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ORIGINAL RESEARCH

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Effects of methylene blue photodynamic therapy on oral carcinoma and leukoplakia cells

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Abstract

Objective: Methylene blue (MB) is a readily available and affordable substrate that can be used as a photosensitizer for photodynamic therapy (PDT). The objective of this study was to determine if PDT with MB can downregulate matrix metalloproteinases (MMPs) related to oral carcinoma.

Methods: Cell cultures of oral squamous cell carcinoma (CA-9-22), oral leukoplakia (MSK-Leuk1), and immortalized keratinocytes (Rhek-1A) were photosensitized with MB and treated with PDT. MMP-9 gene expression was interrogated via qRT-PCR. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to confirm the efficacy of MB PDT.

Results: MMP-9 gene expression was found to be significantly decreased in oral carcinoma, leukoplakia, and immortalized keratinocytes with use of MB PDT.

Conclusion: This work demonstrates that MB-mediated PDT can downregulate MMPs which are critical to the invasion and metastasis of oral cancer. These results suggest that MB PDT could be a clinically significant and cost-effective treatment for oral leukoplakia and carcinoma.

Level of Evidence: NA

KEYWORDS
molecular biology, oral cavity, oropharynx

1 | INTRODUCTION

Every year, over a quarter million cases of oral cancer are diagnosed worldwide, with particularly high incidence in the South Asian nations of India, Pakistan, and Sri Lanka, where oral cancer is the most common malignancy in men.[1] In the United States, there are 18.1 cases of oral cavity and pharynx cancers per 100,000 men and 6.5 cases per 100,000 women annually.[2] Tobacco, alcohol, and betel nut chewing constitute the major risk factors, and approximately half of afflicted patients will not survive past 5 years.[1] Treatment of oral cancer consists of surgery with or without radiation therapy, after which survivors often cope with disfigurement, difficulty eating, and challenges with speech.

Photodynamic therapy (PDT) is an emerging technology for the control of oral carcinoma and its precursors. In the United States, PDT is currently Food and Drug Administration approved for treatment of esophageal carcinoma, Barrett's esophagus, tracheobronchial carcinoma, actinic keratosis, and macular degeneration.[3–6] In the European Union, PDT is approved for treatment of head and neck cancer.[7] PDT spares adjacent normal tissue, often takes place in a single outpatient

session, and can be safely repeated, all while not precluding future radiation or surgery, making it a highly attractive modality.[7,8]

Between 1990 and 2008, our co-author (MAB) used PDT to treat 138 patients with carcinoma in situ or T1N0 squamous cell carcinoma of the oral cavity and found that all patients achieved a complete clinical and pathologic response after a single treatment. The nine cases of local or regional recurrence were salvaged with either repeat PDT or surgery, resulting in a 100% cure rate at 5 years.[7] He also treated 52 patients with T2N0 and T3N0 squamous cell carcinomas of the oral cavity which were less than 1 cm thick. Again, all patients achieved complete clinical and pathologic remission after one treatment, and the six recurrences were successfully salvaged for a 3-year cure rate of 100%.[7] Importantly, oral function was preserved without development of fibrosis or xerostomia. In the first multi-institutional phase II trial of PDT for oral cancer, 96 patients with recurrent or second primary carcinoma of the oral cavity demonstrated a 50% rate of complete histologic response and 79% survival at 1 year.[7]

PDT requires administration of a photosensitizing drug which selectively concentrates in precancerous or tumor cells, after which a wavelength of non-thermal light is used to activate the drug, resulting in tumor cell death. Various photosensitizers have been used, including 5-aminolevulinic acid (5-ALA), its methyl ester (Metvix), Photofrin, BPD, Foscan, and taloporfin sodium.[7,9] MB is a phenothiazine derivative that has previously been used for the treatment of methemoglobinemia and malaria, and also as an injectable dye for sentinel lymph node biopsy.[10,11,12] Proven to have low toxicity, it has usefully served as a photosensitizer when embedded into tumor-specific nanoparticles.[13] The fact that it is readily available and affordable makes it particularly appealing in the treatment of head and neck cancer.

We examined potential pharmacodynamic markers and determined that members of the matrix metalloproteinase family could be useful markers for the impact of MB PDT. Matrix metalloproteinases (MMPs) are enzymes which potentiate tumor invasion and metastasis by degrading the extracellular matrix. Many MMPs are overexpressed in head and neck squamous cell carcinoma (HNSCC), such as MT1-MMP, which influences tumor cell invasion and angiogenesis.[14] For example, MMP-2 mRNA is overexpressed in 32% of oral dysplasias and 47% of oral cancers, and MMP-9 mRNA is overexpressed in 85% of oral dysplasias and 100% of oral cancers.[15] MMP-9 has been identified as a promising biomarker in several cancers.[16] Ikebe et al., studying oral squamous cell carcinoma, demonstrated an association between high gelatinolytic activity and immunohistochemical staining of MMP-2 and MMP-9 and a higher Yamamoto grade of tumor invasion.[17] Relatedly, O-Charoenrat et al. found that MMP-2 and MMP-9 mRNA expression in HNSCC was significantly correlated with the incidence of lymph node metastasis.[18] Based on this evidence, we chose MMP-2 and MMP-9 as representative MMps to demonstrate the effects of MB-mediated PDT.

A triggered decrease in the expression of these enzymes serves to indicate the efficacy of MB PDT. We hypothesized that MB-mediated PDT of both cancerous and precancerous oral squamous cells would decrease MMP levels. This would make it a promising candidate for non-invasive and cost-effective treatment of oral cancers and precancers.

2 | MATERIALS AND METHODS

2.1 | Cell lines

As a translational science cell line project, this study did not require IRB review at our institution. CA-9-22 are derived from squamous cell carcinoma of the human tongue and grown in RPMI supplemented with 10% FBS at 37°C in 5% CO_2 (ThermoFisher, Waltham, MA). MSK-Leuk1 cells were isolated from a dysplastic leukoplakic lesion adjacent to a squamous cell carcinoma of the tongue. These were a kind gift from Dr. Peter Sacks and are grown in Keratinocyte Growth Medium (KBM-2, Lonza, Walkersville, MD) supplemented with bovine pituitary extract, epidermal growth factor, insulin, and hydrocortisone at 37°C in 5% CO_2. Rhek-1A cells are SV40-immortalized human epidermal keratinocytes grown in DMEM supplemented with 10% FBS at 37°C in 5% CO_2 (ThermoFisher). Cell line authentication was carried out by STR genotyping by the Fragment Analysis Facility at Johns Hopkins University followed by BLAST searches of the ATCC, CLIMA, and DSMZ databases. MSK-Leuk1 cells and Rhek-1A cells were phenotyped and found not to match any existing cell line records in any database. CA-9-22 cells were phenotyped and found to match existing cell line records for CA-9-22 cells from the Riken Cell Bank in Japan.

2.2 | MTT cell viability assay

Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) incorporation (MilliporeSigma, St. Louis, MO). Cells were seeded in 96-well tissue culture plates at 5×10^4 cells/well. At 24 h after plating (day 0), MB was added to selected plates, and at 48 h (day 1), selected plates were treated with a 664 nm light (Miravant Systems, Santa Barbara, CA). At days 0, 1, 2, or 3, MTT was added to the culture media at 0.5 mg/ml and incubated at 37°C for 4 h. Mitochondrial dehydrogenases of live cells converted MTT to a water-insoluble purple formazan, which was then solubilized in isopropyl alcohol/DMSO, and the absorbance at 560 nm was read. Six replicates were used at each data point.

2.3 | Photodynamic therapy

Cells were plated in growth media, split after 24 h, and plated at 1 million cells per flask. These were incubated at 37°C for 48 h. Each PDT experiment involved four flasks: untreated, MB only, light only, and full PDT. MB (1 μg/ml) was added to the appropriate flasks, and after 24 h, the appropriate flasks were treated with the 664 nm light at a fluency of 0–10 J/cm^2 (for MTT) or 6.3–6.5 J/cm^2 (in later experiments) and at a fluence rate of 16–38 mW/cm^2. Light was provided

by a DD4 diode (Miravant, Santa Barbara, CA) outputting light at 664 nm with a 400 μm microlens fiber (PDT Systems, Santa Barbara, CA) at a dose rate 32 mW/cm^2. The microlens fiber was positioned above the flasks and plates so that the light spot size matched the area of adherent cells. Twenty-four hours later, cells were trypsinized and pelleted, while supernatants were clarified by centrifugation. Total RNA was isolated from frozen cell pellets using the 5Prime PerfectPure kit (Gaithersburg, MD). First-strand cDNA was synthesized via PCR using the SuperScript III kit with oligo(d)T primer (ThermoFisher).

2.4 | Quantitative (real-time) RT-PCR

Quantitative (real-time) PCR using the Universal Probe Library (UPL) and LightCycler 480 Master Mix for UPL was used to interrogate the cDNA samples using a specific primer/probe set for MMP-9 (Roche Applied Science, Indianapolis, IN). Equivalent cDNA production across samples was analyzed via ß$_2$M primers. Results were analyzed using LightCycler 480 software.

2.5 | Statistical methods

Data were analyzed in a group wise fashion for differences in cell viability via ANOVA testing (one-way) for each experiment, unless otherwise noted. Dunnett's post testing was additionally employed to determine which groups were statistically different from the control groups or if the combination treatment was statistically different from the single agent treatments. One-way ANOVA results are presented as F ratio with degrees of freedom and the p value. Data in the charts were presented as a Mean +/− SEM for each group. P <.05 was used as a cutoff for statistical significance on testing. MTT treatments and qRT-PCR were also compared by a Student's t-test with P < .05 considered statistically significant. All analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA).

3 | RESULTS

3.1 | MTT cell viability assay

We first examined the effects of the different treatments on oral cancer cell viability. Treatment of cancerous cells (CA-9-22) with MB alone, with no light, demonstrated a dose-dependent decrease in cell viability with all concentrations (Figure 1A). CA-9-22 cells were treated with 0, 0.5, 1, 2, or 4 μg/ml MB. By one-way ANOVA analysis with Dunnett's post-test, a statistically significant difference in absorbance in all MB titration treatment groups versus the control group was observed at both day 2 (F (4, 25) = 131.2, P <.0001) and day 3 (F (4, 25) = 399.3, P <.0001). After 3 treatment days, the MB resulted in a decrease in cell viability with all doses. Therefore, we used 0.5 and 1 μg/ml of MB for the remainder of the experiments.

Treatment of CA-9-22 cells with 664 nm light alone at a fluence of 0, 5, or 10 J/cm^2, with no MB, had no effect on cell viability by MTT assay (Figure 1B). At day 2 (24 h after light treatment), there was

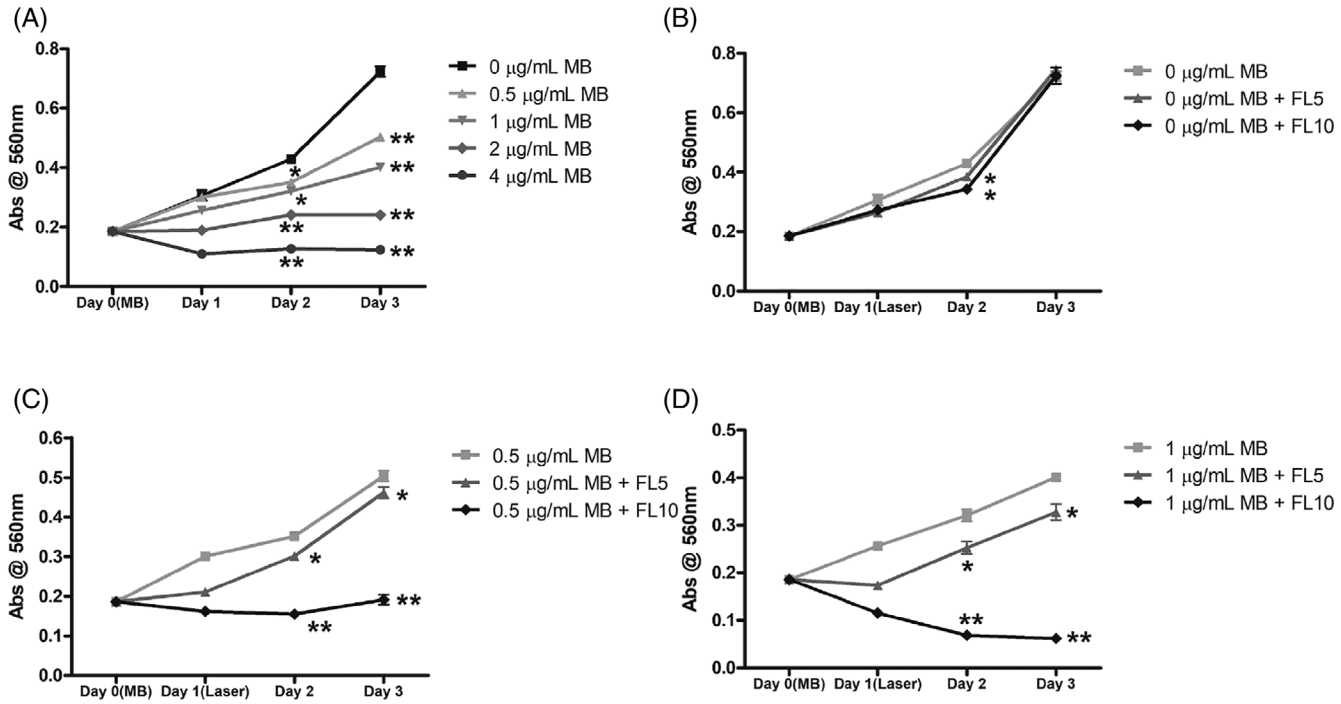

FIGURE 1 Photodynamic therapy with methylene blue causes statistically significant decreases in cancerous cell (CA-9-22) viability by MTT assay. (A): Methylene blue alone, (B): light alone, (C): 0.5 μg/ml methylene blue with light, (D): 1 μg/ml methylene blue with light

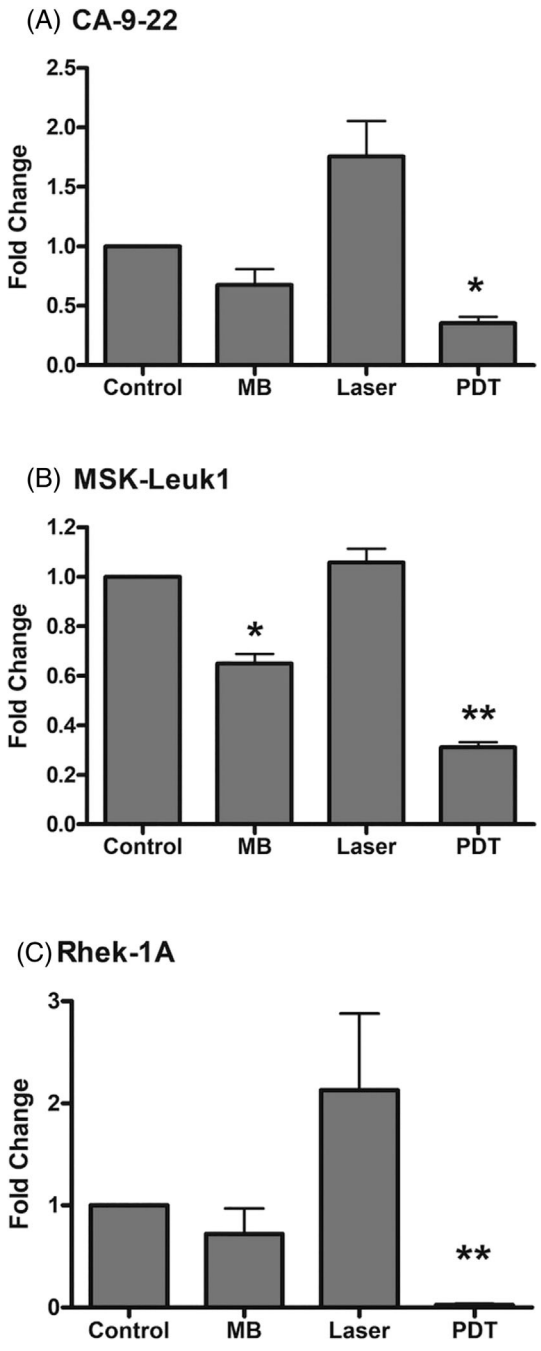

FIGURE 2 Methylene blue photodynamic therapy decreases MMP-9 gene expression across all cell lines. (A): Cancerous cells (CA-9-22); (B): Precancerous oral leukoplakia cells (MSK-Leuk1); and (C): Dermal keratinocytes (Rhek-1A)

a significant decrease in cell viability (P <.05 vs. control) for each fluence; however, by day 3, the decrease was not sustained.

Treatment of cells photosensitized with 0.5 or 1 μg/ml MB using 664 nm light treatment at a fluence of 0, 5, or 10 J/cm^2, resulted in a larger decrease in cell viability than treatment with MB alone (Figure 1C,D). Light treatment combined with 0.5 μg/ml MB significantly decreased viability at fluences of 5 or 10 J/cm^2 by one-way ANOVA analysis at both day 2 (F(2,15 = 165.7, P <.0001) and day 3 (F(2,15 = 159.7, P <.0001; Figure 1C). Light treatment combined with 1 μg/ml MB also significantly decreased viability at both fluences by one-way ANOVA analysis at both day 2 (F(2,15 = 147.7, P <.0001) and day 3 (F(2,15 = 239.6, P <.0001; Figure 1D).

In summary, we found that the photosensitizer MB is itself cytotoxic (Figure 1A), while the concentrations of light we employed for these experiments were not (Figure 1B). The combination of MB and PDT light had a greater dampening effect on cell viability than MB alone (Figure 1C,D), confirming that our simulation of PDT with MB is effectively decreasing cell viability and is cytotoxic at sufficient levels.

3.2 | qRT-PCR for MMP-9

We tested the level and function of MMP-9 with and without MB PDT exposure. PDT with MB decreased MMP-9 gene expression in oral cancer cells (Figure 2A). Cells treated with MB alone (1 μg/ml), light alone (fluence of 6.3–6.5 J/cm^2), neither, or both were pelleted for isolation of total RNA, which was converted to first-strand cDNA. Quantitative RT-PCR was used to interrogate the cDNA samples using MMP-9 primer and probe. The decrease in MMP-9 gene expression from MB PDT was statistically significant compared with the decrease from MB alone (P = .0393).

MB PDT likewise decreased MMP-9 gene expression in precancerous oral cells (Figure 2B). Cells treated with MB alone, light alone, neither, or both were pelleted for isolation of total RNA, which was converted to first-strand cDNA. Quantitative RT-PCR was used to interrogate the cDNA samples using MMP-9 primer and probe. The decrease in MMP-9 gene expression from PDT was statistically significant compared with the decrease from MB alone (P = .0002).

Finally, PDT decreased MMP-9 gene expression in transformed keratinocytes (Figure 2C). Cells treated with MB alone (1 μg/ml), light alone (fluency of 6.3–6.5 J/cm^2), neither, or both were pelleted for isolation of total RNA, which was converted to first-strand cDNA. Quantitative RT-PCR was used to interrogate the cDNA samples using MMP-9 primer and probe. The decrease in MMP-9 gene expression from PDT was statistically significant compared with the decrease from MB alone (P <.0001).

In summary, MB PDT decreased MMP-9 gene expression in cancerous cells (Figure 2A), in precancerous cells (Figure 2B), and in transformed keratinocytes (Figure 2C). This change reached statistical significance in all three cell lines. MB-only and light-only controls were included to illustrate that the decrease in MMP-9 mRNA was specifically attributable to PDT.

4 | DISCUSSION

This study showed that MB PDT significantly decreases gene expression and viability of oral precancerous cells, oral squamous cell carcinoma, and dermal keratinocytes by quantifying MMP-9 expression as a representative marker. MB activated by light was more lethal to cells than MB alone. Based on the decreased gene expression seen with RT-PCR and reduced viability seen with the MTT assay, this study

supports MB PDT as a modulator of MMPs and therefore of oral carcinoma.

Previously, Kofler et al. found that PDT with MB induced decreased cell viability and decreased progeny in clonogenic assays of HNSCC lines.[19] We have shown that MB PDT decreases gene expression and cell viability in both preneoplastic and neoplastic oral squamous cell carcinoma. The finding of decreased viability of Rhek-1A cells, which are preneoplastic, is unique to this study. This has important implications for clinical practice as it would be optimal to use PDT in an outpatient setting for treatment of precancerous lesions. MB is commonly and cheaply available in clinics; therefore, using it in PDT would reduce costs and be particularly useful against precancerous oral lesions in lower-resource countries.

In this study, we also showed the usefulness of MMPs as potential biomarkers for the success of MB-mediated PDT, which could be useful in future clinical trials. Previously, overexpression of MMP-9 has been shown to be potentially useful in the diagnosis of oral cancer as it is highly expressed along the invasive front of oral cancers.[20] Consistent with the function of MMPs in proteolyzing the basement membrane, higher protein levels of MMP-1, MMP-2, MMP-3, and MMP-9 in oral SCC are associated with a higher Yamamoto grade of invasion and with nodal metastasis.[21–23] Two groups have studied the effect of PDT on MMPs in HNSCC specifically. Du et al. applied hypericin-PDT to nasopharyngeal cancer cell lines and a mouse model of nasopharyngeal cancer. They observed that PDT caused upregulation of MMP-1 mRNA and protein expression, and reduction of MMP-9 mRNA and protein expression. They were able to reduce the PDT-induced damper on MMP-9 expression by incubating cells with GM-CSF and concluded that hypericin-PDT downregulates MMP-9 via inhibition of this ligand.[24] Sharwani et al. performed PDT on cell lines derived from human oral squamous cell carcinomas and demonstrated downregulation of both MMP-2 and MMP-9 protein.[25] Both groups' results are consistent with the downregulation seen in our study. These studies support the potential use of the MMP family of proteins as surrogate markers for the efficacy of PDT.

A potential limitation of PDT head and neck carcinogenesis studies is the lack of a suitable animal model.[26] Current animal models would be difficult to employ for MB PDT studies because the instruments are designed for human oral anatomy. In the future, to appropriately perform translational research, one would have to develop well-accepted models and instrumentation for MB PDT of mice and small rodents. This would be a lower priority as patient-derived xenograft models are utilized more in our field.[27] As projects in PDT expand in scope, cellular proliferation findings in studies like this one could be confirmed in organoids or additional studies with cloned cells. However, this could be challenging in premalignant cells as they would have predictably low clonogenicity.

In current clinical practice, facial pediatric vascular anomalies can be treated in office with pulse dye light therapy. This non-invasive treatment has excellent clinical outcomes and low rates of adverse effects.[28,29] Light treatment is easy to use on facial areas that are difficult to treat surgically such as lips, eyelids, and ears, and dosing can be adjusted for better cosmetic outcomes, such as use of lower doses in the periorbital area.[30] Similarly, MB PDT could be employed in these areas for the treatment of head and neck carcinoma. Future investigations could compare PDT and surgery for these carcinomas. Future investigations could also elucidate the molecular pathway by which MB PDT decreases MMPs in preneoplastic oral cavity cells and cutaneous cells. MMP-1 and 9 have been previously found to be identifiable through saliva collection; therefore, another future direction could be to measure modulation of salivary MMPs as a biomarker for the effectiveness of MB PDT in the oral cavity.[31,32]

5 | CONCLUSION

This work demonstrates that PDT with MB, commonly and inexpensively available, can downregulate proteins critical for the invasion and metastasis of head and neck cancer.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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