ISSN 1022-7954, Russian Journal of Genetics, 2009, Vol. 45, No. 3, pp. 304–307. © Pleiades Publishing, Inc., 2009.
Original Russian Text © V.A. Chistyakov, M.A. Sazykina, M.A. Kolenko, G.G. Chervyakov, A.V. Usatov, 2009, published in Genetika, 2009, Vol. 45, No. 3, pp. 349–353.

GENERAL GENETICS

Methylene Blue as a Suppessor of the Genotoxic Effect of Ultraviolet Radiation with a Wavelength of 300–400 nm

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Received December 20, 2007

Abstract—Ultraviolet radiation with a wavelength of 300–400 nm is characteristic of sunlight at the earth surface and causes DNA damage mediated by energy transfer to O_2 with the transformation of the latter in the singlet state. In connection with this, scavengers of reactive oxygen species (ROSs) are potential protectors against the genotoxic effect of this kind of radiation. It was found that the methylene blue dye at doses differing by several orders of magnitude from those that are toxic for humans is able to suppress completely the SOS response induced by UV with a wavelength of 300–400 nm in *Escherichia coli*.

DOI: 10.1134/S1022795409030077

INTRODUCTION

Genotoxic effects of different regions of the ultraviolet (UV) light spectrum are mediated via different mechanisms. The main products of DNA damage caused by 200–280 nm UV light (ultraviolet C) are pyrimidine dimers forming DNA upon absorption of light quanta [1]. Ultraviolet A (320–400 nm) and ultraviolet B (280–320 nm), which are not absorbed by nucleic acids, can induce in cells a cascade of photoreactions accompanied by the generation of reactive oxygen species (ROSs). As this takes place, pyrimidine dimers are effectively repaired by the systems of error-free excision repair, while the products of a free-radical DNA attack, especially such as links with proteins and crosslinks, are repaired far less effectively and with a wide involvement of the error-prone repair systems. Therefore, near ultraviolet is a serous inducer of human skin carcinoma, despite lower energy of its quanta [2–5]. Most of solar ultraviolet reaching the earth surface is represented by 300–400 nm radiation [5]. This range seems to be optimal for selecting protective substances. In practice, mostly sunscreen products, i. e. substances screening the skin from the light, are used to protect aginst the carcinogenic effect of solar radiation [6]. Their efficiency is limited by a variety of factors, mainly by the impossibility of creating a stable film of necessary thickness. The solution of the problem of defence against the negative effects of solar energy must be based on a complex approach that integrates the work of various mechanisms. This determines the importance of searching for compounds able to protect the cell from genotoxic effects of ultraviolets A and B. Obviously, the wider the list of such substances, the more possibilities for producing highly effective complex preparations.

A number of publications describe the antioxidant effect of the methylene blue dye (*N*,*N*,*N*',*N*'-tetramethylthionine) that belongs to the group of thiasines and is widely used in biology and medicine. This substance is able to penetrate easily in living cells and to scavenge reactive oxygen species in concentrations differing by several orders of magnitude from toxic ones [7]. Yet, the photosensibilizing activity of methylene blue was described [8]. Thus, the study of the capacity of this compound to modify destructive effects induced by photoinduction of ROSs is surely of interest.

MATERIALS AND METHODS

A variant of the SOS-lux test previously employed for studying the antimutagenic effects of some natural compounds was used in this work as a system for assessing genotoxicity of the exposure under study [9–11]. The reporter of the SOS response was lux-operon. The *Escherichia coli* strain PT-1 (C600(pPLS-1)) carrying the pPLS-1 plasmid, in which the operon of bioluminescence is under the control of the SOS promoter, was used [12]. To control effects unrelated to SOS induction, the PT-5 (C600(pPLS-5)) strain whose lux-operon is under the control of a consitutive promoter was used [13].

Escherichia coli strains were grown in LB medium. 0.1 ml of overnight *E. coli* culture was placed in 50 ml of the medium and incubated in a thermostat for 1 h at 37°C. One ml of the culture was placed in a 40-mm plastic Petri dish without a cover, which was irradiated with UV light with a wavelength of 300–400 nm using

a unit with a low-pressure mercury lamp as a source of radiation (HG-125). The lamp was located in the focus of a parabolic reflecting mirror, and the UV flow was directed to the exposed object through an aluminium guiding system to diminish the angle of scattering. This made it possible to generate an axis-symmetric and even radiation flow at distances from 20 cm to 2 m; the minimum area of the irradiated surface was 80 × 80 mm, and the uneveness (with 50 mm deviation from the axis) did not exceed 20%.

Methylene blue solution (pure for analysis, Dia-m, Russia) in distilled water was added to a necessary final concentration 30 min prior to irradiation.

After irradiation, the suspensions of microorganisms were kept for a necessary time (Table 1) at 25°C, then luminescence was measured with an LT-01 luminometer (Russia).

The factor of SOS response induction (I^s) was calculated by the formula:

$$I^s = \frac{L_e}{L_k} - 1, \quad (1)$$

where L_k is luminescence intensity of the control sample; L_e is luminescence intensity of the experimental sample.

The sign of significance of the effect of SOS induction was a statistically significant excess of L_e over L_k estimated by the *t*-test.

The antimutagenic potential (A, %) was calculated by the formula:

$$A = \left(1 - \frac{I_a}{I_p}\right)100\%, \quad (2)$$

where I_a is the factor of SOS response induction by the exposure under study in the presence of a protector; I_p is the factor of SOS response induction by the exposure under study.

All experiments were carried out in three independent replications.

Table 1. Dynamics of SOS response induction in the *E. coli* strain PT-1 after exposure to UV radiation (300–400 nm, 390 J/m^2)

Time after exposure, min	Intensity of PT-1 strain bioluminescence, units*	Induction factor, I^S
Before exposure	0.60 ± 0.05	0
30	1.62 ± 0.09	0.7**
60	4.83 ± 0.28	4.2**
90	6.05 ± 0.73	8.4**
120	7.52 ± 0.78	9.8**
150	5.69 ± 0.36	7.5**

Notes: * The mean of four values.
** Differences from the control are statistically significant, $t < 0.05$.

Table 2. SOS response induction in the *E. coli* strain PT-1 by UV with a wavelength of 300–400 nm

Radiation dose, J/m^2	Intensity of PT-1 strain bioluminescence, units*	Induction factor, I^S
0	0.315 ± 0.022	0
65	0.451 ± 0.038	0.43
130	2.32 ± 0.245	5.4**
260	3.09 ± 0.281	7.8**
390	3. 37 ± 0.402	9.7**
780	2.91 ± 0.200	7.3**
1560	2.15 ± 0.089	4.8**
2340	0.725 ± 0.056	1.3**
3120	0.460 ± 0.049	0.46
3900	0.410 ± 0.032	0.3
7800	0.347 ± 0.035	0.1

Notes: * The mean of four values.
** Differences from the control are statistically significant, $t < 0.05$.

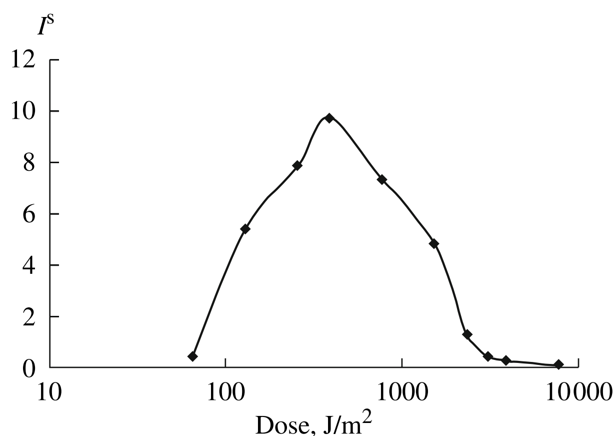
RESULTS

The data on the development in time of a SOS response induced in *E. coli* by UV radiation with a wavelength of 300–400 nm are presented in Table 1. A maximum effect develops within 120 min after irradiation, and the factor of induction reaches the value of 9.8, then gradually decreasing. The fact that the observed enhancement of luminescence intensity is determined exactly by SOS induction follows from the absence of significant differences of this parameter in the PT-5 strain whose lux-operon is under the control of a constitutive promoter (data not shown). Thus, 120-min exposure after irradiation is an optimal time for studying protectors.

As seen from Table 2, an increase in the dose of 300–400 nm ultraviolet radiation leads to an increase of the effect up to the maximum value of the induction factor equal to 9.7 (390 J/m^2). Further increase of the dose leads to gradual attenuation of the effect. Doses over 3120 J /m^2 produce no significant enhancement of bioluminescence of the PT-1 strain. No statistically significant alterations in luminescence of the PT-5 strain were recorded for doses 65 to 7800 J/m^2, suggesting that the obtained effects are determined by SOS response induction rather than by a general activation of bioenergetics of bacterial cells.

Further increase of the dose results in the suppression of luminescence of both strains.

Thus, the dependence of the SOS induction factor on the dose of ultraviolet radiation with a wavelength 300 to 400 nm in the semilogarithmic scale has the



The dependence of the factor of SOS response induction in the *E. coli* strain PT-1 on the dose of UV (300–400 nm).

form of a bell-shaped curve presented in the figure with a maximum at 390 J/m^2. This dose is optimal for studying the effects of protection against the exposure under consideration. The data on methylene blue-mediated modifications of SOS induction by UV (300–400 nm) are presented in Table 3. Concentrations of 0.01–1 nM do not have a statistically significant influence on the level of the induction factor. For higher concentrations a protector effect is recorded. The efficiency of protection by methylene blue increases with an increase of its dose, reaching the maximum (suppression of SOS induction to the level statistically undistinguishable from the background level) at a concentration of 1000 nM. Further tenfold increase of the concentration does not lead to the reduction of the effect.

Table 3. SOS response induction in *E. coli* by ultraviolet radiation (300–400 nm, 390 J/m^2) in the presence of methylene blue

Methylene blue concentration, nM	*A*, %	Induction factor, I^S
0	0	9.0 ± 1.1
0.01	2	8.8 ± 1.0
0.1	0	9.1 ± 0.9
1	19	7.3 ± 0.8
10	86	1.3 ± 0.1**
100	80	1.8 ± 0.2**
1000	91	0.8 ± 0.1**
10000	90	0.9 ± 0.1**

Notes: * The value of the induction factor and a standard error were calculated from the data of three independent experiments.
** Statistically significant protector effect, $P < 0.05$.

DISCUSSION

The SOS-lux test was developed as a method for quantitative assessment of genotoxic effects [11]. We proposed a modification that makes it possible to calculate the true values of SOS induction in the presence of substances inhibiting the activity of bacterial luciferase [13]. The capacity of the test to register the linear growth of SOS response intensity with an increase of the dose of exposure and substances directly interacting with DNA was supported experimentally [12, 14]. The nonlinear pattern of the dose–effect curve obtained in this study seems to be determined by the complexity of free-radical processes leading to DNA damage under the action of UV on bacterial cells. It should be noted that the nonlinear pattern of dose–effect curves is also characteristic for a number of other mutagens whose action is based on the generation of ROSs, in particular, for oxygen under pressure [15, 16]. Nevertheless, the tenfold induction of a SOS response recorded in our experiments permits this parameter to be used for qualitative assessment of the protector activity.

Methylene blue belongs to the group of thiasine dyes. The history of clinical application of this compound covers more than 100 years. It was initially used to treat methemoglobinemia, both congenital and toxicological. It was found at the end of the 20th century that methylene blue has a wide adaptogenic potential; in particular, it protects the cell from destructive consequences of ischemia–reperfusion [17], increases the lifetime of mice [18], restores the cognitive functions after oxidative stress [19, 20], etc.

In the opinion of [7] who described some effects of methylene blue based on which it was assigned to geroprotectors, the adaptogenic properties of this substance are determined by its antioxidant activity. According to the universal mechanism discovered by Skulachev, hydrophobic cations are able to be accumulated in membrane structures carrying a positive charge, for example, in mitochondria and bacterial cells [21]. Methylene blue molecules possess all necessary features for such accumulation [7]. In addition, a comparatively low redox potential of this substance (11 mV) facilitates cyclic transformations between the reduced and oxidated forms [22], which permits this substance to employ the energy of the electron transport chain for deactivating ROSs generated in the cell [7]. It is these features that are likely to explain the fact that the antioxidant properties of methylene blue in experiments with mammalian cells are expressed at 10–1000 nM concentrations, several orders of magnitude lower than

toxic concentrations [7]. The protector effect established by us in respect of 300–400 nm UV is also recorded within this range of concentrations. It also seems to be mediated through the antioxidant mechanism due to similarity in metabolism of mitochondria and aerobic bacteria. Our data suggest that methylene blue in concentrations proved to be safe by experiments with animals [18] and by the 100-year experience of application of this substance in medicine will be able to effectively protect the skin against the genotoxic action of the ultraviolet component of sunlight. Testing this inference with the use of experimental models that are more adequate for humans will become the subject of our further studies.

REFERENCES

1. Pfeifer, G.P., You, Y.H., and Besaratinia, A., Mutations Induced by Ultraviolet Light, *Mutat. Res.,* 2005, vol. 571, nos. 1–2, pp. 19–31.
2. Cadet, J., Berger, M., Douki, T., et al., Effects of UV and Visible Radiation on DNA-Final Base Damage, *Biol. Chem.,* 1997, vol. 378, no. 11, pp. 1275–1286.
3. Pattison, D.I. and Davies, M.J., Actions of Ultraviolet Light on Cellular Structures, *EXS,* 2006, vol. 96, pp. 131–157.
4. Hiraku, Y., Ito, K., Hirakawa, K., and Kawanishi, S., Photosensitized DNA Damage and Its Protection via a Novel Mechanism, *Photochem. Photobiol.,* 2007, vol. 83, no. 1, pp. 205–212.
5. Kligman, L.H., Photoaging: Manifestation, Prevention and Treatment, *Clin. Geriatr. Med.*, 1989, vol. 5, no. 1, pp. 235–251.
6. Lautenschlager, S., Wulf, H.C., and Pittelkow, M.R., Photoprotection, *Lancet*, 2007, vol. 370, no. 9586, pp. 528–537.
7. Atamna, H., Nguyen, A., Schultz, C., et al., Methylene Blue Delays Cellular Senescence and Enhances Key Mitochondrial Biochemical Pathways, *FASEB J.,* 2008, vol. 22.
8. Wagner, S.J., Cifone, M.A., Murli, H., et al., Mammalian Genotoxicity Assessment of Methylene Blue in Plasma: Implications for Virus Inactivation, *Transfusion*, 1995, vol. 35, pp. 407–413.
9. Gus'kov, E.P., Kletskii, M.E., Kornienko, I.V., et al., Allantoin as a Free-Radical Scavenger, *Dokl. Ross. Akad. Nauk*, 2002, vol. 383, no. 2, pp. 105–107.
10. Gus'kov, E.P., Prokof'ev, V.N., Kletskii, M.E., et al., Allantoin as a Vitamin, *Dokl. Ross. Akad. Nauk*, 2004, vol. 398, no. 6, pp. 320–324.
11. Chistyakov, V.A., Cartilaginous Tissue as a Source of Biologically Active Substances, *Vopr. Rybolovstva*, 2004, no. 1(17), pp. 174–178.
12. Ptitsyn, L.R., Horneck, G., Komova, O., et al., A Biosensor for Environmental Genotoxin Screening Based on SOS Lux Assay in Recombinant *Escherichia coli* Cells, *Appl. Environ. Microbiol.*, 1997, vol. 63, no. 11, pp. 4377–4384.
13. Sazykina, M.A., Chistyakov, V.A., and Voynova, N.V., A Method for Assessment of Genotoxicity of Chemical Substances, RF Patent 2179581, 2002.
14. Sazykina, M.A., Evaluation of Trends in Development of Fishery Bioresources in Basins of Azov–Don Watershed Based on Their Components' Genotoxicity Estimation, *Cand. Sci. (Biol.) Dissertation*, Rostov-on-Don: AzNIIRKh, 2003, 161 p.
15. Gus'kov, E.P., Pavlov, Yu.I., Ter-Avanesyan, M.D., and Chistyakov, V.A., The Effect of Hyperbaric Oxygenation on the on the Survivability, Mutagenesis and Recombinogenesis of Single-Cell Organisms, *Tsitol. Genet.*, 1987, no. 1, pp. 10–16.
16. Chistyakov, V.A., Biochemical Mechanisms of Oxygen-Dependent Mutagenesis in Microorganisms, *Izv. Vuzov Severo-Kavkazskii Region*, 2003, suppl., no. 11(11), pp. 57–63.
17. Salaris, S.C., Babbs, C.F., and Voorhees, W.D., 3rd, Methylene Blue as an Inhibitor of Superoxide Generation by Xanthine Oxidase: A Potential New Drug for the Attenuation of Ischemia/Reperfusion Injury, *Biochem. Pharmacol.*, 1991, vol. 42, no. 3, pp. 499–506.
18. Bristol, D.W., TR-540: Technical Report Pathology Tables and Curves, in *Toxicology and Carcinogenesis Studies of Methylene Blue Trihydrate in F344 Rats and B6C3F1 Mice*, National Institutes of Health, Public Health Service, US Department of Health and Human Services, 2006, http://ntp.niehs.nih.gov/go/2095
19. Callaway, N.L., Riha, P.D., Wrubel, K.M., et al., Methylene Blue Restores Spatial Memory Retention Impaired by an Inhibitor of Cytochrome Oxidase in Rats, *Neurosci. Lett.,* 2002, vol. 332, no. 2, pp. 83–86.
20. Riha, P.D., Bruchey, A.K., Echevarria, D.J., and Gonzalez-Lima, F., Memory Facilitation by Methylene Blue: Dose-Dependent Effect on Behavior and Brain Oxygen Consumption, *Eur. J. Pharmacol.*, 2005, vol. 511, nos. 2–3, pp. 151–158.
21. Skulachev, V.P., How to Clean the Dirtiest Place in the Cell: Cationic Antioxidants as Intramitochondrial ROS Scavengers, *IUBMB Life,* 2005, vol. 57, nos. 4/5, pp. 305–310.
22. Kamat, P., Mimitijevic, N., and Fessenden, R., Photoelectrochemistry in Particulate Systems: Electron-Transfer Reactions of Small CdS Colloids in Acetonitrile, *J. Phys. Chem.*, 1987, vol. 91, no. 4, pp. 396–401.