The Screening of Photodynamic Toxicity of Dyes by Means of a Bioassay using Amphibian Embryos

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Abstract

The toxicity and photodynamic toxicity of 10 dyes on Bufo arenarum embryos employing lethality as end point were evaluated. Embryos at the stage of complete operculum (S25) were treated with the dyes at different concentrations and using several incubation and irradiation times. For standardization purposes, the embryos were treated at NOEC values for 48 h and then irradiated with white light for 10 min (72 mW/cm², 43.2 J/cm²). Methylene blue, toluidine blue, rose bengal B, acridine orange and phloxine B were found to be photoactive, whereas trypan blue, remazol brilliant blue R, pyronine Y, indigocarmine and luxol fast blue MBS did not show photochemical toxicity. The survival of control (only irradiated) embryos was not affected. By comparing the NOEC and NOEC+light values the photochemical damage induced by dyes could be easily assessed. These results point out the possibility to evaluate the phototoxic effect of chemicals by means of a simple test employing the amphibian embryo as a whole organism.

Key words: Photodynamic action, amphibian embryos, dyes, photochemical toxicity, photoactive xenobiotics.

Introduction

Photodynamic effects on biological systems are exerted by reactive oxygen species produced by the uptake of a photosensitizer (PS) followed by irradiation with visible light. In the past few years, there has been a great interest in this process and its application for biomedical purposes, the most known example being the photodynamic therapy (PDT) of cancer[5, 42]. The uptake and localization of PSs in cell organelles, photodamage mechanisms, and signaling pathways of apoptotic cell death are now important issues related to PDT research[1, 37, 41].

On the other hand, several common dyes have proved to show photodynamic activity, the most known being thiazine[25, 35, 38, 44-47], xanthene[2, 23], acridine[20] and triarylmethane[6, 18] dyes. It is worth to note that dyes used in industrial activities also present mutagenic, carcinogenic and genotoxic activity[7, 15, 34] which can result in a relevant risk for both human and environmental health. In this context, it is increasingly necessary to evaluate the photochemical toxicity of dyes and other xenobiotics in order to prevent environmental photodamage as well as to explore the potential of new PSs for PDT research. Several test systems for the detection of photodynamic effects and oxidative stress have been reported for example the use of *Allium*[27], *Candida*[19], *Paramecium*[16], *Artemia*[24], *Drosophila*[34] sea urchin[23], intradermal tests[43] and cell cultures[26].

Taking into account that the bioassay using *Bufo arenarum* embryos (AMPHITOX) has been employed successfully for standardized studies on hazard assessment[12, 13, 29], including the possibility to prevent the adverse effects of oxidative stress[9-11, 36], in this work we report the use of the amphibian embryo as a whole organism bioassay to detect chemical and photodynamic toxicity of some common dyes.

Material and Methods

Bufo arenarum adult females weighting around 200-250 g were collected in Lobos (Buenos Aires province). Ovulation was induced by intraperitoneal injection of a suspension of one homologous hypophysis in 2 ml AMPHITOX solution (AS)[12, 13]. Oocytes were fertilized in vitro with a sperm suspension and embryos were maintained in AS until complete operculum, the last stage of embryonic development (S25) (Fig. 1). Batches of 10 animals in 40 ml of AS were treated (by duplicate) with the following dyes: methylene blue (MB), trypan blue (TryB), remazol brilliant blue R (RemBB), indigocarmine (IC), pyronine Y (PY), luxol fast blue MBS (LuxFB), acridine orange (AO), phloxin B (PhB), rose bengal B (RB) and toluidine blue O (TB) (Table 1). Nominal concentrations (w/v) were used, as the purity of dyes samples was very variable.

Different dye concentrations (from 1 to 100 mg/ l), incubation times (from 2 to 4 days), and light doses (from 2.5 min [10.8 J/cm²] to 10 min [43.2 J/cm²]) were used in this work, and the lethal effect as end point of the bioassay was evaluated till 48 h after treatments. For standardization purposes, the embryos were treated for 48 h with a range of

dye concentrations and the No Observed Effect Concentration (NOEC) values were first obtained. Immediately after incubation with dyes, embryos were thoroughly washed with 150 ml of AS, and then irradiated for 10 min with white light (72 mW/cm^2, 43.2 J/cm^2) from an Agfa-Gevaert slide projector (Diamator 1500) equipped with a 150 W lamp. This light dose was found to be appropriate to explore the photodamage exerted by chemicals[36], allowing to determine NOEC+light values.

The projector was placed at a vertical distance of 6 cm from the embryos (10 animals placed into uncapped 3.5 cm plastic dishes with 4 ml of fresh AS, see Fig. 1) and the light beam was filtered through a 4.5 cm water layer to absorb heat (Fig. 2). The light intensity at the site of irradiation was measured with a 13 PEM 001 broadband power energymeter (Melles Griot). The absorption peak of dyes (from 490 to 670 nm) corresponded well to the range of visible wavelengths provided by the light source (from 400 to 750 nm).

Results and Discussion

In a first survey for detecting photochemical toxicity of the dyes used, different concentrations, incubation and irradiation times were analyzed (Table 2). No lethal effects were found in animals only treated with the dyes at the indicated concentrations and maintained in AS without irradiation. Likewise, the survival of untreated control embryos (also maintained in AS but irradiated for 30 min [129.6 J/cm^2]) was not affected. When no photochemical toxicity was observed, the time of exposure to the dyes and the irradiation time were expanded in order to avoid false negative results.

In the case of embryos treated with 10 mg/l MB for 48 h and irradiated for 30 min, 100% lethality was found directly along the irradiation time. Using only 10 min irradiation, the same lethal effect was also observed but at 1 h after irradiation. By means of shorter irradiation time a proportional decrease in lethality was observed (Fig. 3A). The response of embryos to photodynamic treatments with MB and RB showed that the lethal effect could be assessed unambiguously 12-24 h after irradiation (Fig. 3A), and it was dependent on the light dose (Fig. 3A and B).

Based on these results, the evaluation of photochemical toxicity of some selected dyes was standardized by determining the NOEC values for an incubation time of 48 h, and then the response to 10 min irradiation (43.2 J/cm^2) was recorded 24 h later. The NOEC values for chemical toxicity and NOEC+light values for photochemical toxicity were (in mg/L): AO 1 and 0.5; PhB 1 and 0.7; RB 5 and 1; TB 10 and 5, respectively. Plotting NOEC vs. NOEC+light values can be used to represent the position of both photoactive and photoinactive dyes (Fig. 4). Values on the diagonal correspond to dyes without photodynamic activity, whereas photodynamic dyes are located in the area below the diagonal.

Approximately 10000 different dyes and pigments are produced annually worldwide and extensively used in textile, leather, plastic and printing industries, in laboratory activities, and as food, pharmaceutical and cosmetic additives. About 10-15% of the total dyes used in dyeing processes may be found in wastewater[39]. Although ligninolytic enzymes from microorganism (e.g., laccase) can degrade diverse type of dyes[21, 31], the environmental contamination by these compounds[30] and the resulting phototoxicity represent a significant risk for human and wildlife health. In addition, the photochemical properties of some dyes must be also taken into account because of their relation with pharmacological drugs showing undesired photoactivity, some examples being phenothiazine tranquilizers[7, 8] and antimalarial acridines[44]. Some anxiolytic, antirheumatic, antibacterial and antiparasitic drugs have also revealed to be phototoxic[7, 22, 43].

The use of (I) a vertebrate organism such as the *B. arenarum* embryo, and (II) lethality as unambiguous end point of the bioassay allowed us to evaluate more precisely the toxicity of physico-chemical agents and environmental contaminants[11-13, 29]. Research on the adverse effects of xenobiotics on these animals is of special interest, not only because of their potential as suitable sentinels of ecosystem health, but also on account of the relation with the declining of amphibian populations[3, 4].

The photochemical lethality of amphibian embryos induced by the dyes used in this work seems to be related to oxidative stress, which in the case of MB photosensitization could be prevented by previous treatment with antioxidant agents such as glutathione and ascorbic acid[36]. It is known that oxidative stress affects severely ionic channels and Ca^{2+} homeostasis[14, 32] leading to disturbances in signal transmission in the cardiovascular and central nervous system[33, 40]. Extreme photochemical toxicity may produce rapid lethal photodamage even in mammalian organisms such as hairless mice treated with hypericin and high light dose[17]. Although the presence of melanin in the amphibian embryo epidermis could have a major protective role for UV irradiation due to its very high absorption in the UV region[28], the pigment does not interfere with the photochemical lethality by visible light used in this study.

In the present work, the photoactivity of MB, RB, TB, PhB, and AO have been assessed successfully by using the AMPHITOX bioassay[12, 13], showing results that confirm the known photodynamic activity of these model dyes. By comparing the chemical and photochemical toxicity of a given compound, the occurrence of photosensitization can be expressed as the ratio between NOEC and NOEC+light values. This ratio will be higher than 1 for compounds with

photodynamic toxicity. Our results show the possibility to evaluate accurately the photochemical properties of xenobiotics and potential PSs by means of a simple and sensitive toxicity test employing the amphibian embryo as a whole organism.

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References

1. Almeida R.D., Manadas B.J., Carvalho A.P. and Duarte C.B., Intracellular signaling mechanisms in photodynamic therapy. *Biochim. Biophys. Acta*, **1704**, 59 (2004)

Table I

Main characteristics of the dyes used in this work. Physico-chemical parameters were taken from Horobin and Kiernan[15]. *Absorption peaks refer to dyes in aqueous solution

Dye and abbreviation	Colour Index (CI)	Electric charge	Absorption peak (nm)*	Source	Chromophore group
Trypan blue (TryB)	23850	4–	588	Sigma	disazo
Pyronine Y (PY)	45005	+	546	Merck	aminoxanthene
Phloxin B (PhB)	45410	2–	548	Panreac	hydroxyxanthene
Rose bengal B (RB)	45440	2–	548	Sigma	hydroxyxanthene
Acridine orange (AO)	46005	+	492	BDH	acridine
Methylene blue (MB)	52015	+	661	Sigma	thiazine
Toluidine blue O (TB)	52040	+	626	Merck	thiazine
Remazol brilliant blue R (RemBB)	61200	–	600	Serva	anthraquinone
Indigocarmine (IC)	73015	2–	608	Serva	indigoid
Luxol fast blue MBS (LuxFB)	74180	2–	666	Serva	phthalocyanine

Table II

Response of *B. arenarum* embryos to some photoactive and photoinactive dyes. No lethal effects were found in animals only treated with the dyes at the indicated concentrations but not irradiated. Light irradiation of untreated (control) embryos for 30 min was also without effect

Dye	Concentration (mg/l)	Dye treatment (days)	Light irradiation (min)	Lethal effect (at 24 h)
TryB	70	4	15	–
PY	20	2	15	–
PhB	1	2	10	+
RB	5	2	10	+
AO	1	2	10	+
MB	10	2	10	+
TB	10	2	10	+
RemBB	70	4	15	–
IC	100	2	15	–
LuxFB	100	4	15	–
Control	–	–	30	–

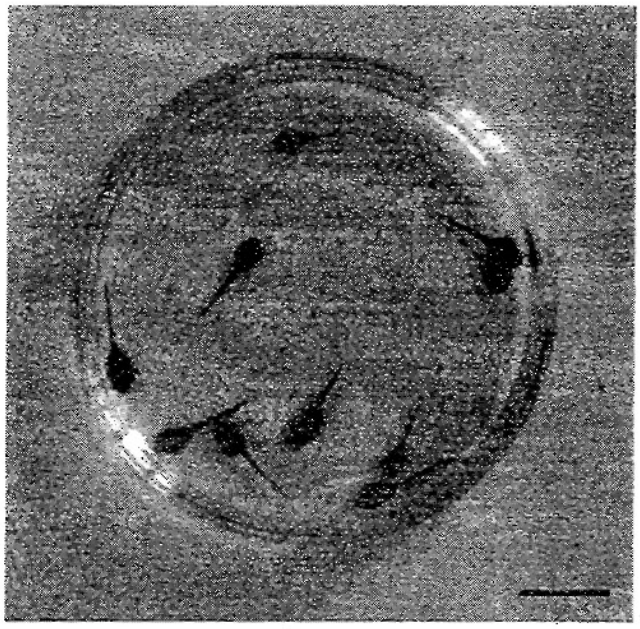

Fig. 1: *Bufo arenarum* embryos at stage 25. Bar: 1 cm.

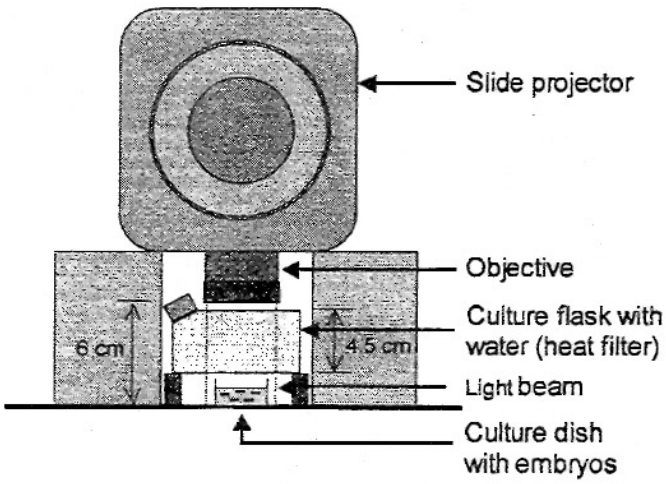

Fig. 2: Device used for light irradiation of *B. arenarum* embryos subjected to photochemical toxicity assays.

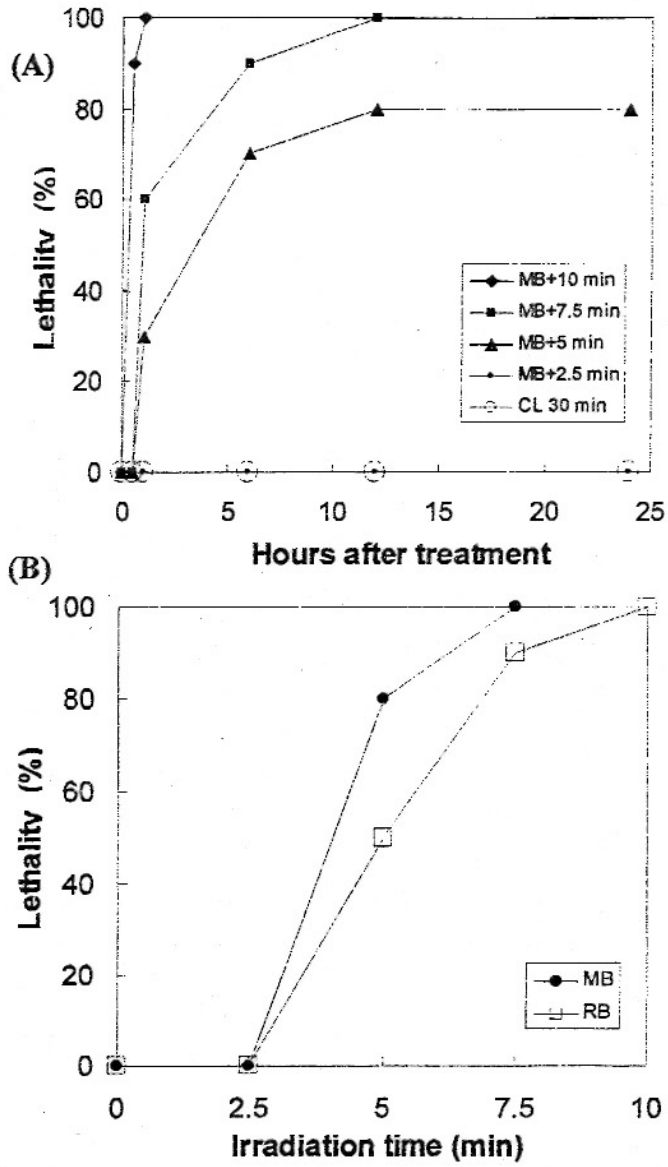

Fig. 3: (A) lethality curves of embryos after methylene blue (MB) photosensitization (10 mg/ l MB for 48 h) followed by different light doses. CL: untreated control embryos only exposed to light for 30 min.

(B) lethality of embryos after 48 h treatment with MB (10 mg/ l) and rose bengal B (RB, 5 mg/ l) followed by different light doses. Observations were recorded 24 h after light irradiation.

2. Banks J.G., Board R.G., Carter J. and Dodge A.D., The cytotoxic and photodynamic inactivation of micro-organisms by rose bengal. *J. Appl. Bacteriol.*, **58**, 391 (1985)

3. Blaustein A.R., Wake D.B. and Sousa W.P., Amphibian declines: judging stability, persistence and susceptibility of populations to local and global extinctions. *Conserv. Biol.*, **8**, 60 (1994)

4. Boyer R. and Grue C.E., The need for water quality criteria for frogs. *Environ. Health Perspect.*, **103**, 352 (1995)

5. Dougherty T.J., An update on photodynamic therapy applications. *J. Clin. Laser Med. Surg.*, **20**, 3 (2002)

6. Gadelha F.R., Moreno S.N., Souza W.D., Cruz F.S. and DoCampo R., The mitochondrion of *Trypanosoma cruzi* is a target of crystal violet toxicity. *Mol. Biochem. Parasitol.*, **34**, 117 (1989)

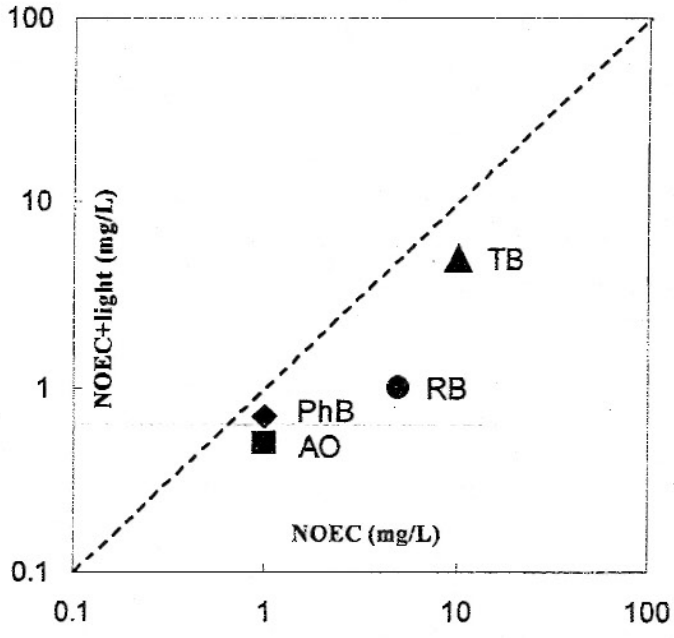

Fig. 4: NOEC and NOEC+light values for *B. arenarum* embryos showing the chemical and photochemical toxicity of some dyes. Embryos were treated either with dyes alone for 48 h (NOEC), or treated with dyes at NOEC and then subjected to light irradiation (43.2 J/cm²) (NOEC+light). Values below the diagonal correspond to compounds with photodynamic activity.

7. Gocke E., Photochemical mutagenesis: examples and toxicological relevance. *J. Environ. Pathol. Toxicol. Oncol.*, **20**, 285 (**2001**)

8. Griebrock J.J., Photomutagenesis by chlorinated phenothiazine tranquilizers. *Proc. Natl. Acad. Sci., USA*, **76**, 469 (**1979**)

9. Herkovits J. and Pérez-Coll C.S., Zinc protection against delayed development produced by cadmiun. *Biol. Trace Elem. Res.*, **24**, 217 (**1990**)

10. Herkovits J. and Helguero L.A., Copper toxicity and copper-zinc interactions in amphibian embryos. *Sci. Total Environ.*, **221**, 1 (**1998**)

11. Herkovits J., Pérez-Coll C.S. and Herkovits F.D., Evaluation of nickel-zinc interactions by means of bioassays with amphibian embryos. *Ecotoxicol. Environ. Safety*, **45**, 266 (**2000**)

12. Herkovits J., Pérez-Coll C.S. and Herkovits F.D., Ecotoxicological studies of environmental samples from Buenos Aires area using a standardized amphibian embryo toxicity test (AMPHITOX) *Environ. Pollut.*, **116**, 177 (**2002**)

13. Herkovits J. and Pérez-Coll C.S., AMPHITOX: A standardized set of toxicity tests employing amphibian embryos. Its potential for customized hazard assessment. In: Multiple stressor effects in relation to declining amphibian populations. ASTM International, 46 (**2003**)

14. Hermes-Lima M., How do Ca^{2+} and 5-aminolevulinic acid-derived oxyradicals promote injury to isolated mitochondria. *Free Radic. Biol. Med.*, **19**, 381 (**1995**)

15. Horobin R.W. and Kiernan J.A., Conn's biological stains. A handbook of dyes, stains and fluorochromes for use in biology and medicine. 10th Ed., Bios Scientific Publishers, Oxford (**2002**)

16. Joshi P.C. and Misra R.B., Evaluation of chemically-induced phototoxicity to aquatic organism using *Paramecium* as a model. *Biochem. Biophys. Res. Commun.*, **139**, 79 (**1986**)

17. Kamuhabwa A.R., Roelandts R. and de Witte P.A., Skin photosensitization with topical hypericin in hairless mice. *J. Photochem. Photobiol., B*, **53**, 110 (**1999**)

18. Kandela I.K., Bartlett J.A. and Indig G.L., Effect of molecular structure on the selective phototoxicity of triarylmethane dyes toward tumor cells. *Photochem. Photobiol. Sci.*, **1**, 309 (**2002**)

19. Knudsen E.A., The Candida phototoxicity test. The sensitivity of different strains and species of *Candida*, standardization attempts and analysis of the dose-response curves for 5- and 8-methoxypsoralen. *Photodermatology*, **2**, 80 (**1985**)

20. Kussovski V.K., Hristov A.E. and Radoucheva T.S., Proflavine-mediated inactivation of *Salmonella dublin* exposed to visible sunlight in natural fresh water. *Microbios*, **105**, 119 (**2001**)

21. Levin L. and Forchiassin F., Ligninolytic enzymes of the white rot basidiomycete *Trametes trogii*. *Acta Biotechnol.*, **21**, 179 (**1999**)

22. Lewis D.F., Ioannides C. and Parke D.V., A retrospective study of the molecular toxicology of benoxaprofen. *Toxicology*, **65**, 33 (**1990**)

23. Marthy H.J., Murasecco-Suardi P., Oliveros E. and Braun A.M., Primary effects of singlet oxygen sensitizers on eggs and embryos of sea urchins. *J. Photochem. Photobiol., B*, **7**, 303 (**1990**)

24. Matthews R.S., *Artemia salina* as a test organism for measuring superoxide-mediated toxicity. *Free Radic. Biol. Med.*, **18**, 919 (**1995**)

25. Mellish K.J., Cox R.D., Vernon D.I., Griffiths J. and Brown S.B., In vitro photodynamic activity of a series of methylene blue analogues. *Photochem. Photobiol.*, **75**, 392 (**2002**)

26. Merlin J., Azzi S., Lignon D., Ramacci C., Zeghari N. and Guillemin F., MTT assays allow quick and reliable measurements of the response of human tumour cells to photodynamic therapy. *Eur. J. Cancer*, **28**, 1452 (**1992**)

27. Molero M.L., Hazen M.J. and Stockert J.C., Photodynamic effect of berberine sulfate on the growth rate of *Allium cepa* roots. *J. Plant Physiol.*, **120**, 91 (**1985**)

28. Parrish J.A., Anderson R.R., Harrist T., Paul B. and Murphy G.F., Selective thermal effects with pulsed radiation from lasers: from organ to organelle. *J. Invest. Dermatol.*, **80**, 75s (**1983**)

29. Pérez-Coll C.S. and Herkovits J., Lethal and teratogenic effects of naringenin evaluated by means of an amphibian embryo toxicity test (AMPHITOX) *Food Chem. Toxicol.*, **42**, 305 (**2004**)

30. Robinson T., McMullan G., Marchant R. and Nigam P., Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresour. Technol.*, **77**, 247 (**2001**)

31. Rodriguez E., Pickard M.A. and Vazquez-Duhalt R., Industrial dye decolorization by laccases from ligninolytic fungi. *Curr. Microbiol.*, **38**, 27 (**1999**)

32. Saito F. and Nakaoka Y., The photodynamic action of methylene blue on the ion channels of *Paramecium* causes cell damage. *Photochem. Photobiol.*, **65**, 902 (**1997**)

33. Sastry P.S. and Subba Rao K., Apoptosis and the nervous system. *J. Neurochem.*, **74**, 1 (**2000**)

34. Smijs T.G.M., Nivard M.J.M. and Schuitmaker H.J., Development of a test system for mutagenicity of photosensitizers using *Drosophila melanogaster*. *Photochem. Photobiol.*, **79**, 332 (**2004**)

35. Stockert J.C., Juarranz A., Villanueva A. and Cañete M., Photodynamic damage to HeLa cell microtubules induced by thiazine dyes. *Cancer Chemother. Pharmacol.*, **39**, 167 (**1996**)

36. Stockert J.C. and Herkovits J., Photodynamic toxicity and its prevention by antioxidative agents in *Bufo arenarum* embryos. *Toxicology*, **192**, 211 (**2003**)

37. Stockert J.C., Juarranz A., Villanueva A., Nonell S., Horobin R.W., Soltermann A.T., Durantini E.N., Rivarola V., Colombo L.L., Espada J. and Cañete M., Photodynamic therapy: selective uptake of photosensitizing drugs into tumor cells. *Curr. Top. Pharmacol.*, **8**, 185 (**2004**)

38. Tremblay J.F., Dussault S., Viau G., Gad F., Boushira M. and Bissonnette R., Photodynamic therapy with toluidine blue in Jurkat cells: cytotoxicity, subcellular localisation and apoptosis induction. *Photochem. Photobiol. Sci.*, **1**, 852 (**2002**)

39. Vaidya A.A. and Datye K.V., Environmental pollution during chemical processing of synthetic fibres. *Colourage*, **14**, 3 (**1982**)

40. Van der Vliet A. and Bast A., Effect of oxidative stress on receptors and signal transmission. *Chem. Biol. Interact.*, **85**, 95 (**1992**)

41. Villanueva A., Vidania R., Stockert J.C., Cañete M. and Juarranz A., Photodynamic effects on cultured tumor cells: cytoskeleton alterations and cell death mechanisms. In: Handbook of Photochemistry and Photobiology. (Nalwa H.S., Ed.) American Scientific Publishers, California, 79 (**2003**)

42. Vrouenraets M.B., Visser G.W., Snow G.B. and van Dongen G.A., Basic principles, applications in oncology and improved selectivity of photodynamic therapy. *Anticancer Res.*, **23**, 505 (**2003**)

43. Wagai N. and Tawara K., Quinolone antibacterial agent-induced cutaneous phototoxicity: ear swelling reactions in Balb/c mice. *Toxicol. Lett.*, **58**, 215 (**1991**)

44. Wagner S.J. and Skripchenko A., Investigation of photosensitizing dyes for pathogen reduction in red cell suspensions. *Biotech. Histochem.*, **78**, 171 (**2003**)

45. Wainwright M. and Crossley K.B., Methylene blue – a therapeutic dye for all seasons? *J. Chemother.*, **14**, 431 (**2002**)

46. Walker I., Gorman S.A., Cox R.D., Vernon D.I., Griffiths J. and Brown S.B., A comparative analysis of phenothiazinium salts for the photosensitisation of murine fibrosarcoma (RIF-1) cells *in vitro*. *Photochem. Photobiol. Sci.*, **3**, 653 (**2004**)

47. Zeina B., Greenman J., Corry D. and Purcell W.M., Cytotoxic effects of antimicrobial photodynamic therapy on keratinocytes in vitro. *Br. J. Dermatol.*, **146**, 568 (**2002**).

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