures are $0.334/85 = 3.9 \times 10^{-3}$, $0.216/65 = 3.3 \times 10^{-3}$ and $0.038/10 = 3.8 \times 10^{-3}$, respectively. Therefore, although sulfoxidation diminishes the *rate* of phenothiazine photodecomposition, the *intrinsic reactivity* of the radical cation towards DNA seems to be the same for TRZ, MRZ and SRZ.

In summary it was found that without UVA irradiation no DNA binding occurred, and only a relatively small genetic effect was found for TRZ in irradiated *E. coli,* possibly by enzymatic oxidation or by UVA-induced damage of membranes which may facilitate the penetration of TRZ.

UVA irradiation in the presence of TRZ or MRZ drastically changes this picture; irreversible binding to DNA occurs and a large genetic effect was observed in *E. coli.* Oxidation of the side chain sulfur, as occurring in metabolism of TRZ, decreases the rate of photodecomposition; at comparable irradiation times TRZ-2-sulfoxide induced less genetic effects in *E. coli* and TRZ-2-sulfone did not induce any genetic effect at all. Apparently metabolism can, in this respect, be considered to be a detoxification process.

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