

# Methylene Blue in Place of Acridine Orange as a Photosensitizer in Photodynamic Therapy of Osteosarcoma

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**Abstract.** We recently established a unique therapeutic modality for musculoskeletal sarcomas, combining acridine orange (AO) with photodynamic surgery (PDS), photodynamic therapy (PDT) and radiodynamic therapy (RDT); excellent results were obtained in the inhibition of local tumor recurrence after intralesional excision. However, AO is not yet approved for clinical use and intravenous injection. Therefore, methylene blue (MB), which has a very similar chemical structure to AO and is already in clinical use for other diseases, was investigated. *In vitro* studies using mouse osteosarcoma (LM8) cells revealed that MB-PDT had a strong cytotoxic effect and that MB was not radiosensitive, showing no effect in RDT. *In vivo* studies showed that MB did not specifically accumulate in mouse osteosarcoma tissue and that it did not inhibit tumor growth. MB is not a better photosensitizer than AO in PDS, PDT and RDT for osteosarcoma.

It has been reported that photodynamic therapy (PDT) is an effective means of inhibiting local tumor recurrence in many early-stage, superficial types of cancer, such as skin (1-3), esophageal (4, 5), bronchial (6, 7) and oral cancer (8-12), but not in deep sarcomas. However, we recently established a quite unique therapeutic modality comprising photodynamic surgery (PDS), photodynamic therapy (PDT) and radiodynamic therapy (RDT) using acridine orange (AO) for musculoskeletal sarcomas (13-24). Basic research data (13-17, 21, 22, 24) and preliminary outcomes of clinical trials (18-20, 23) of this therapy have already been reported. In the clinical trials, diluted AO solution was locally administered to the surgical field after intralesional or marginal resection of sarcomas. However, our

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final aim is intravenous administration of AO, because it selectively accumulates in sarcomas and can kill tumor cells on extracorporeal radiation with low-dose X-rays (16). AO has not been proven as a mammalian carcinogen, but it is a known bacterial mutagen; it is taking time to obtain approval for intravenous administration of AO to humans.

However, methylene blue (MB) has a very similar chemical structure to AO (Figure 1) and is in current clinical use for the treatment of methemoglobinemia (25) and ifosfamide-induced encephalopathy (26, 27). MB is also effective against lower genitourinary tract discomfort caused by cystitis, urethritis and trigonitis. MB is administered by intravenous injection (26, 27).

Therefore, we investigated the possible use of MB as a photosensitizer for PDT or radiosensitizer for RDT using an osteosarcoma cell line and a mouse osteosarcoma model.

## Materials and Methods

### *In vitro* study.

*Assessment of MB-PDT in vitro.* The effect of MB-PDT on a mouse osteosarcoma cell line (LM8) was investigated. The LM8 cell line, which has strong metastatic ability, was derived from Dunn's osteosarcoma (28). Cells were cultured in 96-well plates ( $5 \times 10^3$  cells/well) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C under a 5% CO<sub>2</sub> atmosphere. After 24 h, in pre-confluent cell growth conditions, the medium was replaced with medium containing MB (1.0 or 5.0 µg/ml) with 10% FBS (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). After 10 min, a 10,000 lx light (containing all wavelengths of visible light), obtained from a 500 W xenon lamp source (Sanei Electronics Co., Ltd, Osaka, Japan), was used to excite MB bound to the cells. The control study used four sets of conditions: in MB-free medium with or without light illumination, and in MB-containing medium with or without light illumination. Cell viability in each well was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium inner salt (MTS) assay that measures mitochondrial dehydrogenase activity, in accordance with the manufacturer's instructions (Promega Corporation, Wisconsin, USA). The cell viability ratio was calculated as the viability of the studied cells divided by the viability of cells grown in MB-free medium without light illumination.

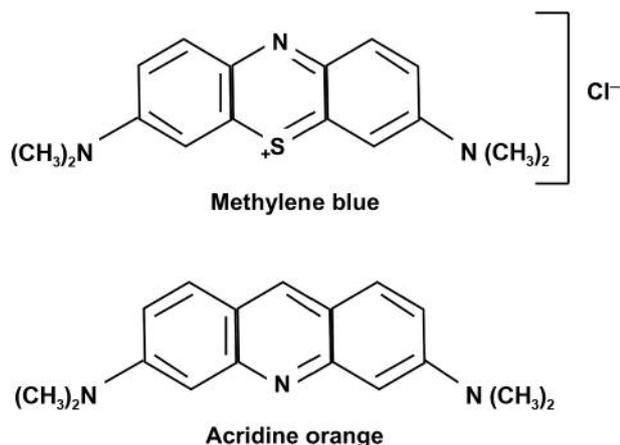


Figure 1. Chemical structures of methylene blue and acridine orange.

The toxicity of MB to LM8 osteosarcoma cells was assessed by adding different concentrations of MB-containing medium to the cells after 30 minutes' illumination.

**Assessment of MB-RDT in vitro.** The effect of MB-RDT on the osteosarcoma cell line was investigated according to the method reported previously (16). LM8 cells were grown on 96-well plates ( $5 \times 10^3$  cells/well,  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ ) in DMEM containing 10% FBS. After 24 h, under pre-confluent cell growth conditions, the medium in the wells was replaced with DMEM containing MB (1.0  $\mu\text{g/ml}$ ) without 10% FBS. After 10 minutes' exposure, the cells (radiation group) were irradiated with X-rays (5 Gy), using a linear accelerator (Clinac® 600C/D: Varian medical systems K.K., Tokyo, Japan). The medium was replaced with MB-free medium with 10% FBS 30 min after irradiation. Cell viability was detected by MTS assay at 0.5, 24, 48 and 72 h after irradiation. The control group contained cells exposed to neither MB nor radiation. The cell viability ratio was calculated as the viability of studied cells divided by the viability of control cells.

**Apoptosis effect of MB-PDT in vitro.** Photodynamic therapy induces apoptosis in tumor cells (29, 30). The TUNEL assay was performed to detect apoptosis induced by MB-PDT. LM8 cells ( $1 \times 10^4$ ) were cultured on coated slides glass (MS-GP: Matsunami-glass Co-Ltd, Osaka, Japan) in DMEM containing 10% FBS. After 24 h, the medium was replaced with medium containing MB (1.0  $\mu\text{g/ml}$ ) and, after 10 min, the cells were illuminated to excite the bound MB (10,000lx light obtained from a 500 W xenon lamp source, Sanei Electronics Co., Ltd.). After a further 30 min, the medium was replaced with MB-free medium. The TUNEL assay was performed 1, 2 and 3 h after MB-PDT, using an automatic staining machine of VENTANA NexEX IHC (Ventana Medical System Inc., Osaka, Japan) with ApopTaq® Apoptosis *in situ* Detection Kit (CHEMICON International Inc., California, USA).

**In vivo study.**

**Mouse tumor model.** The LM8 mouse osteosarcoma cell line was also used for the *in vivo* study. LM8 cells ( $1 \times 10^6$ , isolated by trypsinization from a culture plate) were injected subcutaneously

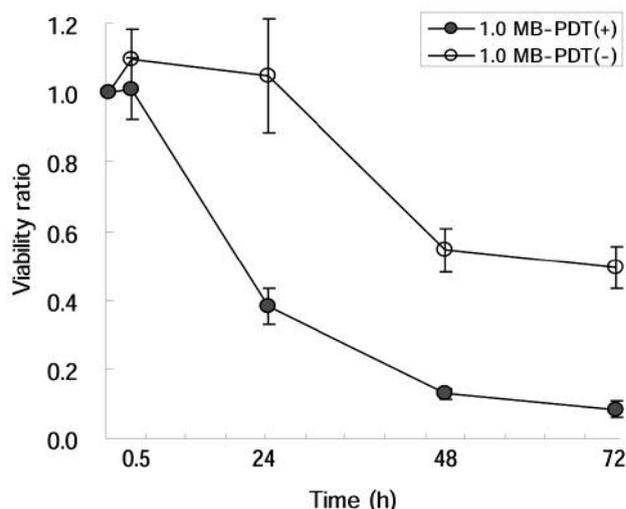


Figure 2. *In vitro* growth inhibition effect of 1.0  $\mu\text{g/ml}$  MB-PDT on the LM8 osteosarcoma cell line. 1.0 MB-PDT(+): illumination group, 1.0 MB-PDT(-) : non-illuminated group, Data are means $\pm$ SD.

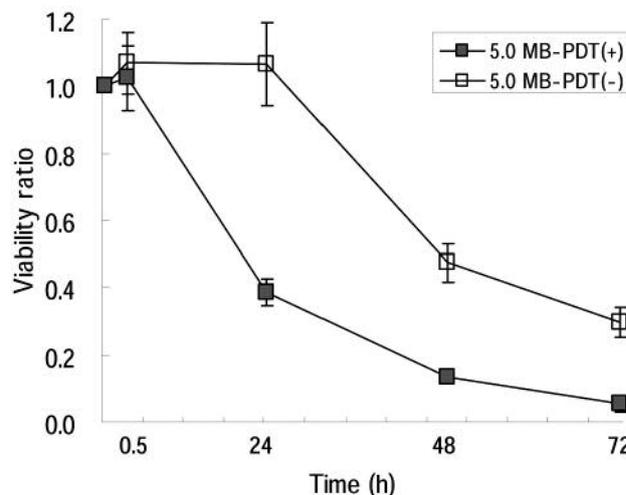


Figure 3. *In vitro* growth inhibition effect of 5.0  $\mu\text{g/ml}$  MB-PDT on the LM8 osteosarcoma cell line. 5.0 MB-PDT(+): illumination group, 5.0 MB-PDT(-) : non-illuminated group, Data are means $\pm$ SD.

into the back of black C3H mice (4 weeks old, male; Oriental Bioservice, Kyoto, Japan). Tumors were treated with MB-PDT 2 weeks after injection, by which time they had reached 8 mm in diameter. At that size, all tumors were free of visible necrosis.

**Assessment of the effect of MB-PDT on tumors.** The tumor-bearing mice were randomly divided into three groups. The control group was intraperitoneally injected with phosphate-buffered saline (n=5). The remaining mice were injected with 12.5 mg/kg MB, then half (n=5) were treated with PDT (MB-PDT(+ group) and half (n=5; MB-PDT(-) group) were not. In the MB-PDT(+) group, 10,000 lx light from a 500 W xenon lamp was delivered to the tumor for 15

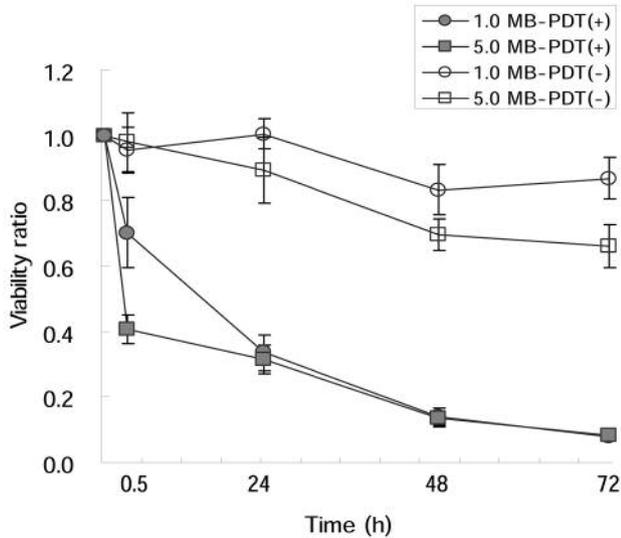


Figure 4. *In vitro* growth inhibition effect of short exposure to MB in the LM8 cell line. 1.0 MB-PDT(+): illumination group with 1.0  $\mu\text{g/ml}$  methylene blue, 1.0 MB-PDT(-): non-illuminated group with 1.0  $\mu\text{g/ml}$  methylene blue, 5.0 MB-PDT(+): illumination group with 5.0  $\mu\text{g/ml}$  methylene blue, 5.0 MB-PDT(-): non-illuminated group with 5.0  $\mu\text{g/ml}$  methylene blue. All the medium containing MB were changed to MB-free medium after illumination. Data are means $\pm$ SD.

minutes, 2 h after injection. To assess the effect of MB-PDT, tumor volume (maximum diameter  $\times$  minimum diameter<sup>2</sup>/2) was calculated and the survival rate for each group evaluated.

**Statistical analysis.** The association among the variables was determined by one way ANOVA with Fisher's PLSD *post hoc* test. *P*-values less than 0.05 were considered significant.

## Results

### *In vitro* study.

**Effect of MB-PDT.** The viability ratios of LM8 cells cultured in MB-containing medium followed by light illumination (MB-PDT(+)) group were significantly lower at 24, 48, and 72 h compared with control cells cultured in MB-free medium (Figures 2 and 3). The viability ratios of cells cultured in MB-containing medium without illumination (MB-PDT(-)) group were also lower, demonstrating MB toxicity.

To reduce the effects of MB toxicity, the exposure time to MB was shortened by replacing the medium with MB-free medium 30 min after illumination. The viability ratios of tumor cells without illumination (MB-PDT(-)) group increased, while the photodynamic effects on tumor cells treated with MB-PDT (MB-PDT(+)) group did not decrease the photodynamic effects (Figure 4). These cells showed cytoplasmic ballooning, which was previously observed in mouse osteosarcoma cells after treatment with AO-PDT (14, 15).

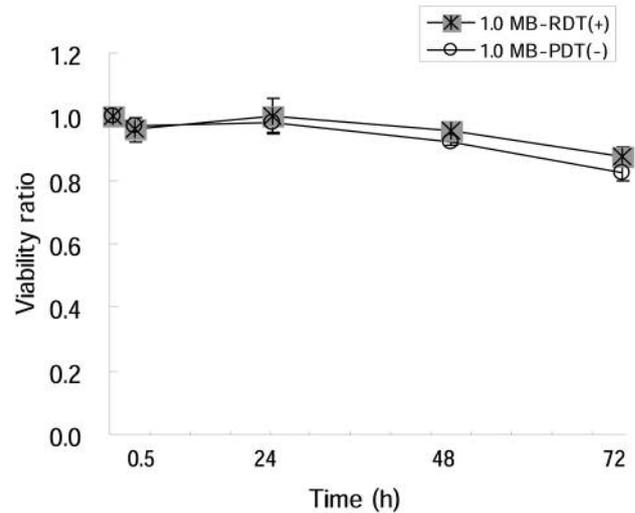


Figure 5. *In vitro* growth inhibition effect of MB-RDT on the LM8 cell line. 1.0 MB-RDT(+): irradiation group with 1.0  $\mu\text{g/ml}$  methylene blue, 1.0 MB-RDT(-): non-irradiated group with 1.0  $\mu\text{g/ml}$  methylene blue. Data are means $\pm$ SD.

**Effect of MB-RDT.** The viability ratio of LM8 cells exposed to 1.0  $\mu\text{g/ml}$  MB followed by radiation with 5 Gy X-rays MB-RDT(+) group did not significantly decrease (Figure 5), suggesting no effect of MB-RDT on osteosarcoma cells. This result was different from that observed with AO-RDT using 5 Gy X-rays (6).

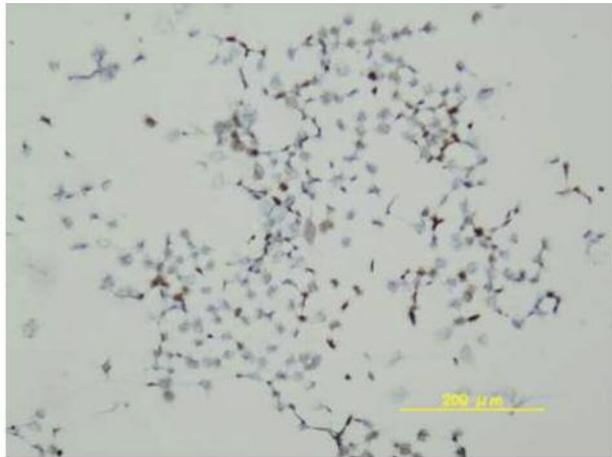
**Apoptosis study.** The apoptosis effect *in vitro* was assessed by the TUNEL method at 1, 2 and 3 h after MB-PDT. At 2 or 3 h, LM8 tumor cells showed positive staining, suggesting that apoptosis had been induced (Figure 6).

### *In vivo* study.

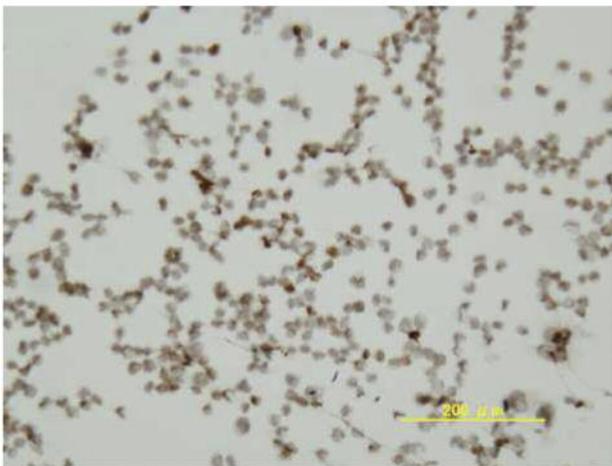
**Tumor growth inhibition.** The LM8 tumor volume increased at the same rate in the MB-PDT(+) group, the control group and the MB-PDT(-) group (Figure 7), showing that intraperitoneal injection of MB did not affect tumor growth. Neither did MB-PDT improve the survival rate compared with that of the control group (Figure 8). Macroscopic findings showed that osteosarcoma tissues inoculated in the back were not stained blue with MB (Figure 9).

## Discussion

We recently established a new adjuvant therapeutic modality using AO excited by photon energy for reduction of surgery in high-grade malignant musculoskeletal tumors (13-24). Diluted AO solution is applied to the area of the resected tumor by local administration after intralesional or marginal tumor resection in order to detect and kill remaining tumor cells. However, it would be better to



1 h



2 h



3 h

TUNEL assay

Figure 6. Cytological findings of TUNEL assay in LM8 cells, 1, 2 and 3 h after MB-PDT.

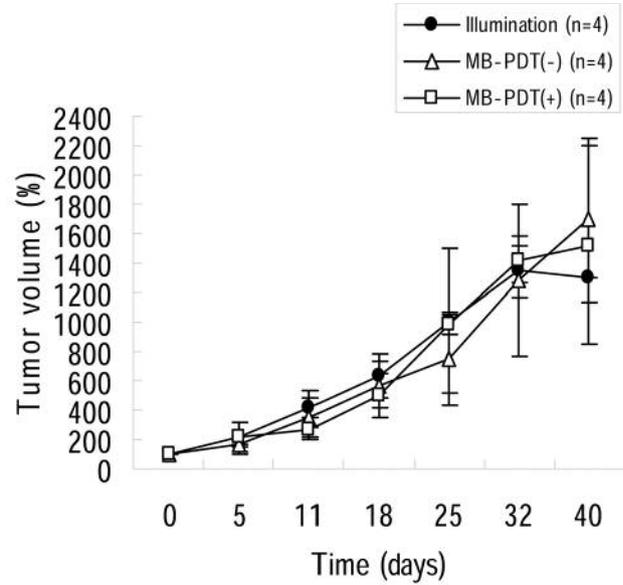


Figure 7. In vivo growth inhibition effect of LM8 osteosarcomas after intraperitoneal injection of 12.5 mg/kg MB followed by 15 min of illumination (MB-PDT). Illumination: illumination only group, MB-PDT(-): intraperitoneal injection of MB, MB-PDT(+): intraperitoneal injection followed by illumination (MB-PDT). Data are means±SD.

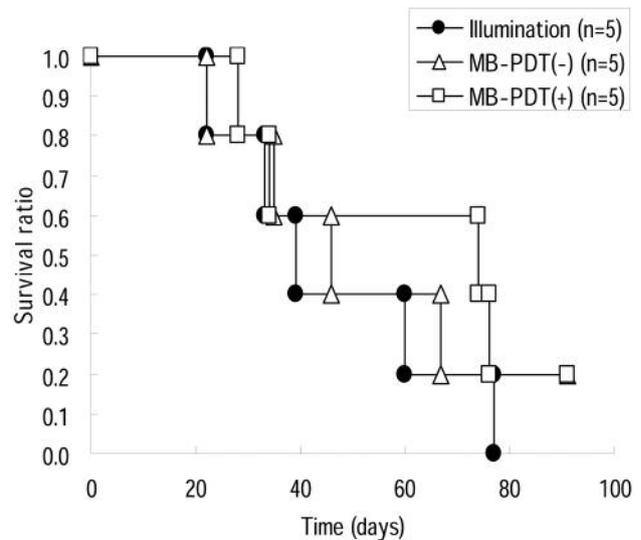


Figure 8. Survival of mice inoculated with LM8 osteosarcomas after intraperitoneal injection of 12.5 mg/kg MB followed by 15 min of illumination (MB-PDT). Illumination: illumination only group, MB-PDT(-): intraperitoneal injection of MB, MB-PDT(+): intraperitoneal injection followed by illumination (MB-PDT). Data are means±SD.

homogeneously expose all tumor cells to AO, with systemic delivery via intravenous injection. Unfortunately, it will take time to obtain approval for the intravenous administration of AO to humans.



Figure 9. Macroscopic findings of LM8 osteosarcoma inoculated in the back of a C3H mouse.

MB has a very similar chemical structure to AO and is already approved for systemic administration by intravenous injection because of its clinical use in other diseases. It has various biological applications, such as a vital staining dye for parathyroid (31), an anti-fungus agent in golden fish, and an anti-malaria agent (32), and has also been used against ifosfamide-induced encephalopathy in humans. It has been approved by The Food and Drug Administration (FDA, USA) for methemoglobinemia and for lower genitourinary tract discomfort caused by hypermotility, cystitis, urethritis and trigonitis, and can be administered by intravenous injection. Therefore, we investigated the possibility that MB could be used in place of AO in PDS, PDT and RDT.

Our results showed that while MB is cytotoxic to osteosarcoma cells, it also has potential as a photosensitizer for PDT: after washing out of MB, tumor cells treated with PDT died within 72 h, but those without PDT did not (Figure 4). Its cytotoxic mechanism is not clear, but morphological changes of tumor cells with cytoplasmic ballooning after MB-PDT were similar to those seen in osteosarcoma cells after AO-PDT. The TUNEL assay confirmed the induction of apoptosis; therefore, the tumor cells died by necrosis with cell rupture and apoptosis. In AO-PDT, necrosis and apoptosis are considered to be induced by

oxidation of intracytoplasmic activated oxygen, which is formed by triplet AO excited with photon energy of xenon light. Therefore, MB might also be excited by xenon light.

It seems likely that AO-PDT and MB-PDT have different cytotoxic mechanisms, because the main targets of AO in the cells are lysosomes or acidic vesicles, as well as DNA and RNA, while MB mainly binds to DNA and RNA (17, 21, 33). Photochemical interactions of MB and its analogs have been studied (34-39), and clinical trials in bladder tumors of PDT with MB were performed in the 1980s and '90s (40, 41), but indicated that the therapy was ineffective.

To our knowledge, there are no reports describing radiosensitivity associated with MB, and our results also showed none, in contrast to AO. If MB was radiosensitive, tumor cells remaining after PDT could be killed by RDT with low-dose X-rays and multiple pulmonary metastatic lesions could be cured by extracorporeal irradiation with X-rays after intravenous injection.

MB-PDT did not inhibit tumor growth *in vivo*, unlike AO. The survival rate of mice with osteosarcoma was not improved after MB-PDT. MB did not accumulate in osteosarcoma tissue (Figure 9), unlike AO, which selectively accumulates in tumor tissue and is visible by fluorescence microscopy after illumination with blue light.

Based on our results, we conclude that MB is not a suitable substitute photosensitizer for AO in PDS, PDT and RDT for osteosarcoma.

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