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ORIGINAL ARTICLE

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Photochemotherapy of experimental colonic tumours with intra-tumorally applied methylene blue

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Abstract *Introduction:* Phototoxicity of intra-tumoral injected methylene blue (MB^+) was studied in 48 experimental colonic tumours in comparison with photosan-3, Zn-phthalocyanine and tetrasulphanated ClAl-phthalocyanine. *Methods:* In mice, xenotransplanted subcutaneous tumours about 1 cm in diameter were treated photodynamically twice, with different sensitisers. The irradiation was performed at the sensitiser-specific wavelength, and a densitiy of 100 mW/cm^2 and a dose of 100 J/cm^2. *Results:* Light alone without sensitiser did not induce any effect in mice tumours. Surprisingly, Al-phthalocyanine could only be used for intratumoral injections because of toxic effects after intravenous applications in nude mice. Using MB^+ (1%), 75% of the tumours were destroyed by a single photodynamic treatment (PDT). In addition, toxicity of MB^+ was most intense when compared with Zn-phthalocyanine and photosan-3. However, after the second PDT, there was no statistically significant difference among these sensitisers. Dark toxicity of MB^+ (1%) could be well demonstrated by sufficient sensitiser incorporation without irradiation, which led to a stationary tumour volume up to 3 weeks after injection. *Conclusion:* Intra-tumoral MB^+ PDT is a potential treatment for inducing necrosis in vivo. With regard to tumour tissue, the selectivity of MB^+ is high and depends on a precise local injection of the dye.

Key words Methylene blue · Photodynamic therapy

Introduction

The medical interest in the use of light as a therapeutic tool has become increasingly important. Photodynamic therapy (PDT) is a promising treatment modality for inducing localised destruction of small areas of benign or malignant neoplasia of the gastrointestinal tract. It involves the systemic or topical administration of a photosensitiser, followed by subsequent light exposure. The wavelength of light, which activates the sensitiser, is usually emitted by lasers or appropriately filtered lamp sources. To obtain a maximum depth of tissue penetration, a strong absorption in the red or infra-red region is a major advantage. PDT results in the damage of tumour cells [1] and/or the destruction of tumour vascularisation [2, 3]. If the sensitiser accumulates, preferably in malignant tissue, a selective tumour therapy becomes possible that distinguishes PDT from other chemically based forms of cancer treatment [4, 5].

The principles of PDT are complex and include biochemical, physical and biological aspects [6]. In addition, the biological effects of cytotoxicity induced by light are different from those induced by heat. The substantial point has been the fact that some sensitisers selectively localise within the tumour cells by sensitiser retention. However, in solid extracranial tumours, the sensitiser concentration in the tumour and the surrounding tissue has rarely been reported to rise above 3:1 [4]. Also, a small difference in sensitiser concentration between the vascular stroma of normal and tumour tissue was observed [4].

Theoretically, high sensitiser concentrations in tumour tissue can be obtained by direct application of the photosensitiser into the tumour. However, the principles of sensitiser distribution are difficult and depend on the chemical properties of the dye.

Cationic molecules, such as thiazines, are under discussion because of their selective uptake by mitochondria of carcinoma cells [7]. In addition, lysosomes can act as a trap for cationic sensitisers [8, 9]. The thiazinium dye methylene blue (MB^+) is well known to generate singlet oxygen [10] and is a potential photonuclease [11]. Recently, benzophenothiazines, which are similar to MB^+, have been shown to be effective photosensitisers in murine sarcomas [12, 13]. However, it has been reported that direct tumour

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cell killing rather than destruction of the vasculature is the primary mode of tumour eradication [12].

The photodynamic mechanism of MB^+ is complex. In addition to the generation of singlet oxygen, it has been suggested that MB^+ mediates cell cytotoxicity via the generation of hydroxyl radicals, which change the intracellular Ca^{2+} homeostatic mechanisms [14]. This could be proved in cell cultures on a subcellular level by a non-linear redox reaction of MB^+ during light exposure [15].

The purpose of this study was to investigate the cytotoxiticy of MB^+ as a sensitiser in experimental colonic tumours. We have used this model system for the demonstration of PDT efficiency with 5-aminolevulinic acid [16]. The PDT efficiency of MB^+ was compared with phthalocyanines and photosan-3.

Materials and methods

Chemicals and laser properties

In this study, methylene blue 1% (MB^+, Neopharma, Germany) was used without further purification. MB^+ was injected directly into the tumour. The porphyrin drug photosan-3 (500 µg/mouse, Seehof Laboratorium, Germany), liposomal zinc phthalocyanine (ZnPc 0.03 mg/kg, Giba-Geigy, Switzerland) and the water-soluble tetrasulphanated chloro-aluminium-phthalocyanine ($ClAlS_4Pc$ 18 mg/kg, Cancer Research Centre, Moscow, Russia[1] was administered intravenously with isotonic sodiumchloride (NaCl 0.9%). $ClAlS_4Pc$ was also injected intra-tumorally (18 mg/kg) because of biological problems after intravenous application. The tumour irradiation was performed with an argon-laser pumped dye-laser at the sensitiser-specific wavelength, with a density of 100 mW/cm^2 and a dose of 100 J/cm^2.

Tumours and animals

All animal experiments were carried out according to our standards and were authorised by the responsible authorities (Regierungspräsidium Tübingen, AZ 37-9185.81-3). Human colonic tumour cells (adeno-carcinoma, G-3) were transplanted subcutaneously in both flanks of 50 female nude mice (NMRI nu-nu; body weight about 25 g). However, in two mice, tumour transplantation was not successful. When the tumour in the remaining 48 mice had reached a diameter of about 1 cm (usually after approximately 4 weeks), initially, PDT treatment was started in four groups of eight mice each. The tumours were fully vascularised and vital, except for the central region, where a slowly growing necrotic area was found. In the first group, MB^+ was injected intra-tumorally in one of both induced tumours. The dye was applied by means of several injections at various sites of the tumour, the total volume being 0.1 ml. The second tumour in the same mouse served as a non-treated control (Fig. 1). The other three groups were treated with one of the following sensitisers: ZnPc, photosan-3, and $ClAlS_4Pc$. These sensitisers were given intravenously and incubated for 24 h. In the $ClAlS_4Pc$ group, 50% of the mice died immediately after intravenous injection of the sensitiser (data not shown). As a substitute, $ClAlS_4Pc$ was injected directly into the tumour in an additionally xenotransplanted group of eight mice and incubated without any problems for 3 h. Before laser irradiation, the skin was carefully dissected to expose the subcutaneous tumour for subsequent treatment under general anaesthesia with Ketamin-ratiopharm (Ratiopharm, Germany).

[1] Experimental sensitiser, kindly supplied by Prof. Dr. Steiner Institute for Laser Technology in Medicine, Ulm

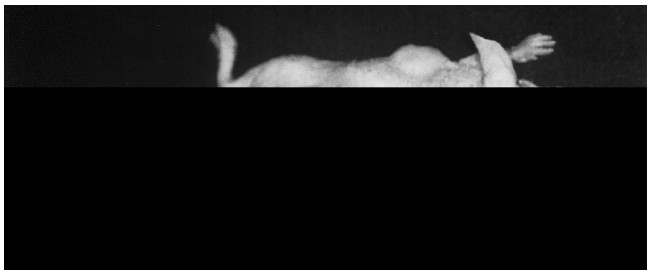

Fig. 1 Nude mouse with xenotransplanted tumours in both flanks. The tumour in the right flank served as control

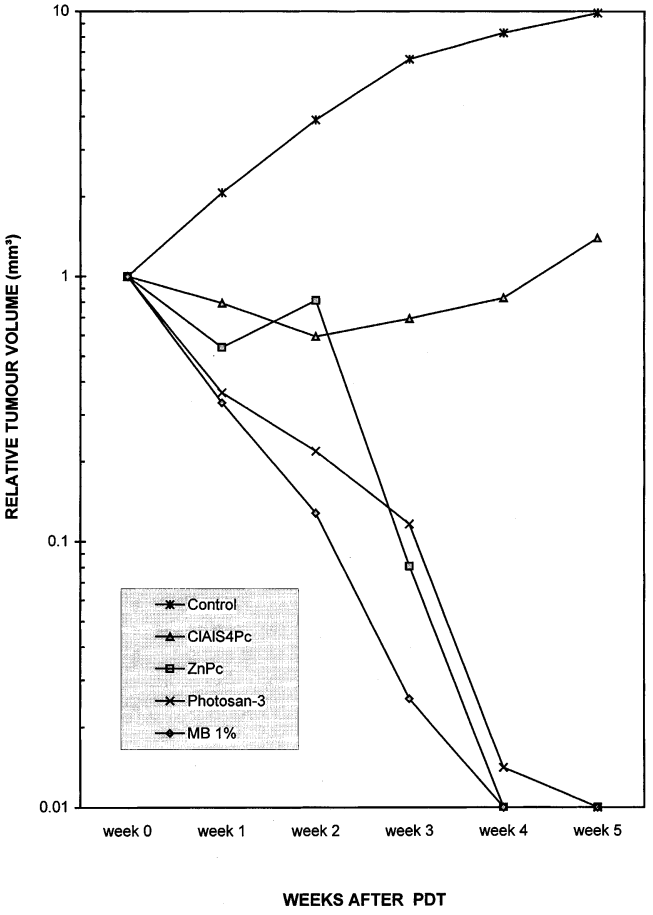

Fig. 2 Median of the tumour volume of the untreated and photodynamic treatment (PDT)-treated xenotransplanted colonic tumours in mice. The values of the different sensitisers were calculated relatively to the tumour volume at the beginning of the experiment (week 0)

The MB^+-stained tumours were irradiated after an incubation period of 60 min at a wavelength of 665 nm. The ZnPc-, photosan-3- and $ClAlS_4Pc$-sensitised tumours were irradiated at 670 nm, 630 nm and 672 nm, respectively. If the tumours were not completely destroyed after the first PDT, we performed a second PDT 2 weeks after the first treatment. The treatment modalities in the different groups remained the same, as described above. After 5 weeks observation time, all mice were sacrificed via an overdose of anaesthesia, since the control tumours became too large (up to tenfold greater than the initial volume at week 0, Fig. 2).

In a second experiment, we tested dark and light toxicity of MB^+ in two groups of eight tumour-bearing mice, each with a single intra-

Table 1 Tumour volume of experimental colonic tumours after photodynamic treatment (PDT) with different sensitisers

Time after PDT	Tumour volume (median and range, mm^3)				
	Photosan-3 (i.v) ($n = 8$)	$ClAlS_4Pc$ intra-tumoral ($n = 8$)	ZnPc (i.v) ($n = 8$)	MB^+ (1%) intra-tumoral ($n = 8$)	Untreated controls ($n = 32$)
Week 0	64 (45–150)	59 (14–105)	37 (18–41)	40 (123–16)	45 (24–55)
Week 1	51 (36–130)	48 (28–128)	20 (5–34)	13 (63–4)	85 (60–130)
Week 2	31 (3–98)	33 (7–136)	30 (0–90)	5 (0–16)	154 (84–195)
Week 3	17 (0–162)	40 (9–119)	3 (0–92)	1 (0–9)	255 (180–310)
Week 4	2 (0–54)	51 (14–119)	0 (0–78)	0 (0–50)	310 (188–360)
Week 5	0 (0–96)	88 (18–187)	0 (0–105)	0 (0–102)	420 (260–530)

Table 2 Tumour volume in experimental colonic tumours after methylene blue (MB^+) treatment. *PDT* photodynamic treatment

Time after PDT	Tumour volume (median and range, mm^3)		
	MB^+ (1%) PDT Intra-tumoral ($n = 8$)	MB^+ (1%) intra-tumoral without irradiation ($n = 8$)	Irradiated control tumours without MB^+ ($n = 16$)
Week 0	32 (24–41)	23 (6–30)	35 (24–50)
Week 1	4.9 (3–9)	21 (6–34)	73 (39–110)
Week 2	0 (–)	24 (2–41)	133 (84–180)
Week 3	1.3 (0–9)	27 (12–58)	220 (128–297)

tumoral MB^+ injection. One group was irradiated at a wavelength of 665 nm; the other group was not exposed to light. The control tumour on the other flank tumour was not incubated with any sensitiser, but was irradiated at 665 nm. The observation period in these two groups was 3 weeks.

The therapeutic response was quantified by measurements of the tumour volume atweekly intervals. Tumour shape can be estimated by half ellipsoid; therefore, tumour volume was calculated by Vol. = $2/3 \cdot \pi \cdot a \cdot b \cdot c$. To eliminate differences of tumour growth, the volume of each tumour was normalised to the value at the beginning of the experiment (week 0). The statistical significance of the therapeutic effect of different treatment modalities was examined by unpaired two-sided Wilcoxon's test. We compared the PDT effect of MB^+ (1%) with the dark toxicity of MB^+ (1%) and the PDT effects of each of the sensitisers (Tables 1, 2).

Since irradiation of the deep-stained tumours during PDT was suspected to induce thermal effects, the surface temperature was measured during PDT by an infrared camera in all sensitiser experiments. Dehydration of the tissue was avoided by applying small amounts of isotonic sodium chloride given at intervals of 1 min.

Results

The sensitiser application and photodynamic therapy was well tolerated by the mice with the exception of the mice with intravenous application of $ClAlS_4Pc$, which induced shock in 50%. Each treatment modality proved to be highly effective ($P < 0.01$ in comparison with the controls from the first to the fifth week).

The phototoxic effects of the different sensitisers are shown in Fig. 2. Clinically, the PDT effect was best with the sensitiser MB^+. In this group, tumour volume decreased to a median ratio of 13:85 in comparison with the control tumours during the first week. At the end of the fourth week and the second PDT, the median ratio was 0:310 due to the treated tumours. In seven of eight animals, a complete macroscopic regression of the tumours was found at the end of the third week. However, one tumour in this group recovered in the fifth week. In one of eight animal tumours, volume could be reduced to 7% of the initial volume, but did not completely vanish. Histological examination of the two residual tumours proved complete destruction of the main tumours. However, small rests of vital tumour cells initiated new tumour growth (Fig. 3).

PDT with ZnPc and photosan-3 was demonstrated to be an effective tumour treatment with high statistical significance compared with the untreated tumours ($P < 0.01$). Both sensitisers developed the strongest cytotoxicity after the second PTD. In the ZuPc group four of eight and in the photosan 3 group five of eight mice, the xenotransplanted tumours could be destroyed after the third week (Table 1). In the rest of animals, tumour growth could be delayed, but not stopped. In comparison with MB^+, PDT with ZnPc differed statistically in the second week ($P < 0.05$). Photosan-3 showed significant differences in comparison with MB^+ in the third week ($P < 0.05$).

PDT with intra-tumorally applied $ClAlS_4Pc$ was completely different from MB^+ over the whole experiment and did not destroy any of the tumours. Tumour volume could be reduced up to the third week and then slowly increased to a mean of 94 mm^3 in comparison with 62 mm^3 at the beginning of the experiment.

Irradiation of the experimental tumours alone, without intra-tumoral MB^+ injection did not show any macroscopic cytotoxicity or inhibition of tumour growth and was statistically not different from the untreated controls. However, when MB^+ (1%) was injected, tumour growth was inhibited or at least delayed for up to 3 weeks after injection (Fig. 4; Table 2). The difference when comparing with the untreated tumours was highly significant ($P < 0.01$). Irradiation of the incubated tumours initiated macroscopical tumour destruction. This experiment demonstrated the additional effect of phototoxicity of MB^+ with a maximum in tumour debulking 2 weeks after PDT. The phototoxic effect was also very different when compared with the dark toxicity of MB^+ ($P < 0.01$).

[2] a, b, c are the semiaxis of the tumour ellipsoid

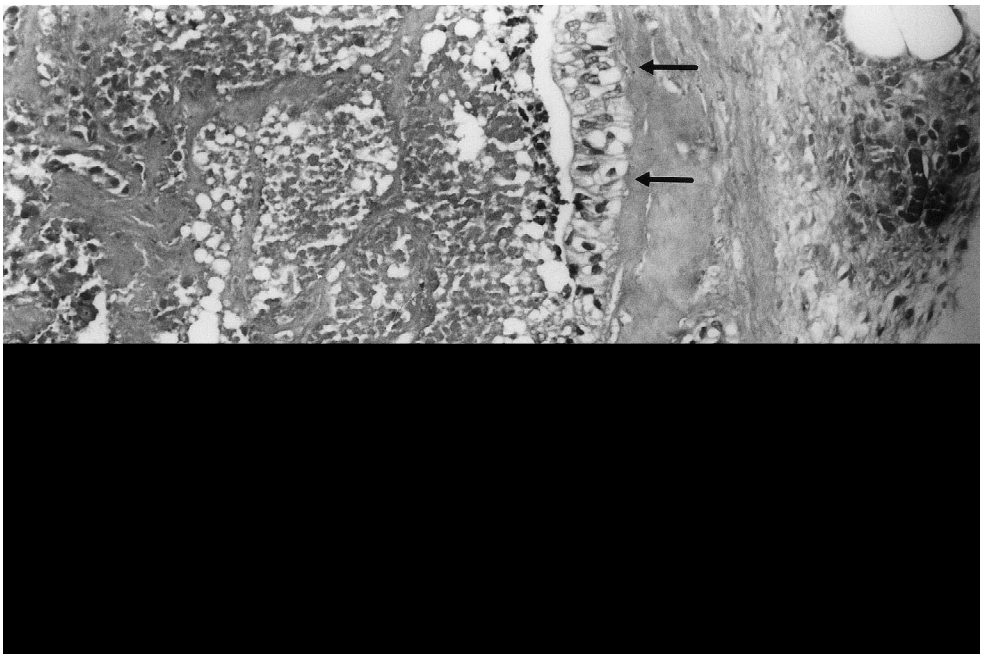

Fig. 3 Microscopical examination of a residual tumour. The area shows inactivated cells, but also small amounts of vital (→) tumour cells with rests of interstitial methylene blue (MB^+) and fibrotic tissue

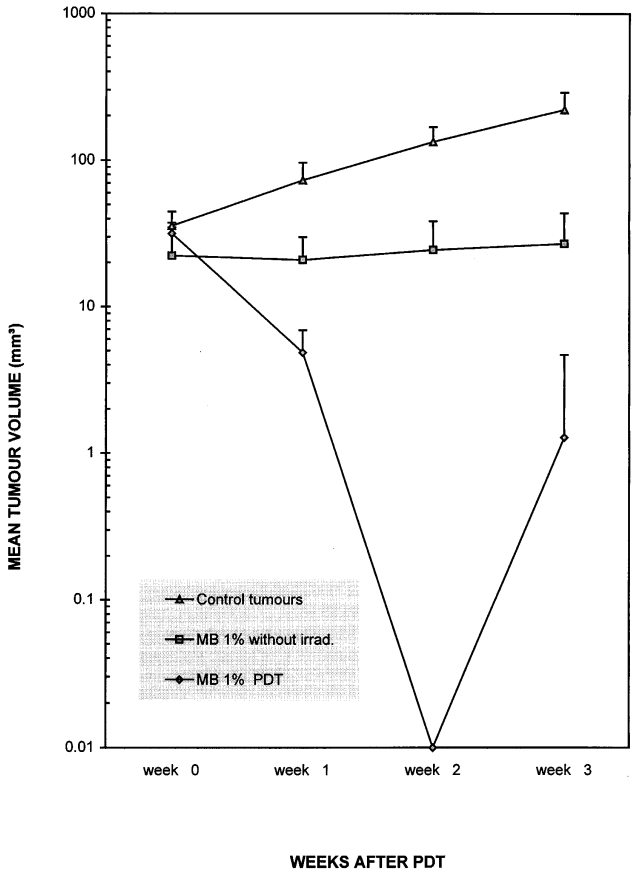

Fig. 4 Mean tumour volume and standard deviation of untreated controls in comparison with 1% methylene blue (MB^+)-incubated, but not irradiated, tumours as well as 1% MB^+-incubated and -irradiated (photodynamic treated) tumours

The increase in temperature during PDT was expected to be maximal at the surface where the irradiance was highest. The increase in temperature during PDT was remarkably low and could be estimated to be a maximum of 1 °C, compared with surrounding muscle tissue.

Discussion

This work describes cytotoxic and phototoxic properties of MB^+ in an experimental colonic tumour compared with those properties of ZnPc, $ClAlS_4Pc$ and photosan-3. As we could show, the toxic effect of MB^+ involves darkness – and light-induced mechanisms. MB^+ is characterised by a low toxicity and high tumour specificity [17–19]. As an important vital dye, it can be used for intraoperative in vivo staining of different tumours [20, 21]. As a redox indicator, MB^+ is rapidly reduced to stainless MBH, which is not photodynamically active when given to the organism intravenously [22]. Therefore, PDT with systemically applied MB^+ failed [23]. However, local administration of MB^+ was successful in the intra-luminal treatment of inoperable oesophageal tumours [24] and in the topical treatment of psoriasis [25].

MB^+ (1%) injected directly into tumours was most effective, leading to a stationary tumour volume up to 3 weeks after injection. These results are in accordance with those reported by Lewis [26], who fed sarcoma-bearing mice with Nile blue. They found up to a 20-fold inhibition of sarcoma growth in comparison with the controls. They also described the extent of tumour retardation to depend on the kind and amount of Nile blue fed to the host. Jacobi [27] treated patients with inoperable cancer with pills containing MB^+. He claimed that the

treatment prolonged their lives, although it failed to cure the disease.

Additional irradiation of MB^+-incubated tumours increased cytotoxicity up to complete destruction of the tumours. The PDT compared with 5-ALA, which we recently reported [16], the photochemically induced tumour necrosis was markedly enhanced. Although PDT with MB^+ during the first 3 weeks tended to be more effective than with ZnPc or photosan-3, the significance of the differences was weak. The increasing cytotoxicity after the second PDT with these sensitisers may be explained by accumulation of these long acting chemicals. $ClAlS_4Pc$, known as a long-acting sensitiser in organisms, could not be proved in PDT as reported [28]. The failure of this drug in mice after intravenous application could not be explained.

The relationship between dark toxicity and phototoxicity of MB^+ is well shown in the work of Menezes et al. [29]. In experiments on strains of *Escherichia coli,* MB^+ induces a type of prelesion in DNA that transforms into a single-strand break under alkaline conditions. These prelesions were reversible if the dye was washed out, but became irreversibly stable after illumination with white light. Although thermal effects were excluded in our experiments, hyperthermia was shown to act synergistically with MB^+ in the case of *Escherichia coli* [30]. The role of temperature seems to be that of facilitating the incorporation of the dye. In clinical routine, this synergism may be used to increase the PDT effect.

PDT with the sensitiser MB^+ overcomes the problem of an adequate sensitiser concentration in the tumour area. Intratumoral MB^+ injection does not require further preparation. However, the mechanisms of distribution and localisation of MB^+ after injection still remain unknown. With regard to tumour tissue, the selectivity of MB^+ is high and depends on a precise local injection of the dye. This technique is more appropriate for early and small tumours than for advanced ones. Repeated performance of PDT is possible, and we recently demonstrated this in the case of oesophageal tumours [31]. Moreover, MB^+ was shown to be of potential use in PDT, not only for cancer, but also against microbial cells. Millson et al. [32] describe the successful, photochemically induced killing of *helicobacter pylori* on host mucosal epithelium following topical administration of MB^+.

The perspectives for an introduction into the clinical setting seem to be promising, although it has to be emphasised that this technique has not yet advanced beyond the experimental stage. Further studies are needed to define the clinical value of PDT in the management of tumour therapy.

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