Basic Research on the Biology of Meta-tetra(hydroxyphenyl) Chlorin for Photodynamic Therapy in Gynaecology: Somatic Genotoxicity Assayed with *Drosophila melanogaster*

P.M. SCHWEIZER[a], H. WALT[b], M. FEHR[b], U. HALLER[b]

[a]*Institute for Medical Radiobiology, University of Zürich and Paul Scherrer Institute, CH-8029 Zürich, Switzerland*
[b]*Research Division of Gynaecology, Department of Gynaecology and Obstetrics, University Hospital, CH-8091 Zürich, Switzerland*

Correspondence to: P.M. Schweizer, Institute for Medical Radiobiology of the University of Zürich and the Paul Scherrer Institute, August Forel Str. 7, CH-8029 Zürich, Switzerland

Abstract. The well-established SMART test, a somatic mutation and recombination assay of Drosophila, was applied to assess the possible genotoxicity of sublethal meta-tetra(hydroxyphenyl) chlorin (mTHPC) photodynamic therapy (PDT) to clonogenic cells in situ. The SMART assay monitors the loss of heterozygosity (LOH) at selected cell-marker loci in clonogenic cells of the larval wing primordia. No evidence of genotoxicity was observed under conditions that killed between 38 and 86% of the exposed test larvae. Since the SMART assay is based on the oral uptake of the suspected genotoxic agent, the uptake kinetics of mTHPC by the assay's specific target cells must be known. Therefore, relevant studies are being carried out at present in order to draw final conclusions from this negative test result for genotoxicity.

INTRODUCTION

Photodynamic tumour therapy (PDT) is based on the light-induced activation of photo-reactive drugs, thus generating highly reactive oxygen species that are toxic to the cells. Local restriction of photodynamic impacts to the target tissue is achieved by systemic or local administration of the photodynamic agents and, subsequently, strictly limited illumination of the target tissue area. The development of new photosensitizers is regularly followed by extended preclinical studies. Such studies are now being carried out on the bio-reactivity of second-generation photosensitizers. Extensive testing of chlorins such as meta-tetra(hydroxyphenyl) chlorin (mTHPC) by in vitro experiments that analysed the dark toxicity and the DNA modifications of tumour cells found neither of these effects (1). The authors were also interested in the potential risk of genotoxicity and somatic mutagenicity of photosensitizers. In PDT, light scattering will inevitably leave a marginal zone of subcytotoxically treated cells. These could potentially constitute a source of late complications arising from DNA alterations where the cell impact was sublethal. Activated oxygen, referred to as singlet oxygen, is a DNA-damaging agent (2). Its relatively short lifetime in solution, between micro- and milliseconds (3), limits its interaction with biomolecules to the vicinity of the photoreagents from which it is produced. The risks of mutagenic effects from PDT will depend largely on the spatial distribution of photoreactive agents within the cytoplasmic and nuclear compartments of the cells (3–5). Several studies have actually demonstrated the DNA-modifying activity of photo-oxidative reactions. Following photo-oxidation by haematoporphyrin derivatives, DNA alterations such as alkali-labile sites (6), single- and double-strand breaks (7–10), induction of sister chromatid exchanges (SCE) (11, 12) and chromosome aberrations were observed (13). Most interestingly, the inhibition of DNA repair functions by photo-oxidative reactions has also been documented

recently (10, 14). Consequently, mutagenic and clastogenic effects in subcytotoxically affected cells could also be produced indirectly as a consequence of an impaired capacity of the metabolism to maintain DNA integrity (10). For the present study of the genotoxicity of mTHPC and its photo-oxidative reaction, the well-established and sensitive somatic mutation and recombination assay of *Drosophila melanogaster* known as SMART was chosen (15–17). This test monitors the consequences of the loss of heterozygosity (LOH) due to a broad spectrum of mutational events such as point mutations, deletions, homologue recombination, chromosome loss and non-disjunction as well as gene conversion (15). Loss of heterozygosity is recognized as an important cause of tumour-suppressor neoplasia mediated by gene loss in humans (16). In the fruit fly *D. melanogaster*, which is heterozygous for appropriate recessive mutations that alter the morphology of the cuticle in a cell-autonomous fashion, the loss of heterozygosity in somatic cell lineages becomes visible as spots of mutant characters (genetic mosaics) on the body surface of the adult flies.

The authors addressed the question as to whether a subcytotoxic PDT treatment with mTHPC that killed 38–86% of the larvae would yield an enhanced frequency of LOH-mediated genetic mosaics among the survivors compared to untreated controls.

MATERIALS AND METHODS

The SMART test protocol has been described comprehensively by Graf et al (15). The test is based on feeding potential mutagens to larvae of the fruit fly *D. melanogaster* that are transheterozygous for the recessive wing-cell marker mutations multiple wing hairs (mwh) and flare (flr). The induced loss of heterozygosity at either or both of the loci in clonogenic cells is expressed in the resulting clones as mutant spots on the phenotypically wild-type wing surface of the hatched adults. Homologous recombination proximal to the marker loci gives rise to mwh/flr twin spots (Fig. 1). Single spots expressing the distal marker mwh result primarily from the recombination between the two marker positions mwh and flr, from terminal deletions covering the distal marker mwh or from mutation of the wild-type allele. Single spots expressing the phenotype of the proximal marker flr can
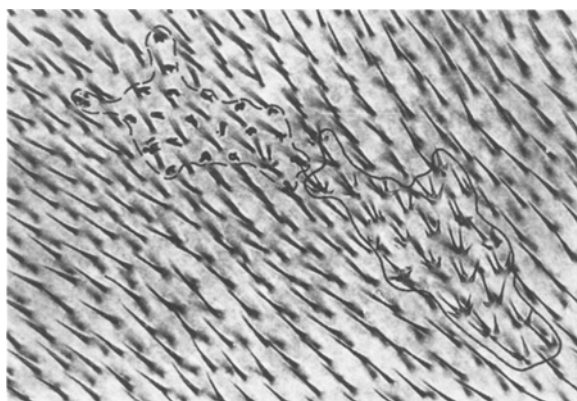

Fig. 1. Microscopic view of a wing area from a SMART assay fly displaying representative examples of clonal expression of the recessive cell-marker mutations multiple wing hairs (mwh) and flare (flr) following loss of heterozygosity (LOH) in wing precursor cells of a fly heterozygous at the respective loci.

result from double recombination proximal and distal to the respective locus flr, lethality of one of the twin products of a proximal recombination event, gene mutation or gene conversion (15).

F_1 larvae from crosses of the homozygous mwh/mwh strain carrying the visible recessive mutation multiple wing hairs (mwh 3–0.00) and of the balanced strain flr3/TM3, ri pp e bx34 Ser, carrying the wing-hair mutation flare (flr 3–38.8) (15) were chronically exposed to the photosensitizer mTHPC (Foscan®, Scotia Pharmaceuticals Ltd, Guildford, UK) by being fed up to 45 mg mTHPC per ml of instant food. After an incubation period of 64 h (84 h after egg laying), the larvae were exposed to laser illumination at 652 nm 8.45 J cm^{-2} for 16 min. The irradiation was carried out using either an argon ion-pumped dye laser (Coherent Innova 310 and CR 599, GMP, Switzerland) or a diode laser (Applied Optronics, USA). The lethality rate of the larvae in several test tubes pooled for this study was between 38 and 86%. The lethality rate was recorded 24–48 h after exposure to the laser illumination when the surviving larvae underwent pupation. In additional series, the larvae were exposed to Ringer solvent alone, to laser illumination alone, to mTHPC incubation in the dark and to 4 Gy of X-rays (84 h after egg laying, 100 kVp X-rays, 1.4 Gy min^{-1}). In the last three series mentioned above, the survival of the larvae was not affected compared to the series subject to exposure to Ringer's solvent alone. X-rays of 4 Gy were applied to demonstrate the operation of the assay. The X-ray dose was monitored by a PTW-SN4 dosimeter. After

metamorphosis, the hatched adults were collected and the wings were examined for mutant spots at a magnification of 400 × following the method and criteria of Graf et al (15).

The statistical validation of the data obtained in this study was based on the computer-assisted program LIMIT for the routine evaluation of the Drosophila wing-spot test SMART (17). This program validates the mosaic frequency in control and treated series with respect to a null hypothesis H(O) postulating no difference between a control and a treated series, and the alternative hypothesis H(A) that assumes an a priori mosaic frequency m times the spontaneous level. Acceptance or rejection of either hypothesis is based on the conditional binomial distribution.

RESULTS

Figure 1 illustrates representative examples of clonal expressions in the wing margin of the recessive cell-marker mutations multiple wing hairs (mwh) and flare (flr) following radiation-induced LOH in the wing precursor cells of the larvae. Table 1 summarizes the total number of wings analysed and the scores of mosaic spots per wing in control and treated series, as well as the diagnosis for genotoxicity according to the LIMIT statistics program of the SMART genotoxicity assay (16). The particularly relevant clone classes are the large mwh single spots identifying LOH due primarily to distal mitotic recombination, terminal deletions and gene mutations, and the wmh/flr twin spots identifying the homologous recombination proximal to the marker loci. Small single spots result from LOH in development later than 84 h after egg laying (20). No effects on their frequency could be expected under the present treatment conditions. Single flr spots result from double recombination proximal and distal to this marker gene. Their spontaneous frequency is therefore very low (18) and will be affected only by strong recombinogenic agents. It is evident from the data compiled in Table 1 that the SMART assay is sensitive enough to detect the somatic mutagenicity and recombinogenicity associated with a moderate X-ray dose of 4 Gy and, according to the authors' previous study (19), even with X-ray doses as low as 1 cGy. Conversely, neither of the other treatments,

eg laser illumination alone, mTHPC dark toxicity or PDT treatment (mTHPC+laser), led to a recognizable enhancement of spot frequencies. H(0) was accepted at a significance level of 2a=0.05 in all these latter series and with respect to all indicators.

DISCUSSION

The biological effects of PDT are complex and their comprehensive elucidation requires diverse systematic studies. Every photosensitizer reacts differently and its targets are mainly subcellular organelles within the cytoplasm. To establish PDT effects in greater detail, the dark toxicity and reactivity of mTHPC in vitro in human tumour cells was studied using two test systems, namely the colony-forming test and the cell-viability test (21, 22). These tests clearly demonstrated the effectiveness of mTHPC as a somatic photosensitizing substance. The decision to test the genotoxicity of PDT with a relevant assay was a logical consequence of the earlier analysis. The Drosophila SMART assay is well established in terms of toxicology and cross examinations of the effectiveness of drugs in the SMART test, and other established tests for carcinogenicity reveal an excellent correlation (23). The presented data yielded no evidence of a genotoxic effect from PDT treatment mediated by mTHPC. Considering that the PDT treatment caused substantial lethality among the exposed larvae (38–86%), the possibility of insufficient uptake of mTHPC by the larvae can be excluded. However, the time-dependent and spatial pharmacokinetics of mTHPC has not yet been studied in the specific target tissue of the SMART assay. This study is currently in progress and its outcome is needed to draw final conclusions about the non-genotoxicity of PDT mediated by mTHPC in the SMART assay. A fluorescence analysis of the mTHPC uptake is being used in addition to cell pathology methods to identify necrotic and/or apoptotic cell decay after PDT treatment. Since the SMART assay has proved itself capable of identifying the genotoxic activity of a large variety of agents via oral application (23), the authors' preliminary inclination is to suggest that the failure to observe genotoxic activity following the reported mTHPC PDT treatment is not merely a consequence of an insufficient uptake. On the basis of these results, the authors are optimistic that

Table 1. Observed spots per wing in mwh flr+/mwh+flr heterozygous SMART assay flies: results from untreated controls and after exposure of larvae to conditions of laser irradiation (84 h after egg laying, 16 min, 8.45 J cm⁻², mTHPC (68 h, 45 mg ml⁻¹), mTHPC and laser irradiation (68 h, 45 mg ml⁻¹, 16 min, 8.45 J cm⁻²) or 4 Gy of acute 100 kVp X-ray irradiation (84 h after egg laying, 1.4 Gy min⁻¹)

Treatment	Type of spot	Number of		Spots/wing	Two-sided test of H(0): Signif level (2α=0.05)	
		Wings	Spots		Probability	Conclusion
Control	Small mwh singles	100	20	0.200 (0.136–0.261)		
Laser		100	17	0.170 (0.109–0.233)	0.743	H(0): accepted; not genotoxic
mTHPC		200	28	0.140 (0.104–0.174)	0.285	H(0): accepted; not genotoxic
mTHPC+Laser		200	42	0.210 (0.169–0.241)	0.976	H(0): accepted; not genotoxic
4 Gy		50	14	0.280 (0.168–0.403)	1.40	H(0): accepted; not genotoxic
Control	Large mwh singles	100	5	0.05 (0.017–0.092)		
Laser		200	3	0.030 (0.007–0.060)	0.727	H(0): accepted; not genotoxic
mTHPC		200	5	0.025 (0.009–0.041)	0.426	H(0): accepted; not genotoxic
mTHPC+Laser		200	10	0.050 (0.029–0.061)	1.00	H(0): accepted; not genotoxic
4 Gy		50	34	0.680 (0.459–0.746)	0.000	H(0): rejected; sig. genotoxic
Control	flr singles	100	0			Inconclusive
Laser		100	0			Inconclusive
mTHPC		200	0			Inconclusive
mTHPC+Laser		200	0			Inconclusive
4 Gy		50	3	0.060 (0.017–0.090)	0.074	H(0): accepted; not genotoxic
Control	mwh/flr twin spots	100	1	0.010 (0.003–0.019)		
Laser		100	1	0.010 (0.003–0.019)	1.00	H(0): accepted; not genotoxic
mTHPC		200	2	0.010 (0.001–0.015)	1.00	H(0): accepted; not genotoxic
mTHPC+Laser		200	6	0.030 (0.017–0.035)	0.527	H(0): accepted; not genotoxic
4 Gy		50	10	0.200 (0.129–0.219)	0.00	H(0): rejected; sig. genotoxic
Control	Total of spots	100	26	0.260 (0.188–0.328)		
Laser		100	21	0.210 (0.142–0.283)	0.560	H(0): accepted; not genotoxic
mTHPC		200	35	0.175 (0.134–0.213)	0.164	H(0): accepted; not genotoxic
mTHPC+Laser		200	58	0.29 (0.244–0.331)	0.737	H(0): accepted; not genotoxic
4 Gy		50	61	1.220 (1.033–1.383)	0.000	H(0): rejected; sig. genotoxic

The exact 95% confidence intervals of binomial distribution are given in parentheses.

D. melanogaster has the potential for being used in additional preclinical studies in PDT. Since various forms of malignant tumour also occur in this insect that is so convenient to study, the authors have started a series of experiments to investigate the action of PDT on neoplasms using this model.

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