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Photodynamic therapy using methylene blue in lung adenocarcinoma xenograft and hamster cheek pouch induced squamous cell carcinoma

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ABSTRACT

Background: Photodynamic therapy (PDT) is used to treat early proximal bronchial cancer during a flexible bronchoscopy. The technique relies on the excitation of a photosensitizer by an appropriate wavelength, which is delivered into the bronchus in close contact with the tumor.
Objective: To assess methylene blue (MB) as a PDT agent for the treatment of respiratory tract cancer in animal models.
Methods: MB-induced PDT was performed on 7 subcutaneous NCI-H460 lung adenocarcinoma xenografts in nude mice and 9 induced squamous cell cancer in the hamster cheek pouch model. In mice, PDT was carried out on right-sided tumors after intratumoral injection of methylene blue 1% (w/v) and illumination at 630 nm at 200 J/cm (Diomed PDT 630), with the left tumor used as control (illumination alone or MB alone). The tumoral volume was assessed before and 15 days after PDT.
Results: Fourteen xenografts were treated in mice, including seven treated with MB-PDT, producing a 52% mean tumor volume regression (1568 mm^3 *vs.* 544 mm^3) compared to seven control cases in which tumor volume increased ($p = 0.007$; Mann-Whitney test). Nine cheek pouch induced carcinomas were treated in the hamster group, with a mean volume decrease of 85.8% (from 44.8% to 100%) (initial mean volume = 210 mm^3 *vs.* post PDT mean volume = 97 mm^3). Histology analysis showed 4/9 complete responses.
Conclusion: Intratumoral MB appears efficient as PDT agent for cancer treatment in animal models. Further studies are needed to assess the safety and efficacy of MB-associated PDT for the treatment of lung cancer in humans.

1. Introduction

Antitumor photodynamic therapy (PDT) is a treatment technique based on the association of photosensitive molecules (*i.e.*, photosensitizer) and an appropriate light wavelength for the photosensitizer [1]. The action of the light, at the correct wavelength, induces the formation of singlet oxygen $^1O_2$ in the cell that absorbed the photosensitizer, in the presence of oxygen. A singlet oxygen is a powerful oxidant that reacts with numerous cellular constituents, leading to direct cellular damage such as necrosis or apoptosis [2–4]. In humans, the photosensitizer is usually injected intravenously and concentrates in the tumor cells. Apart from potential undesirable side-effects, the principal inconvenience of systemic administration of the photosensitive agent is represented by the variable accessibility and concentration of the agent in the target cells.

Methylene blue (MB), a powerful fluorophore, possesses photodynamic properties at 630 nm. Photo-excited at 200 J/cm^2, MB generates oxygen singlets creating the photodynamic effect [5]. In an earlier study, PDT using local application of MB was successfully tested in three patients with inoperable esophageal cancer [6]. In nude mice with colic tumor cell xenografts, this treatment

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was shown to be efficacious with a complete response rate of 79% after one course of therapy [7].

Our hypothesis was that the local application of MB coupled with phototherapy was also efficacious in the treatment of cancerous lesions of the respiratory tract. The objective of our study was to demonstrate the efficacy of PDT coupled with the local application of MB, using two tumor models, the bronchial cancer cell xenografts in nude mice and the hamster cheek pouch cancer model.

2. Material and methods

2.1. Study design

The study was designed to demonstrate the potential effect of methylene blue as a photosensitizer in cancer of the respiratory tract. First we used the NCI-H460 lung cancer cell line, which is derived from pleural effusion of a patient with large cell lung carcinoma [8–11], for the xenograft model in nude mice. Secondly, we used the cheek-pouch model, which represents a carcinogen-induced cancer model that recapitulates the natural evolution of a squamous carcinoma, from reserve cell hyperplasia to invasive carcinoma [12,13].

2.2. Animals

The animals were housed in groups of three in pre-sterilized cages placed in a positive/negative ventilation housing system (PNCT3HR30EZ model, Allentown Inc., Allentown, New Jersey, USA). The ambient temperature was 22 °C. The circadian rhythm was maintained. The animals had unlimited access to food and water. The study started following 5 days of acclimatization.The animal experiment was undertaken in accordance with ethics rules of the University of Rouen and those of the regional committee for animal ethics (N/03-12-10/25/12).

2.2.1. The mice

Twelve female nude mice (Nu/Nu, Janvier, Le Genest Saint Isle, France), weighing 10 g and aged 6 weeks, were used for the NCI-H460 cell xenografts. Each mouse received, under isoflurane gas anesthesia (Abbott Laboratories, Chicago, USA), a subcutaneous injection of NCI-H460 cells in each flank. Each injection (100 μl) contained 3.10^6 cells/100 μl.

2.2.1.1. NCI-H460 cell preparation. The cells were cultured in a RPMI 1640 medium containing 10% fetal bovine serum. The culture medium was enriched with antibiotics: penicillin (500 U/ml), streptomycin (0.1 mg/ml), and neomycin (0.2 mg/ml). The cells were conserved at 37 °C in a 5% CO_2 atmosphere.

The adherent NCI-H460 cells were washed with PBS. They were then exposed to a solution of 0.25% trypsin and 0.2% EDTA in an incubator for 5 min. The trypsinization was stopped *via* a medium containing 10% fetal bovine serum.

The cells in suspension were centrifuged at 500 g and 4 °C for 4 min. The supernatant was withdrawn, and the cells were re-suspended in a medium containing 10% fetal bovine serum. The cells were re-suspended at a concentration of 3.10^6/100 μl in a RPMI medium.

2.2.1.2. Treatment of the xenografts in the mice. The lesions that developed on the right flank received a photodynamic therapy (MB-PDT = local methylene blue + illumination), while the lesions on the left flank received either a local injection of MB alone or, solely, illumination using a diode laser.

2.2.2. The hamsters

2.2.2.1. Carcinogenesis of the hamster's cheek pouch. Carcinogenesis was obtained *via* an implant impregnated with dimethylbenzanthracene (DMBA) (Sigma Aldrich, Saint-Louis, USA) according to the technique described by Heller [12]. The implant was positioned in the right cheek pouch under general anesthesia *via* intraperitoneal injection of a mixture of ketamine 30 mg/Kg (Laboratoire Merial, Lyon, France) and xylazine 10 mg/Kg (Laboratoire Bayer, Leverkusen, Germany) following isoflurane gas induction (Abbott Laboratories, Chicago, USA).

According to the method of Wani [13], 4 weeks following implantation, the right cheek pouches were everted and stained with a solution of arecaidine (Sigma Aldrich Laboratory, Saint-Louis, USA) at 1 g/l of mineral oil (Aguettant Laboratory, Lyon, France), with three applications per week.

2.2.2.2. Treatment of the cheek cancer lesions. Nine male Syrian hamsters, (Golden Syrian Hamsters, Janvier, Le Genest Saint Isle, France), aged 6 weeks (100 g), were included in the protocol of MB-PDT. The methylene blue was injected into the cancer lesion in the right cheek pouch followed by illumination with the phototherapy device.

2.3. The photodynamic therapy material

The device consisted of a laser source DIOMED 630 PDT (DIOMED, Cambridge, Great Britain) and a fiberoptic cylindrical diffuser (PDT-DCYL410 and 420, DIOMED, Cambridge, Great Britain). The silica fiber had a proximal connection for a conventional SMA connector to the laser source and a distal cylindrical diffuser tip, designed to diffuse uniform light energy along the length of the distributor.

2.4. The photodynamic therapy

The treatment was administered under general anesthesia *via* intraperitoneal injection of a mixture of ketamine 30 mg/Kg (laboratoire Merial, Lyon, France) and xylazine 10 mg/Kg (Bayer, Leverkusen, Germany) following isoflurane gas induction (Abbott Laboratories, Chicago, USA). For mice, the treatment was started when the tumor that developed in either one of the flank had reached a minimal initial volume of 500 mm^3. All hamsters presenting with macroscopic cheek pouch lesions were sampled for histological analysis to ensure that all lesions corresponded to invasive carcinomas.

On Day 0, the lesions were photographed and measured. The initial tumor volume of each lesion was calculated (initial tumor volume $VTi = A \times B^2/2$, where A was the largest diameter and B the smallest diameter).

According to the size of the lesion, 50–150 μl of 1% (v/w) methylene blue (concentration 31.3 mM), (Aguettant laboratory, Lyon, France) was injected into the cancerous lesion. The methylene blue was injected gently using a 29 Gauge needle and 0.5 ml syringe (Penta®—fine R.0, Campli, Italy) in the center of the tumor, either in the mouse and the hamster models. The injection (50–150 μl) induced immediate blue coloration of the tumor and was stopped when the whole cutaneous surface of the lesion turned blue, in order to avoid spreading of the dye beyond the visible lesion.

We used 20 mm long cylindric diffusers to treat the lesions in the mice and 10 mm cylindric diffusers for the lesions in the hamsters. After fiber calibration, the total power delivered by the diffusers was set to 600 mW or 400 mW, for the 20 mm and 10 mm cylindric diffusers, respectively. The total illumination time was 500 s and 750 s, for hamster and mice's lesions, respectively. The PDT fiber, delivering 200 J/cm optical energy density, was applied on the sur-

Table 1
Evolution of nude mice H-460 xenograft tumors after treatment with methylene-blue associated photodynamic therapy, illumination alone or intra-tumoral methylene blue injection alone.

Nude mice H-460 xenografts	TUMORAL LESION	TUMOR VOLUME (mm^3)			
		Day 0	Day 5	Day 10	Day 15
Mouse 1	RIGHT: PDT + MB	3564	1270.5	4512.5	1666
	LEFT: MB alone	3726	4151.5	15,300	20,808
Mouse 2	RIGHT: PDT + MB	1440	486	320	907.5
	LEFT: MB alone	1656	3901.5	4536	907.5
Mouse 3	RIGHT: PDT + MB	2944	352	256	405
	LEFT MB alone	1267.5	4630.5	4630.5	4630.5
Mouse 4	RIGHT: PDT + MB	864	486	544	Dead
	LEFT: Illumination alone	196	196	7488	
Mouse 5	RIGHT: PDT + MB	320	220.5	100	320
	LEFT: Illumination alone	2025	3564	4600	8437.5
Mouse 6	RIGHT: PDT + MB	1960	1089	29.9	Dead
	LEFT: Illumination alone	320	1859	778.2	
Mouse 7	RIGHT: PDT + MB	1568	87.5	750	Dead
	LEFT: Illumination alone	1666	1296	800	

Table 2
Evolution of Hamster cheek-pouch tumors after treatment with methylene-blue associated photodynamic therapy.[a]

Hamster cheek-pouch	TUMOR VOLUME (mm^3)			
	Day 0	Day 5	Day 10	Day 15
Hamster 1	56	22.5	6	0
Hamster 2	1331	735	Euthanized	
Hamster 3	18	0.5	0.5	Sacrificed[b]
Hamster 4	75	22.5	18	Sacrificed[b]
Hamster 5	22.5	1	0.75	Sacrificed[b]
Hamster 6	352	56	112.5	Sacrificed[b]
Hamster 7	0.5	0.25	0	0
Hamster 8	0.75	5	10	0
Hamster 9	40	13.5	0	0

Abbreviations: PDT; Photodynamic therapy; MB; Methylene Blue.

[a] Only the right cheek pouch in each hamster was considered for the tumoral induction, and therefore, only this cheek pouch was treated with methylene-blue photodynamic therapy.

[b] Hamsters 3, 4,5 and 6 have been sacrificed after evaluation at Day 10 in order to be able to analyse the histological damages of MB-PDT on the residual lesions, as the tumor volume was decreasing. Complete response was obtained in hamster 1 at Day 15. Hamster 2 has been euthanized between Day 5 and Day 10 for ethical reason.

face of the lesion and moved on all of the tumoral surface, with illumination time of 50 s on each spot, for a total of 750 s or 500 s.

2.5. Therapeutic evaluation

The treatment of the lesions was evaluated on Day 5, 10, and 15.

Each lesion was photographed and measured as described above, following isoflurane gas induction, allowing for post-treatment tumor volume assessment on Day 5, 10, and 15. The final tumor volume on Day 15 was called the Vtf. The aspects of the lesion were also noted (necrotic, hemorrhagic, or indurated).

At the end of the protocol, the animals were euthanized by an intra-cardiac injection of thiopental (1 ml, Hospira laboratory, Lyon, France). The lesions were excised, measured, weighed, and conserved in formalin solution (4%, Labonord laboratory, Templemars, France) for 48 h before histological examination.

2.6. Statistical analysis

The relative variation of tumor volume ($\Delta V = [Vtf\text{-}VTi]/VTi$) was compared between the group of lesions treated with PDT and the control lesions, in mice, using the non-parametric Mann-Whitney test. A p value <0.05 was considered significant.

3. Results

3.1. The nude mice

The 12 nude mice developed neoplastic lesions on the two flanks following the xenograft of NCI-H460 cells. Five mice died during the course of the experiment, without evaluation of the therapeutic effects. The cause of the death occuring before PDT cannot be precised. Seven mice remained evaluable for efficacy testing of the photodynamic therapy (PDT) on their right lesion. Fifteen days after the xenograft, the initial mean volume was 1679 mm^3 (median 1504 mm^3). Among them, as control, 3 animals received an injection of methylene blue alone on their left lesion, and 4 animals received illumination alone on their left lesion.

3.1.1. Macroscopic analysis

Table 1 show the evolution of tumor volume during follow-up after treatment administration, either with MB-PDT, or with MB or illumination alone.

Two days after the treatment with MB-PDT, 2 mice developed skin necrosis on the treated lesion. Spontaneous healing was observed before Day10. Three mice died on Day 10 and Day 11 after MB-PDT (Table 1).

The right lesions treated with MB-PDT regressed 52% on average (1568 *vs.* 544 mm^3).

The relative variation of tumor volume (ΔV) differed significantly between the lesions treated with MB-PDT and the control lesions (−73% *vs.* +76%, p = 0.007, Mann-Whitney test) (Table 3).

3.1.2. Histological analysis

Histological analysis revealed cancerous lesions in all cases. Necrotic areas were found in the samples taken both from the right lesions (treated with PDT), and the left lesions (controls).

3.2. The hamsters

The initial median volume of the tumor that developed in the cheek pouches was 40 mm^3 (min = 0.5 mm^3; max = 1331 mm^3). Seven lesions presented with an initial volume less than or equal to 75 mm^3 (Table 2).

No animal lost weight during the treatment period, and there were no complications such as death or infection.

3.2.1. Macroscopic analysis of the lesions treated with MB-PDT

The treated lesions presented with a necrotic appearance, at times hemorrhagic as of Day 5. The tissue adjacent to the treated lesion did not show any abnormality.

Table 2 shows tumor volume evolution of the cancerous lesions of the cheek pouch following treatment, from Day 0 to Day 15. The size of the lesions decreased 85.5% on average (95%CI = [45–100]), and 94.2% (95%CI = [76–100]) for lesions less than 75 mm^3. One healthy cheek pouch underwent MB-PDT, as control, showing a necrotic zone in the area in which the treatment was administered.

3.2.2. Histological analysis

We found residual cancer cells in 5 out of 9 animals treated, while a complete histological response was observed in the other 4 animals. As shown in Fig. 1, different histological abnormalities were identified: areas of vascular necrosis, fibrosis, and sub-acute inflammation.

Healthy cheek pouches treated with PDT also showed alterations at the histological level, notably inflammatory infiltrates with areas of suppurative necrosis and diffuse muscular alterations (Fig. 1C).

4. Discussion

Methylene blue, used *in situ* by intratumoral injection, and associated with an immediate illumination of 630 nm at 200 J/cm, appears to be a powerful photodynamic agent. In this study, it was able to induce a significant volume reduction of the cancerous lesions treated, whether it was subcutaneous adenocarcinoma xenografts, in mice (in 6 out of 7 treated lesions), or cancerous lesions induced in the hamsters' cheek pouch, in which histological complete response has been obtained in 4 out of 9 animals.

We observed a 73% decrease in tumor volume for the lesions treated with photodynamic therapy at 630 nm and 200 J/cm coupled with an intratumoral injection of MB 1%. These results were consistent with those obtained by Orth in nude mice [7], despite the fact that the size of the lesions (320–3564 mm^3) was more heterogeneous and larger in our study, and that the amount of energy delivered was 200 j/cm, *vs.* 100 j/cm delivered in Orth's study. In spite of this difference, we did not note any irreversible or lethal side-effects following this treatment, with 2 mice showing skin necrosis in tumoral areas, followed by complete healing in less than 8 days. In the hamsters showing complete response, the histological analysis of the treated tumors revealed inflammatory infiltrates as well as areas of tissue/vascular necrosis and chorion fibrosis compatible with a photodynamic effect identical to that obtained with the classical photosensitizing agents, such as porfimer sodium [5].

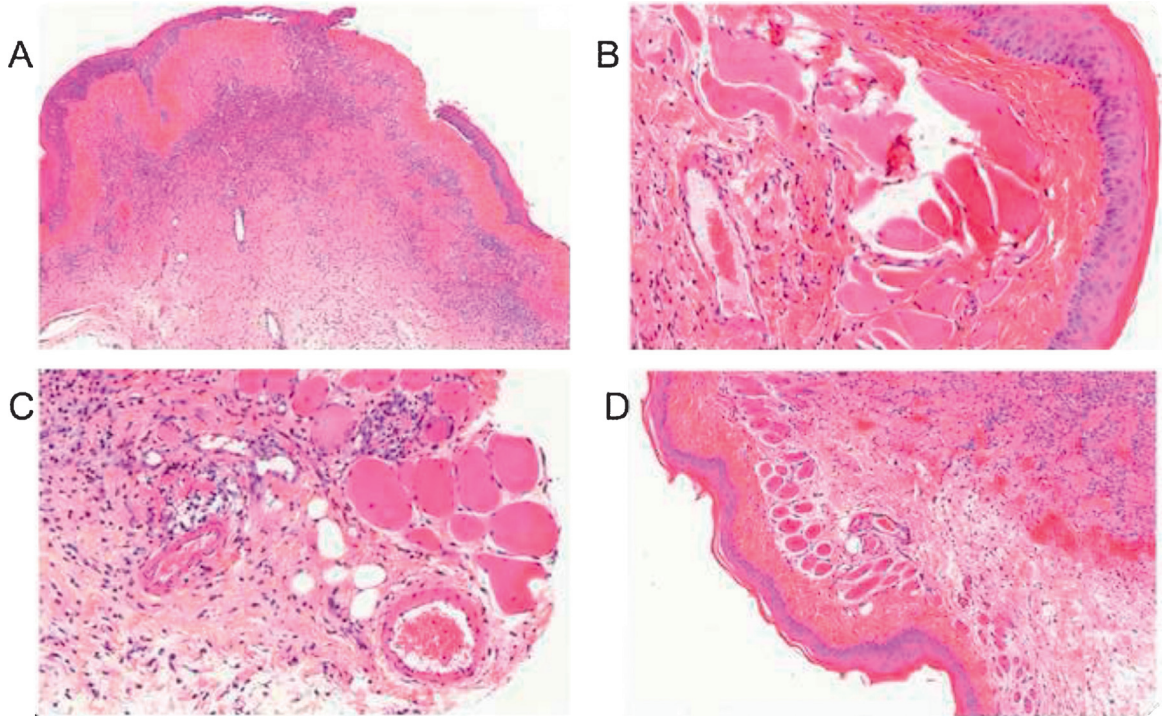

Fig. 1. Histological slices from hamster's cheek pouch after PDT (all animals were euthanatized on Day 15). A to C: squamous carcinomas after methylene blue-induced PDT; A/epithelial ulceration (X5 magnification); B/subepthelial fibrotic changes (X20); C/vascular necrosis (X20); D: modification of healthy control pouch after methylene blue-induced PDT showing inflammatory infiltrates with areas of liquefactive necrosis with pus formation and diffuse muscular alterations (X10).

Table 3
Evolution of xenograft mean tumor volume (mm^3) between Day0 and Day15 according to the treatment received (n: number of mice).

	MB-PDT	MB alone	Illumination alone
Day0	1808.6 (n = 7)	2216.5 (n = 3)	1051.7 (n = 4)
Day5	570.2 (n = 7)	4227.8 (n = 3)	1728.7 (n = 4)
Day10	1191.6 (n = 7)	8155.5 (n = 3)	3416.5 (n = 4)
Day15	415.6 (n = 4)	10818.8 (n = 3)	8437.5 (n = 1)

Abbreviations: MB-PDT: Methylene blue—associated photodynamic therapy; MB: methylene blue.

Previous studies have shown that direct intra-tumoral injection of the dye results in high intra-tumoral concentration of the sensitizer. In their study, Mannino et al. [14] demonstrated a similar tumoral uptake according to intra-tumoral or intra-veinous administration of a sensitizer (m-THPC (Temoporfin)), and the absence of extra-tumoral spreading of the dye. In our study, the evaluation of the dye distribution relies on the visual appreciation of the methylene blue on the tumoral surface. Because MB coloration disappears rapidly from the tumor after treatment, the distribution of the dye in the inner side of the tumor cannot be evaluated at the time of residual tumor excision. Therefore, the homogeneity of the photosensitizer distribution into the tumor cannot be controlled, which represents a limitation of the study, and may explain why no complete response was obtained in the mouse model.

The technique used in the present study involves the direct injection of the dye in the center of the tumor until the whole surface of the lesion turned blue, which also rises the question whether a mechanical effect due to the injected volume of the dye may have affected the tumor growth. This appears improbable in the present study as none of the tumor injected with MB without illumination showed a decrease in tumor volume with time. Five mice died before the PDT treatment and 3 mice died between D10 and D15 after treatment (Table 1). The delay between MB injection and death suggests that death were not due to an immediate toxic effect of the MB injection, but rather to the tumoral progression of non-treated (*i.e.* control) lesion. Altogether, this suggests that the injection of the dye into the lesion did not affect the tumor by a reduction of the tumoral vascular supply due to compression or by a direct toxicity effect.

As far as healthy tissue is concerned, treatment with MB-PDT resulted in histologically confirmed necrotic lesions, consistent with those observed with PDT associated with photosensitizers systemically applied [15]. This indicates that MB-PDT may have non-specific effects on cancer, compared to the surrounding tissue.

On the other hand, PDT using intratumoral MB may have several advantages: (a) because methylene blue is applied locally, its utilization would not require the skin protection measures needed for UVA-UVB and visible sunlight exposure, as with most systemic PDT agents; (b) the local application may allow for better access of the photosensitizing agent to the tumor; (c) the cost of methylene blue is currently very low, and methylene blue would be even more cost-effective for the health system since it would not require any adjunct prescriptions (protective creams, hospitalization, *etc.*). However, as the intratumoral homogeneity distribution of the photosensitizer maybe critical, the evaluation of efficacy and of potential advantages of local utilization of MB-PDT in human tumors will require specific dedicated studies.

For future use in humans, the intratumoral injection of MB implies a precise localization of the treatment areas, as well as a technique of application that would limit the diffusion of the dye to the pathological areas. This strategy could be feasible for the proximal respiratory tract where the lesions could be precisely detected and targeted for dye injection, using endoscopic techniques able to precisely localize the lesions, such as autofluorescent endoscopy or Narrow Band imaging [16]. MB could also be injected safely into peripheral lung nodules using navigational bronchoscopy. This has been performed for subpleural localisation of small tumors before thoracoscopy [17] In addition, methylene blue has been succesfully applied to the *in vivo* microscopic imaging of distal, intraparenchymal lesions of the lungs *via* the probe-based confocal laser endomicroscopy technique and navigational bronchoscopy, suggesting that the dye may also be used for the PDT treatment of distal lung tumors [18].

Altogether, our results suggests that intratumoral methylene blue for PDT of superficial bronchial cancers in humans could be feasible. Evaluation of efficacy and of potential advantages of local utilization of MB-PDT (for example avoidance of skin protection measures, better access of the photosensitizing agent, or cost-effectiveness) will require specific dedicated studies.

5. Conclusion

In these experimental models, photodynamic therapy coupled with the intratumoral injection of methylene blue appears to be an efficacious treatment for the local management of cancer lesions. Further studies in humans are needed to assess its *in vivo* efficacy for the treatment of both superficial bronchial cancers, and distal lung cancers in inoperable patients.

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