The susceptibility of trichophyton rubrum to photodynamic treatment

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Chapter VII

DEVELOPMENT OF A TEST SYSTEM FOR MUTAGENICITY OF PHOTOSENSITIZERS USING *DROSOPHILA MELANOGASTER*

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Two *Trichophyton rubrum* mircroconidia, destroyed by photodynamic treatment with Sylsens B and red light shortly after germination

ABSTRACT

In the past few years, there has been an increase in the application of photosensitizers for medical purposes. A good standardized test system for the evaluation of the mutagenic potentials of photosensitizers is therefore an indispensable device. In the standard Ames test, white light itself was proven to be mutagenic and the result influenced by the light source. Lack of a reliable positive control is another problem in many genotoxicity test systems used for the evaluation of mutagenicity of photosensitizers. Based on the validated somatic mutation and recombination test, known as SMART, and using Drosophila melanogaster, we developed the Photo-SMART and demonstrated that methylene blue, known to induce photomutagenicity, can act as a positive control in the presented test system. The SMART scores for the loss of heterozygosity caused predominantly by homologous mitotic recombination. The Photo-SMART can be used to detect photogenotoxicity caused by short-lived photoproducts or by stable photoproducts or both. We demonstrated the Photo-SMART to be a good standardized test system for the evaluation of mutagenic potentials of the photosensitizer Sylsens B. We demonstrated that Sylsens B was mutagenic using the Photo-SMART. For hematoporphyrin, the results of the Photo-SMART indicate the absence of mutagenicity.

INTRODUCTION

In the past few years, there has been a great improvement in the development of new photosensitizers and their application for medical purposes (1,2). At present, it is therefore necessary to evaluate the different phototoxic effects of photosensitizers in preclinical studies. With respect to this subject, the reported DNA damage and inhibition of DNA repair functions by photooxidative reactions is an interesting aspect (3). Mutagenic potentials of photosensitizers used in PDT are an important issue to which much attention has been paid (4-6). The need for a good and standardized test system for photomutagenicity is an essential qualification.

Till date, many different test systems for the detection of photochemical genotoxicity have been reported (7), but most of them have certain limitations. In the well-known and internationally accepted Ames test, although correctly adjusted to use light sensitization (8,9), white light was proven to induce mutagenicity as well (10). Another problem is the lack of a reliable positive control for the photomutagenicity test system.

An important qualification of the photomutagenicity test system is the possibility to detect within one system photogenotoxicity caused by either the production of short-lived products, such as reactive oxygen species (ROS), or the production of stable photoproducts. The aim of the present study was to introduce a test system that in the first place complies with these terms, in which white light itself does not display mutagenicity and that offers a standard positive control. We investigated the possibility of methylene blue (MB, Table 7.1), known to induce photomutagenicity (7,11) to act as a reliable positive control in our test system. Furthermore, the test system we developed, based on the validated somatic mutation and recombination test (SMART) (12,13) and referred to as Photo-SMART, uses a whole organism, *Drosophila melanogaster*. More than 180 chemicals were tested in the SMART (13), and the results were in accordance with the data given by other tests. The SMART is an important test in *D. melanogaster* for the identification and characterization of potential genotoxic compounds and scores for loss of heterozygosity (LOH) (14). LOH may occur due to different types of recombination, deletions, point mutations, loss of chromosomes or nondisjunction. In flies heterozygote for the recessive eye color marker *white*, new somatic mutations of the wild-type *white* gene, formed early in the larval development, become visible as white spots in the otherwise normally red colored eyes. Increased sensitivity of the test was obtained by introduction of the

tumor suppression gene *warts* (14,15) and evaluated with the chemical mutagen methylmethanosulfonate and X-rays (14). Loss of the wts^+ function in wts/wts^+ cells results in overproliferation and apical hypertrophy of epithelial cells, producing a wart-like phenotype on all external parts of the flies including the eyes.

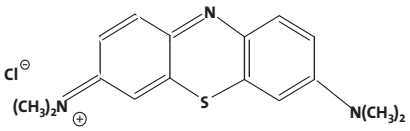
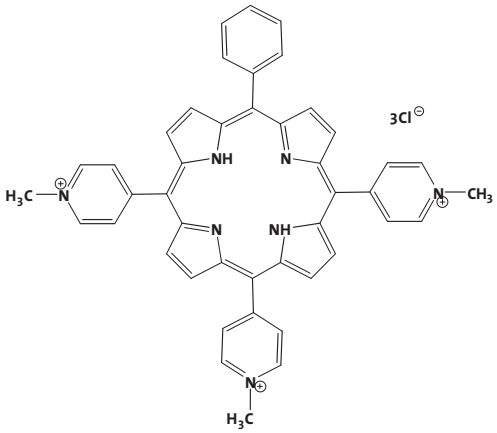
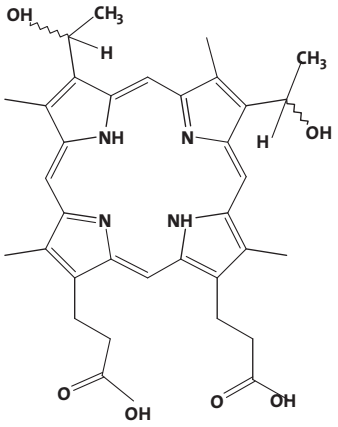
Chemical Structure	*Selected name*	*Abbreviation*
	Methylene Blue	MB
	5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]-porphine trichloride	Sylsens B
	Hematoporphyrin	HP

Table 7.1. Formulae of the porphyrin photosensitizers, Sylsens B and HP and the phenotiazine MB

Use of a whole organism is an important additional amenity of the test. This is especially true because it was recently demonstrated that *D. melanogaster* could be used to assess photodynamic activity of many different photosensitizers as well (G. M. T. Smijs, unpublished). It is important to realize that the Photo-SMART is meant to detect certain biological effects caused by photosensitizers under comparable circumstances. To evaluate the Photo-SMART, the mutagenetic potentials of the well-known photosensitizers hematoporphyrin (HP) and Sylsens B were investigated. The absorption maximum for HP is situated at 392 nm, for Sylsens B at 424 nm and for MB at 661 nm. For formulae and related names, see Table 5.1. In all cases, presence or absence of mutagenicity was determined using experimental conditions such that between 40% and 60% light-dependent kill occurred.

MATERIALS AND METHODS

Strains and crosses

The following *D. melanogaster* strains were used: a strain carrying the X-linked eye color marker white (*w*) and a strain carrying on the third chromosome $wts^{MT4\text{-}1}$, a lethal *warts* allele, balanced over TM3, characterized by multiple inversions and marked by the dominant mutation *stubble* (*Sb*). The warts allele $wts^{MT4\text{-}1}$ was induced with the alkylating agent ethylmethanesulfonate in a chromosome carrying the eye color markers starlet and pink, the bristle marker inturned and the wing morphology marker radius incomplete (14,16).

The following cross was made: *wts*/TM3 males with *w* females.

Chemicals

HP was from Porphyrin Products Inc. (Logan, UT), Sylsens B was synthesized and kindly provided by ARC Laboratories BV at the Department of Bio-Organic Photochemistry, Leiden University, The Netherlands (purity, checked with nuclear magnetic resonance, was more than 99.5%) and MB was purchased from J.T. Baker (Deventer, The Netherlands). Tween-80 was obtained from Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands), whereas all other chemicals were from J.T. Baker.

The following solvent was used for the photosensitizers: a sodium-potassium phosphate buffer, pH 7.0 (2.79 g Na_2HPO_4 and 2.27 g KH_2PO_4/L distilled water). Stock solutions of the photosensitizers HP (20 mM), Sylsens B (20 mM) and MB (5 mM) were stored at 4°C for no longer than 1 week.

Light source

Illuminations were performed with a lamp from MASSIVE (no. 74900/21), 1 x maximum 500 W, 230 V, R7s, IP 44. To avoid heating of the samples to be illuminated, the white light produced by the lamp was passed through water layer first before reaching the samples. Light intensity was measured with IL1400A photometer equipped with a SEL033/F/U detector (International Light, Newburyport, MA). The amount of UVA and UVB in the white light produced by the lamp was measured with an UVX radiometer; UVP, Upland, CA). The amount of UVA appeared to be less than 0.1%, and the amount of UVB appeared to be 0.007%.

Photo-SMART

The method used and referred to as Photo-SMART is a modification of the SMART, which is a combination of the *w/w*$^{+}$ eye test (10) and the *wts/wts*$^{+}$ tumour suppressor system (15).

w females (40) flies were mated with *wts*/TM3 males (35) flies and allowed to lay eggs in bottles on food supplemented with the photosensitizer dissolved in solvent before mixing it to the standard food (2 mL/50 mL food). After 24 h, the parent flies were discarded, and the eggs were allowed to develop for another 75 h. The larvae were then isolated with a 20% sucrose solution, washed twice with a 0.7% NaCl solution (using a 30 µM Millipore filter) and illuminated in a total volume of 2 mL of 10% sucrose solution in 6 cm diameter culture dishes (Greiner, Alphen aan de Rijn, The Netherlands). Reduction of the light through the plastic lid was evaluated and found to be negligible. Light fluency and concentration range were always such that a light-dependent kill of 40–60% occurred. These conditions have to be established in preliminary experiments using the same experimental setup and parental cross.

After illumination or dark period, treated larvae were transferred to bottles containing normal food supply and left to develop at 25°C. After 10 days, the number of flies present in the bottles was counted and males were removed and females were collected (genotypes, *w/w*$^{+}$ and *w/w*$^{+}$*; wts/wts*$^{+}$) for scoring of mutagenicity. The scoring of etherized flies was carried out in a liquid consisting of 90 parts of ethanol, one part Tween-80 and nine parts water. Inspection of the eyes for the presence of white spots and warts tumors (Fig. 7.1) was performed using a Nikon stereomicroscope at a magnification of 50–75x. Large white spots are seen as white parts in the red eyes and small white spots as dark ommatidia in a red eye. Similarly, small warts tumours are seen as dark ommatidia. Spots separated from each other by at least four

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nonmutated ommatidia were counted as independent events, and the smallest size of white clone expected to be counted accurately is 2 (13,17). The white spots and warts tumours were scored together. An increase in white and warts clone frequency was only accepted as a positive response if it was significantly higher than that of both the concurrent and pooled experimental controls.

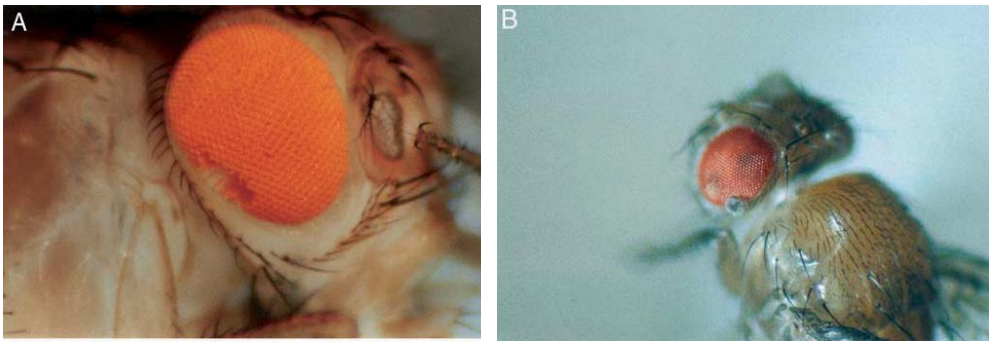

Figure 7.1. Drosophila eye (A) showing a large white spot in the lower left quadrant and an eye (B) showing a warts tumor.

Statistical test

The statistical significance of the differences between spot frequencies in the experiment (light induced) and control (dark) was calculated using the X^2 test for proportions as described by Frei and Würgler (18).

RESULTS

White light

For both genotypes scored in the Photo-SMART, *w/w⁺; wts/wts⁺* and the *w/w⁺*, no mutagenicity was established for the white light itself. For the different light doses used, namely 180, 360 and 1080 kJ/m², the scored spot percentage was not significantly [X^2 method, (18)] higher than that scored for the corresponding dark controls (Tables 7.2–7.4).

MB concentration (mM)	*0*	*0.01*	*0.025*	*0.05*	*0.1*	*0.2*
A: ***w/w⁺;wts/wts⁺***						
N_t	400	290	320	420	514	620
n_t	50	75	99	166	245	372
N_c	400	600	600	600	600	600
n_c	66	84	78	92	80	96
χ^2	1.91	14,7	33,9	60,4	110,6	152,7
B: ***w/w⁺***						
N_t	400	222	276	410	480	446
n_t	33	45	65	115	154	163
N_c	400	400	400	260	400	400
n_c	40	44	44	33	36	40
χ^2	0.49	7,9	18,4	16,3	52,78	60,8

Table 7.2. Test of statistical significance of the data for MB using the X^2 test as described by Frei and Würgler (18). The result for the *w/w⁺; wts/wts⁺* system is given in A (corresponding the filled and open squares in Fig. 7.3) and for the *w/w⁺* in B (corresponding filled and open diamonds in Fig. 7.3)[a]
[a] N_t = number of eyes scored in the test, i.e. the number of eyes of the flies that were submitted to light treatment (1080 kJ/m²). n_t = number of spots scored in the test, i.e. the number of spots scored in the eyes of the flies that were submitted to light treatment (1080 kJ/m²). N_c = number of eyes scored in the control, i.e. the number of eyes of the flies that were kept in the dark. n_c = number of spots scored in the control, i.e. the number of spots scored in the flies that were kept in the dark. X^2 (α = 0.05; ν = 1) = 2.706.

HP concentration (mM)	*0*	*0.1*	*0.3*	*0.6*	*0.8*
A: ***w/w⁺;wts/wts⁺***					
N_t	400	180	300	200	100
n_t	40	28	63	26	20
N_c	400	200	200	200	200
n_c	44	20	22	18	22
χ^2	0.11	0.76	6,48	1,11	3,24
B: ***w/w⁺***					
N_t	400	144	300	200	190
n_t	28	8	15	10	24
N_c	400	200	300	200	200
n_c	28	16	14	10	16
χ^2	0.02	0,409	0	0	1,6

Table 7.3. Test of statistical significance of the data for HP using the X^2 test as described by Frei and Würgler (18). The result for the *w/w⁺; wts/wts⁺* system is given in A (corresponding filled and open squares in Fig. 7.5) and for *w/w⁺* in B (corresponding filled and open diamonds in Fig. 7.5)[a]
[a] N_t = number of eyes scored in the test, i.e. the number of eyes of the flies that were submitted to light treatment (360 kJ/m²). n_t = number of spots scored in the test, i.e. the number of spots scored in the eyes of the flies that were submitted to light treatment (360 kJ/m²). N_c = number of eyes scored in the control, i.e. the number of eyes of the flies that were kept in the dark. n_c = number of spots scored in the control, i.e. the number of spots scored in the flies that were kept in the dark. X^2 (α = 0.05; ν = 1) = 2.706.

Sylsens B Concentration (mM)	*0*	*0.1*	*0.4*	*0.6*	*0.8*
A: ***w/w⁺ ;wts/wts⁺***					
N_t	400	400	400	400	162
n_t	54	111	133	155	58
N_c	400	300	300	300	300
n_c	48	48	44	54	53
χ^2	0.25	10	22,6	24,0	13,6
B: ***w/w⁺***					
N_t	400	400	380	400	210
n_t	32	80	104	115	71
N_c	400	200	180	200	160
n_c	28	22	28	34	26
χ^2	0.15	5,8	6,7	6,9	10,0

Table 7.4. Test of statistical significance of the data for Sylsens B using the X^2 test as described by Frei and Würgler (18). The result for the *w/w⁺; wts/wts⁺* system is given in A (corresponding filled and open squares in Fig.7. 6) and for the *w/w⁺* in B (corresponding filled and open diamonds lines in Fig. 7.6)[a]
[a] N_t = number of eyes scored in the test, i.e. the number of eyes of the flies that were submitted to light treatment (180 kJ/m²). n_t = number of spots scored in the test, i.e. the number of spots scored in the eyes of the flies that were submitted to light treatment (180 kJ/m²). N_c = number of eyes scored in the control, i.e. the number of eyes of the flies that were kept in the dark. n_c = number of spots scored in the control, i.e. the number of spots scored in the flies that were kept in the dark. X^2 (α = 0.05; ν = 1) = 2.706.

Methylene blue

The experimental conditions used in the photomutagenicity test, the Photo-SMART, were determined in a number of preliminary experiments. These experiments comprise light dose and concentration variations using the experimental setup as described for the Photo-SMART. The results of these experiments yielded a number of combinations of light dose and MB concentration, each resulting in a light-dependent kill of about 50%. Based on the final results, as shown in Fig. 7.2, and the practical circumstances, a light dose of 1080 kJ/m² was chosen for MB. At this particular light dose and final MB concentration of 0.2 mM, a light-dependent kill of 50% was observed. The practical circumstances referred to comprise the solubility of the photosensitizer and the heat production of the lamp. When using higher MB concentrations, it is the dark toxicity that becomes increasingly important.

In a total of three different experiments, we determined the mutagenicity of MB in the Photo-SMART as described using a light fluency of 1080 kJ/m² and a concentration range that varied up to 0.2 mM (Fig. 7.3). Before scoring of the mutagenicity, the survival of *D. melanogaster* at each different MB concentration was determined. The

results were in concordance with the results obtained in the preliminary toxicity experiments. Up to 0.025 mM MB, we found a light-dependent survival of 100%, 0.050 mM yielded 90% survival, 0.1 mM 65% and 0.2 mM 50%. The dark toxicity is negligible for MB concentrations up to 0.1 mM and ranges between 20% and 25% for 0.2 mM MB.

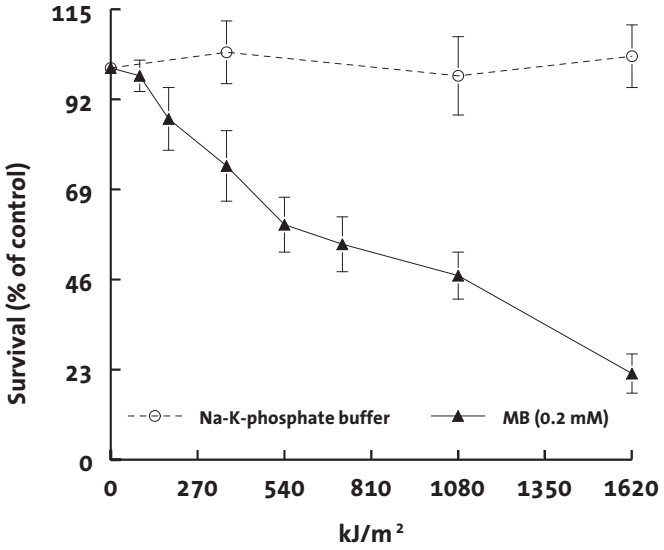

Figure 7.2. Survival curves for *Drosophila melanogaster* exposed to increasing levels of white light at a constant concentration of MB of 0.2 mM, using the parental cross *wts*/TM3 males with *w* females. White light intensity variations were performed at a fixed MB concentration of 0.2 mM. In the control experiment, the solvent, phosphate buffer of pH 7.0, was used instead of MB. The number of flies in the control, no light administration, was 184 for MB and 250 for the solvent. The values given here are the mean of three experiments and the standard deviation and represent the number of flies counted 10 days after the start of the experiment.

As can be seen from Fig. 7.3, there is definitely a concentration dependent increase in spot percentage in both genotypes that were scored, the *w/w*$^+$ and *w/w*$^+$*; wts/wts*$^+$ system.

Significance of the results obtained for the light-induced mutagenicity and the dark control values was determined according the X^2 method as described by Frei and Würgler (18). A summary is given in Table 7.2 for both the *w/w*$^+$ and *w/w*$^+$*; wts/wts*$^+$ system (A) and the *w/w*$^+$ (B). In both cases, significance (for α = 0.05; ν = 1, the value for X^2 is 2.706) is evident for all the concentrations submitted to the test.

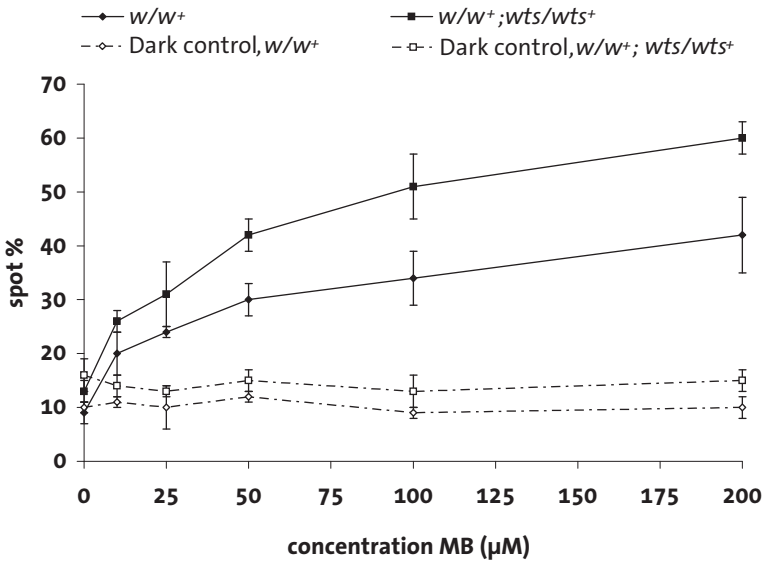

Figure 7.3. Light-induced increase in the percentage of white spots and warts tumors for MB according to the Photo-SMART.
Light conditions: white light, 1080 kJ/m² (UVA, 0.1%, UVB, 0.007%). The values given are the mean of three different experiments and the standard deviation and represent the percentage of white spots (*w/w⁺*), or white spots and warts tumors (*w/w⁺; wts/wt*), of the number of counted eyes. The number of eyes scored in one experiment varied from 100 to 200 for a given concentration and genotype.

Other photosensitizers

To evaluate the test system for photomutagenicity further, the two porphyrin photosensitizers, Sylsens B and HP, were submitted to the system to investigate their mutagenetic potentials.

From the preliminary experiments, different combinations of light dose and photosensitizer concentration were obtained, all resulting in a 50% kill. Practical circumstances, solubility properties of the photosensitizers and the presence of dark toxicity with higher concentrations, will eventually determine the combination to be used in the Photo-SMART. Figure 7.4 gives the final result of the preliminary experiments concerning the light conditions to be used in the Photo-SMART. As can be seen from this figure, in both cases, a photosensitizer concentration of 0.7 mM was used, and a variable light dose of up to 2160 kJ/m² for HP and 1620 kJ/m² for Sylsens B was used. At this photosensitizer concentration, light conditions giving 50% light-dependent kill appeared to be 360 kJ/m² for HP and 180 kJ/m² for Sylsens B. For higher photosensitizer concentrations, solubility problems became important for both sensitizers, and especially for Sylsens B the dark toxicity.

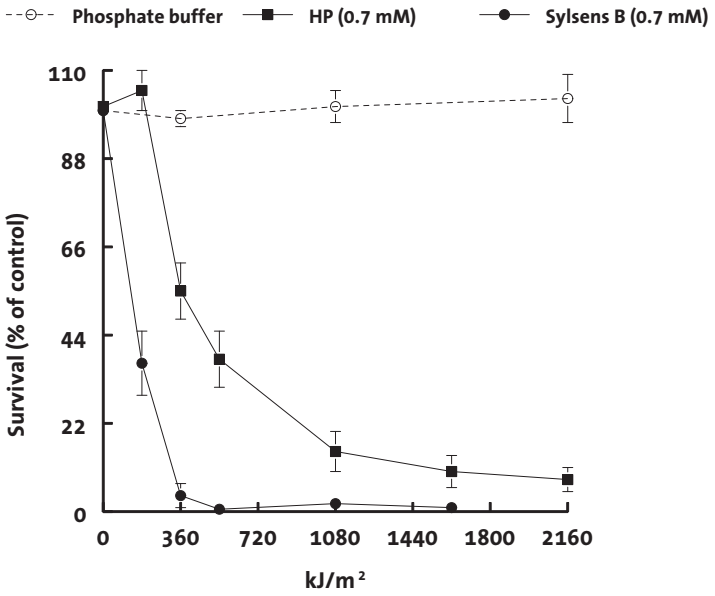

Figure 7.4. Survival of *Drosophila melanogaster* as a function of various doses of white light at a constant concentration of HP or Sylsens B of 0.7 mM, using the parental cross *wts*/TM3 males with *w* females. White light variations were performed at a fixed photosensitizer concentration of 0.7 mM. In the control experiment, the solvent, phosphate buffer of pH 7.0, was used instead of photosensitizer. The number of flies in the control, no light administration, was 225 for HP, 215 for Sylsens B and 250 for the solvent. The values given are the mean of three experiments and the standard deviation and represent the number of flies counted 10 days after the start of the experiment.

Two separate mutagenicity experiments were performed for both HP and Sylsens B varying the sensitizer concentration up to 0.8 mM. The mean result is given in Fig. 7.5 for HP and in Fig. 7.6 for Sylsens B. In the case of Sylsens B, an increase in spot percentage can be seen as a result of an increasing photosensitizer concentration. Significance was determined as described (17), and the results are summarized in Table 7.3 for HP and in Table 7.4 for Sylsens B.

Table 7.3 shows that for HP in the *w/w⁺; wts/wts⁺* system, only the concentrations 0.3 and 0.8 mM led to a significant spot enhancement in the presence of white light (for α = 0.05; ν = 1, the value for X^2 is 2.706). In the other cases (0.1 and 0.6 mM), no significant spot enhancement could be detected. For the *w/w⁺*, no significant increase in spot percentage could be detected for HP for the whole concentration range, when compared with the dark control values.

From Table 7.4, it may become clear that in the case of Sylsens B, there is definitely a significant difference in spot percentage for both the *w/w⁺; wts/wts⁺* system (A) and the *w/w⁺*, (B).

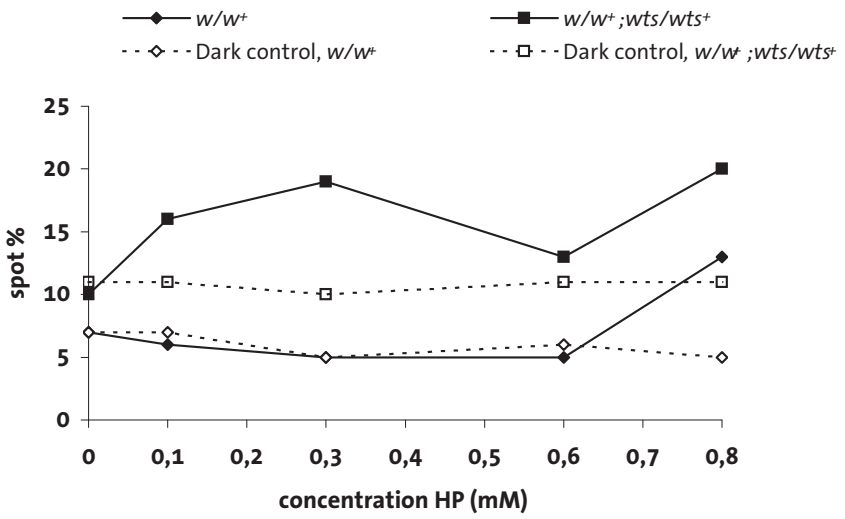

Figure 7.5. Percentage of white spots and warts tumors at a constant dose of white light and increasing concentrations of HP Light conditions: white light, 360 kJ/m² (UVA, 0.1%, UVB, 0.007%). The values given are the mean of two different experiments and represent the percentage of white spots (*w/w⁺*), or white spots and warts tumors (*w/w⁺; wts/wt*), of the number of counted eyes. The number of eyes scored in one experiment varied from 100 to 200 for a given concentration and genotype.

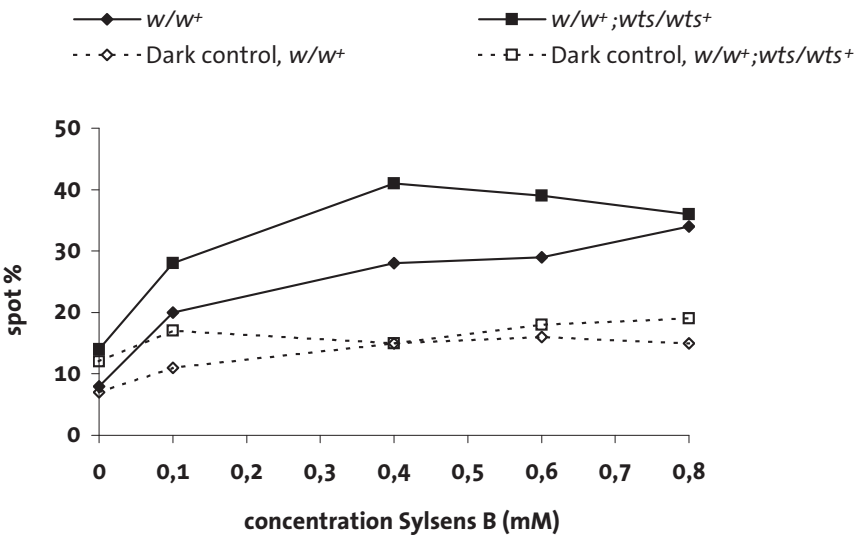

Figure 7.6. Percentage of white spots and warts tumors at a constant dose of white light and increasing concentrations of Sylsens B. Light conditions: white light, 180 kJ/m² (UVA, 0.1%, UVB, 0.007%). The values given are the mean of two different experiments and represent the percentage of white spots (*w/w⁺*), or white spots and warts tumors (*w/w⁺; wts/wt⁺*), of the number of counted eyes. The number of eyes scored in one experiment varied from 100 to 200 for a given concentration and genotype.

The determination of the light-dependent survival of *D. melanogaster*, before scoring of mutagenicity, yielded for HP 100% survival for 0.1 mM, 90% for 0.3 mM, 70% for 0.6 mM and 60% for 0.8 mM. For Sylsens B, the light-dependent survival values were 90% for 0.1 mM, 70% for 0.4 mM, and between 40% and 60% for the highest concentrations of 0.6 and 0.8 mM. Dark toxicity for HP was found to be negligible in the given concentration range. For Sylsens B, the dark toxicity is negligible up to a Sylsens B concentration of 0.4 mM, and between 10% and 15% for the highest concentrations of 0.6 and 0.8 mM.

DISCUSSION

Clearly, white light itself did not induce mutagenetic events, leading to an increase in spot percentage, in the presented Photo-SMART. The presented data therefore provide the evidence of a genotoxic effect from PDT mediated by MB in Drosophila eye tissue. As mentioned before, the amount of UVA and UVB produced by the light source used in the experiments was measured. The amounts of UVA and UVB were such that no mutagenic effects in Drosophila were to be expected [(19,20); M. J. M. Nivard, unpublished]. Moreover, the experimental setup is such that the sample spot is situated beneath glass. A glass filter containing water (5 cm thickness) is used to absorb the infrared light from the lamp.

It is important to realize that the presented test system is meant to provide a method for the evaluation of the mutagenic potentials of medical photosensitizers. This category of photosensitizers will comprise preferably no chemicals that possess substantial absorption peaks in the UV part of the spectrum because of the low penetration capacity of the UV light in tissue.

The results obtained for MB with the Photo-SMART, as presented in Fig. 7.3, show that there is definitely a concentration and light-dependent increase in spot percentage. An increase in spot percentage was shown to be significantly higher (Table 7.2) than the spot percentage obtained for the dark control (for every tested photosensitizer concentration).

The Photo-SMART as described here is, to our opinion, a valuable tool to evaluate mutagenetic potentials of photosensitizers. As mentioned in the Introduction, this test system provides reliable information about biological effects caused by photosensitizers. The test offers the possibility to compare the positive biological

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effects, the light-induced toxicity, with the negative effects, the mutagenicity. Because the light treatment in the Photo-SMART is completely included within the test system, this system offers the possibility to detect not only mutagenic potential of stable photoproducts but also the mutagenic potential of short-lived photoproducts, such as ROS. Using a whole organism in this test may of course have great advantages compared with the customary genotoxicity tests. It is commonly accepted now, in considering mutagenicity testing, to regard a negative finding in an *in vivo* procedure as the definitive indicator of the lack of genotoxic potential, whatever the effects seen in *in vitro* studies.

An additional advantage of the Photo-SMART is the fact that *D. melanogaster* was recently proven to be susceptible to PDT (G. M. T. Smijs, unpublished) and thus can act as a valuable tool in preclinical PDT studies. Measurements of photodynamic efficacy, uptake and clearance and the mutagenic potential of chemicals can thus be performed using the same test organism. This offers the possibility to correlate pharmacokinetic studies to photodynamic and mutagenic studies.

The results obtained for HP and Sylsens B in the Photo-SMART led to the conclusion that there is definitely an indication for mutagenicity for Sylsens B under the given conditions (Table 7.4, Fig. 7.6). For HP, however, the presented data (Table 7.3, Fig. 7.5) give no elucidation concerning the presence of mutagenicity. Additional experiments, leading to a larger total amount of scored eyes (N_t and N_c), will be necessary to present the necessary explanation. Another possibility may comprise experiments using a lower HP concentration in combination with a higher light dose. In that case, however, photobleaching characteristics of HP might interfere with the thus obtained results (21).

Considering all the data obtained here using the Photo-SMART, it is obvious that the introduction of the warts tumor makes the test system more sensitive, as was reported before (14,15). Important in this aspect is the fact that the tumour suppressor gene warts (*wts*) is involved in cell cycle regulation, and homologues of the *wts* gene are also found in lower organisms (22). The possibility of isolating the easily detectable *wts* tumors, produced in the Photo-SMART, and the possibility to submit them to further study, offers a valuable advantage of this test.

We conclude that because white light was found to be not mutagenic and the fact that a whole organism is used as test organism, the presented Photo-SMART may be seen as a valuable addition to the current range of photogenotoxicity test systems.

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