



Photodynamic therapy down-regulates the invasion promoting factors in human oral cancer

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Summary Squamous cell carcinomas of the head and neck are characterized by their high tendency for invasion and metastasis. Several studies have identified the roles of matrix metalloproteinases (MMPs), vascular endothelial growth factors (VEGF) and urokinase plasminogen activators (uPA) in this process. Photodynamic Therapy (PDT) is an emerging treatment currently in clinical practice for the treatment of early cancer. Here we evaluate, *in vitro*, the influence of PDT on the expression of these molecules. A series of human keratinocyte cell lines derived from human oral squamous cell carcinomas (OSCC) were used as the PDT 'targets' in this study. Each cell line was subjected to sublethal dose of PDT. Activity of MMP-2, MMP-9, MMP-13, uPA and VEGF were evaluated at protein levels using zymography and ELISA on culture medium. For uPA, a chromogenic assay was performed. Gelatin zymography results revealed that, in control medium, MMP-9 and MMP-2 were secreted in proform. MMP-2 was highly expressed by H376 cells while VB6 and UP cells relatively show similar MMP-2 with comparatively low expression. For MMP-9, the latent type was highly expressed by VB6 cells and only slightly by H376, while active-MMP-9 was expressed by VB6 cell line only. Following PDT, both active and latent MMP-2 and MMP-9 were down regulated by UP and VB6 cells ($p < 0.001$), while H376 showed an increase in active-MMP-2. These observations were supported by ELISA. This study has demonstrated that, PDT causes the suppression of factors responsible for tumour invasion which may be of therapeutic value.

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Introduction

Head and neck cancer is a worldwide problem with different histopathologies, clinical behaviours and response to treatment.¹ Over 90% of head and neck cancer patients reported consist of squamous cell carcinoma.² In the European Union, there are approximately 84,400 new cases of head and neck cancer reported every year, and, in the UK alone, around 10.2 per 100,000 males and 5.4 per 100,000 females are diagnosed every year.³ Globally, there were estimated to be over 500,000 cases of head and neck cancer in 2000. In the US, 40,000 new cases were diagnosed, with approximately 12,500 deaths.⁴ The invasion of surrounding healthy tissues by malignant cells is one of the essential steps in tumour progression. The key molecules usually involved in this process are the tumour promoting enzymes, such as matrix metalloproteinases (MMPs), urokinase plasminogen activator (uPA) and the vascular endothelial growth factor (VEGF). Many studies have shown that MMP-2 and MMP-9,^{5,6} MMP13,^{7,8} uPA^{9,10} and VEGF¹¹ are expressed in head and neck squamous cell carcinomas.

MMPs have a dual role in tumour growth and metastasis. They promote tumour growth by degrading most of extracellular matrix (ECM) components, such as collagen and fibronectin (FN), and enhancing angiogenesis.¹² VEGF is one of the main regulators of angiogenesis, which is now accepted to play a vital role in the continued growth and metastasis of solid tumours.^{13,14} Based on these considerations, a positive correlation between malignancy and expression/suppression of these factors would be expected.

Photodynamic therapy (PDT) is currently under clinical investigation for the treatment of early cancer. The biological effects of PDT are primarily mediated by the generation of reactive oxygen species, such as singlet oxygen, that result in cytotoxic effects on treated cells. Apart from a death response, cells can also undergo a rescue response after PDT and restore their activities which can be monitored by physiological responses.^{15–17} Clinically, a marked softening of the skin of patients with sclerodermatic disease is observed after PDT treatment.¹⁸ This antisclerotic effect of PDT could be explained by its effects on collagen and its degrading enzymes. A study by Karrer et al., demonstrated an induced effect for PDT on MMP-1 and MMP-3 synthesis in human dermal fibroblast cells.¹⁹ However, this later study and a few others do not involve keratinocyte malignant cells, which may be the initial PDT target. Therefore, we used both malignant and non-malignant human keratinocytes, in culture, to investigate the influence of PDT on the

secretion of MMP-2, MMP-9 and MMP-13, uPA and VEGF.

Materials and methods

Photosensitiser

The chemotherapeutic drug, *meta*-tetrahydroxyphenyl chlorin (*m*-THPC; temoporfin; Foscan[®]) 4 mg/ml stock was kindly provided by Biolitec Pharma Ltd (Edinburgh, UK) and stored according to the manufacturer's instructions. Further dilution (0.25–4 µg/ml) was performed in culture medium.

Cells and cultures

Cell lines derived from oral squamous cell carcinomas (OSCC), H376²⁰ and VB6²¹ and UP, a human HPV16-transformed epidermal keratinocyte cell line,²² were retrieved from liquid nitrogen and cultured to confluence in keratinocyte growth medium (KGM; Gibco, UK) supplemented with 10% fetal calf serum (FCS, Gibco, UK). KGM included Dulbecco's minimal essential medium (DMEM)/F12 (3:1, v/v) supplemented with 10 ng/ml epidermal growth factor (EGF; Sigma, UK), 0.5 µg/ml hydrocortisone (Sigma, UK), 0.5 µg/ml insulin (Sigma, UK), 1.8×10^{-4} M adenine (Sigma, UK), 1×10^{-10} M cholera toxin (Sigma, UK), 100 IU/ml penicillin (Gibco, UK), 100 µg/ml streptomycin (Gibco, UK) and 2.5 µg/ml Fungizone (Sigma, UK). All cells were allowed to attach for 24 h before replacing the medium with fresh KGM. The cultures then were maintained in a humidified incubator at 37 °C with 5% CO₂/95% air atmosphere.

Viability assay

Cell viability was determined by the MTT colorimetric assay; MTT (Chemicon, UK). MTT is based on cellular conversion of the tetrazolium compound (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; MTT) into a purple formazan product. Optical readings were performed at 570 nm using a microplate reader (Multiscan, MCC 340; Flow Laboratories, Herts, UK).

Cell sensitisation and irradiation

In order to determine the sublethal doses of PDT, the UP, VB6 and H376 cells were subjected to range of PDT doses. Briefly, cells were harvested, counted and seeded into 96-well plates at a cell concentration of 3×10^4 cells in KGM per well. Cells were allowed to attach for 24 h at 37 °C before

Table 1 Sub-lethal PDT dose determined from phototoxicity curve

	Incubation time (h)	Foscan ($\mu\text{g/ml}$)	Fluence (J/cm^2)	Fluence rate (mW/cm^2)	Survival (%) \pm S.D.
UP	24	1.0	0.5	25	88 ± 3
VB6	24	0.5	0.5	25	87 ± 2
H376	24	0.25	0.25	25	85 ± 3

m-THPC was added, in darkness, at concentrations between 0.25 and 4 $\mu\text{g/ml}$ in low-serum (1%) medium. After 24 h of incubation, cells were washed twice with PBS and fresh serum-free DMEM was added. Cells were then exposed to 652 nm diode red laser light (Diomed Ltd, Cambridge, UK). The laser beam was transmitted through a microlens fibre, giving a fluence (energy) rate of 25 mW/cm^2 and fluence density between 0.25 and 4 J/cm^2 . Cells were considered to be "controls" when they were not subjected to *m*-THPC or laser light. The obtained results were then used to construct the dose-response curve for each cell line, from which the sublethal doses of PDT were determined as follows: 0.5 J/cm^2 (time 20 s), 0.5 J/cm^2 (time 20 s), and 0.25 J/cm^2 (time 10 s) for UP, VB6 and H376 cells, respectively. The doses of *m*-THPC for each cell line were also determined from the toxicity curves (Table 1). These doses of light and *m*-THPC applied had resulted in approximately 15% (maximum) cell death in all cell lines, compared with untreated control cells, as determined by MTT assay (data not shown).

Preparation of cell supernatants for zymography

Following PDT, cells were washed twice with PBS, detached with trypsin/EDTA and seeded into 24-well tissue culture plates, at a concentration of 1×10^5 cells per well in 0.5 ml of additive free medium (AFM) with 10% FCS. AFM was KGM without additives and contained DMEM/F12 (3:1, v/v) for 24 h before being washed twice in PBS, and new 0.5 ml of serum free AFM was added. The supernatant of each cell line was sampled after 24 h, at which time cells were detached and counted. Conditioned medium was cleared of cells and debris by centrifugation at $4000 \times g$ for 10 min, followed by protein estimation using the colorimetric BCA protein assay reagent (Pierce Warriner, UK). Cell media of non-treated cultures was considered as controls.

Gelatin zymography

MMP-9 and MMP-2 activities were analysed using sodium dodecylsulphate (SDS)-PAGE substrate gels.

Gelatin (BLOOM 300; Sigma, UK) was added to at a final concentration of 1 mg/ml and poured using a pre-assembled Vertical gel electrophoresis apparatus (Mini-PROTEAN® II cell; BioRad). To each gel, supernatant samples with equal protein, as determined by BCA protein assay, were diluted 1:1 in non-reducing sample buffer, 62 mM Tris-HCL (pH 6.8), 10% glycerol, 2% sodium dodecylsulphate and 0.1% bromophenol blue, and loaded into the wells using capillary disposable pipette tips. Molecular weight markers (Kaleidoscope prestained standards; BioRad, UK) was prepared according to the manufacturer's instructions and run on each gel. Samples were run at 100 V through stacking gel stage and the current then elevated to 120 V until the samples approached the resolving gel base. The gels were separated from plates with care and washed twice in 2.5% Triton X-100 for 30 min at 37 °C to remove SDS. The gels then allowed to equilibrate in developing buffer containing 50 mM Tris-HCL, 0.2 M NaCl, 5 mM CaCl_2 and 0.02% Triton X-100 overnight at 37 °C. Gels were stained with 0.5% Coomassie Blue G250 in 30% (v/v) methanol, 10% (v/v) glacial acetic acid for 30 min at room temperature before being destained with Coomassie de-stain (30% (v/v) methanol, 10% (v/v) glacial acetic acid) until the enzyme activity bands were obviously clear as white bands against a blue background. Stained gels were captured under illumination using the UVP Image store 5000 (Ultra-Violet Products, UK) and obtained images saved on a personal computer (PC). The images were analysed quantitatively using the Scion image software (Scion, USA). The intensity bands was measured by densitometric analysis, and comparisons were made within each gel to determine the relative changes in MMP activity. Data for each zymogram were expressed as a relative change in MMP activity, and these relative changes were compared with repeat experiments. Direct comparison between separate gels was not made as the intensity of background staining was variable.

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for human MMP-9 (total) and MMP-2 (total), pro MMP-13 and VEGF were purchased from R&D Systems (Oxford, UK). The assay is based on a two site ELISA 'sandwich' format. Cell supernatants

were prepared as for zymography, and 100 μ l sample was added to each well and immobilized MMP or VEGF detected by the horseradish peroxidase labeled antibody directed to MMP-9, MMP-2, MMP-13 or VEGF. The reaction was stopped by addition of an acid solution (2 M H₂SO₄) and the intensity of the resultant colour change read at 450 nm on a spectrophotometer (Multiscan, MCC/340; Flow Laboratories, Herts, UK). The concentration was determined by interpolation from a standard curve using known concentration of MMP-9, MMP-2, MMP-13 or VEGF standards as supplied.

Assay of plasminogen activator (PA)

The activity of PA in 50 μ l of cell supernatants (prepared as for zymography) was indirectly estimated by hydrolysis of a specific chromogenic substrate H-D-Val-Leu-Lys-paranitroanilide 2HCl (2.5 mg/ml; Cs -2251; Chromogenix, UK) in the presence of Human Glu-plasminogen (15 μ M; Enzyme Research Labs, UK) in Tris-HCl buffer, 150 mM, pH 7.4 at 37 °C for 2 h. The intensity of formed H-D-Val-Leu-Lys-OH + pNA was measured spectrophotometrically at 405 nm using a microplate

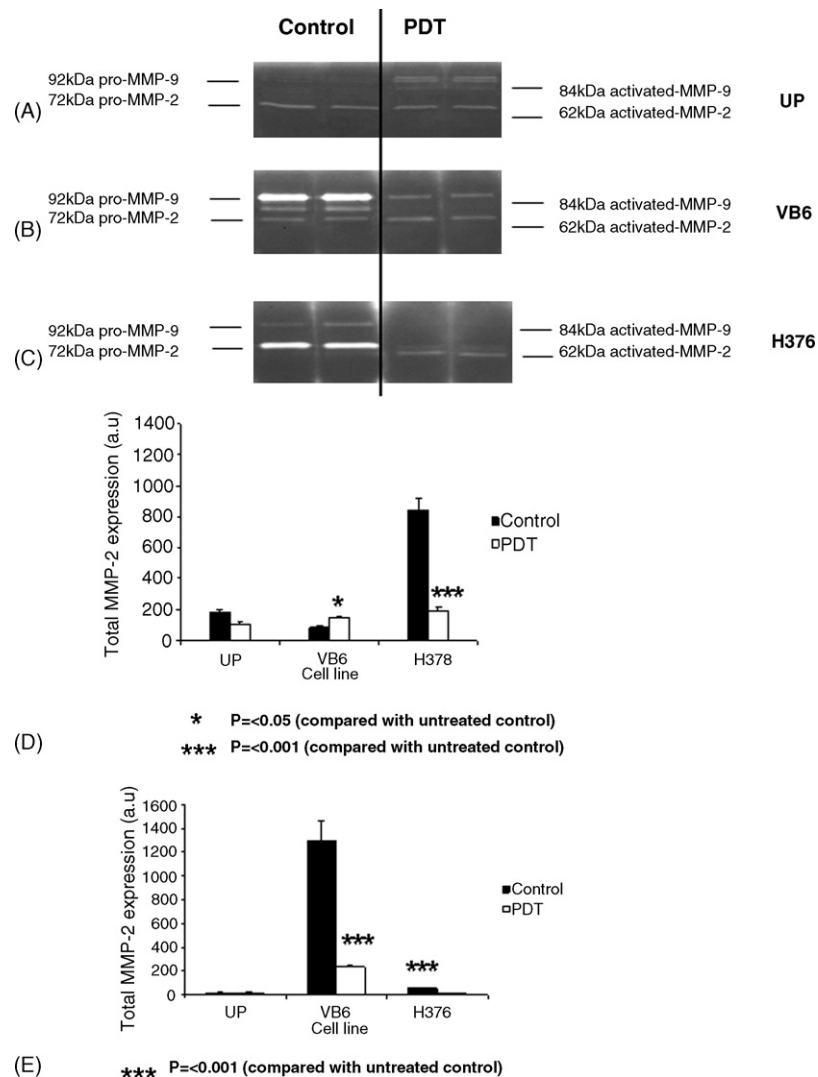


Figure 1 Zymography for MMP-2 and MMP-9. After treatment with sublethal doses of PDT (as determined from toxicity curve), cells were grown for 24 h in DMEM before supernatant sampling and cell counting. Samples containing equal protein were run on each gel with mw markers. The intensity of the bands was measured by densitometric analysis, and comparisons were made within each gel to determine relative changes in MMP activity. Comparison between zymograms should not be made due to variation in intensity of background staining. Zymogram showing PDT effect on MMP secretion by UP (A), VB6 (B) and H376 cells (C). Control samples were supernatants of the untreated cells. Densitometric analysis of zymograms showing MMP-2 (D) and MMP-9 (E) activity by UP, VB6 and H376 cells after exposure to PDT compared with untreated controls. Mean values of three experiments are given. (Error bars = \pm standard deviation).

reader (Multiscan, MCC/340; Flow Laboratories, Herts, UK). The concentration of uPA was estimated by interpolation from standard curve using known concentrations of uPA standards. As this assay measures both tissue plasminogen activator and uPA, therefore, the results cannot be attributed to either tissue plasminogen activator or urokinase plasminogen activator separately.

Statistical analysis

A two-sided ANOVA and an unpaired Student's *t* test were preformed. Results were considered significant when the value of $p \leq 0.05$. Each experiment was performed three times in triplicate.

Results

PDT regulates MMP-2 activity

The effect of PDT on the activity of MMP-2 by UP, VB6 and H376 cells was examined using substrate zymography assay. The supernatant samples of equal protein were run in 12% acrylamide separating gel and stained with Coomassie blue. The MMP-2 bands (Fig. 1A–C) revealed various profiles of MMP-2 lysis zones corresponding to the molecular weight expressed by each cell line. Supernatants of control cells, gelatinases of 72 kDa corresponded to the pro-MMP-2 were found to be highly expressed by H376 cells and VB6, while UP cells showed a relatively low expression. No band of gelatinases was detected corresponding to 62 kDa active-MMP-2 for all cell lines. After exposure to PDT, the total activity of MMP-2 was down-regulated 1.8-fold, 4.3-fold ($p < 0.001$) by UP and H376 cells, respectively, while in VB6 cells, total MMP-2 was upregulated 1.8-fold ($p < 0.05$) (Fig. 1D).

In order to support these results, an ELISA kit against MMP-2 was used, taking care to ensure that the same protein loading concentrations were used as in the zymography. The results showed that, following PDT treatment, the levels of total MMP-2 proteins were reduced by 9.6-fold and 2.2-fold only by the H376 ($p < 0.001$) compared to the control (Fig. 2A). No significant difference was found between PDT treated cells and the controls in UP and VB6 cells.

PDT modulates MMP-9 activity

The zymography profiles of MMP-9 lysis zones corresponding to molecular weights of 92 kDa (latent form) and 84 kDa (active form) gelatinases were

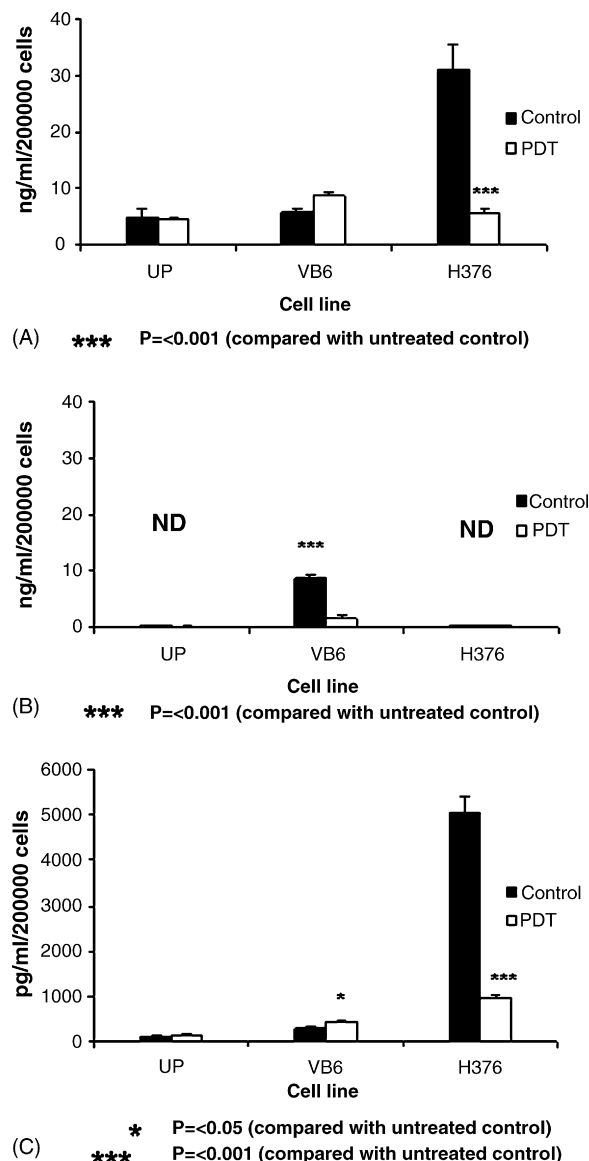


Figure 2 ELISA for MMP-9 (A), MMP-2 (B) and MMP-13 (C) after treatment with sublethal doses of PDT. Cell supernatants were prepared as for zymography. Sample (100 μ l) was added to each well and the concentration determined by interpolation from standard curve using known concentrations of MMP standards. Control samples were supernatants of the untreated cells. (Error bars = \pm standard deviation). ND = not detectable.

observed. In the untreated control, gelatinases (Fig. 1) of 92 kDa were highly expressed by VB6 cells yet only slightly by the H376 cells, while the 84 kDa was expressed by the VB6 cell line alone. After PDT treatment, both 92 and 84 kDa MMP-9 were reduced, with a significant decrease in expression by VB6 cells ($p < 0.001$). These results were confirmed and quantified by ELISA. Fig. 2B showed that, following PDT, the total MMP-9 levels were 5.4 times less in VB6 cells compared to the control ($p < 0.001$). No significant differences between

the control and PDT treated samples of UP and H376 cells was found.

PDT modulates MMP-13 activity

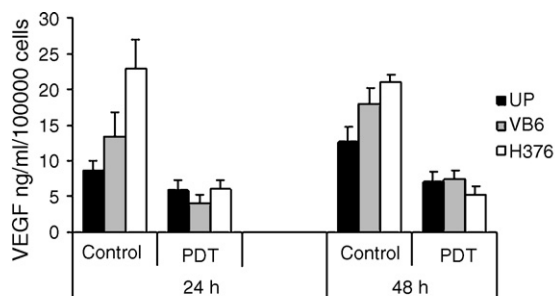
Commercial ELISA kit against pro-MMP-13, were used on wells loaded with cells supernatant following PDT. The result (Fig. 2C) revealed that in the untreated controls, MMP13 was remarkably expressed only by the H376 cells. After exposure to PDT, MMP-13 levels were significantly reduced 19.45-fold ($p < 0.001$) by the H376 cells and increased 1.6-fold ($p < 0.05$) by VB6 cells in comparison to the control samples.

PDT decreases VEGF activity

The effect of PDT on VEGF levels in the conditioned medium was determined by the ELISA assay (Fig. 3). The results showed that the level of VEGF in the medium of PDT treated cells, compared to the untreated controls over the 24 h culture period, were 3.3 and 3.8 times less in VB6 and H376 cells, respectively ($p < 0.05$). This was reduced to 2.4-fold and 4-fold ($p < 0.01$) when the cells were cultured for 48 h. No significant change in expression of VEGF by UP cells following PDT was found.

PDT decreases uPA activity

A chromogenic assay of uPA protein activity on supernatants of PDT treated cells revealed that



	VB6	H376
PDT vs untreated control (24 h)	$P < 0.05$	$P < 0.05$
PDT vs control (48 h)	$P < 0.01$	$P < 0.01$

Figure 3 ELISA for VEGF. After treatment with sublethal doses of PDT, cells were grown for 24 h or 48 h before supernatant sampling and cell counting. Cell supernatants were prepared as for zymography. Sample (100 μ l) was added to each well and the concentration determined by interpolation from standard curve using known concentrations of VEGF standards. Control samples were supernatants of the untreated cells. Mean values of three experiments are given. (Error bars = \pm standard deviation).

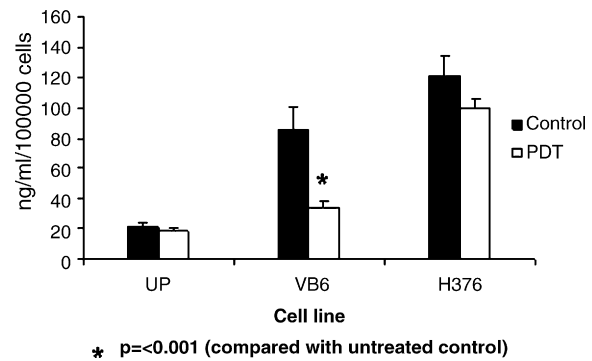


Figure 4 Chromogenic assay for uPA after treatment with sublethal doses of PDT. Cell supernatants were prepared as for zymography. Sample (50 μ l) was added to each well and the concentration determined by interpolation from the constructed standard curve prepared using range of human uPA substrate uPA standards. Control samples were supernatants of the untreated cells. Mean values of three experiments are given. (Error bars = \pm standard deviation). ND = not detectable.

total uPA activity was significantly decreased only by the VB6 cells ($p < 0.05$) in comparison to the untreated controls (Fig. 4).

Discussion

In the present study, we examined the effect of m-THPC-PDT on the expression of several known tumour promoting factors in head and neck cancer. While the results do not confirm whether PDT reduces the invasive potential of malignant head and neck cells, the treatment method does reduce the expression/activity of molecules known to play a key role in the potential of malignancy of such cells *in vitro*. It is striking that this suppression of invasiveness was observed at sub-lethal doses, which kill no more than 15% of cells, which implies the effectiveness of this emerging therapy. A reduction in the *in vitro* invasion of the cell lines was associated with the decreased activity of MMP-2, MMP-9, uPA and VEGF in most of the cell models used. However, one must remember that in the present study, while 2D culture systems were indeed useful for initial investigative studies of *in situ/in vivo* tissue organization, more relevant results would be obtained from 3D culture models e.g. investigating the effects of tumour cell invasion in collagen gels.

MMP-2 and MMP-9 are released from cells initially in inactive proform (zymogen) which is approximately 10 kDa larger weights than the activated form of the enzyme. On gelatinase zymography, MMP-2 and MMP-9 activity was occasionally seen as a doublet, reflecting the inactive and active

enzyme. In this study, both decrease and increase in intensity of both bands were noted following treatment with PDT. An increase of the active band (MMP-2) by H376 malignant cell line can be explained, as the proform of enzyme becomes activated during the process of denaturation and renaturation after gel electrophoresis. In addition, MMPs are often secreted with their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) which dissociated from MMP and lead to partial activation of MMP.²³ On the other hand, PDT also could have an effect on MMP/TIMP complexes and may lead to an imbalance between MMPs and their TIMP, while the decrease in the inactive form of MMP band by most of cell lines tested could be due to a reduction in synthesis of the matrix protein following exposure to PDT. This conflicts with the findings of other researchers,²⁴ who have explored the effect of Photofrin-mediated PDT on the expression and/or activity of MMPs, thus, an enhanced effect was found on MMP-9, but MMP-2 profiles were unchanged following PDT. One potential explanation of this conflict could relate to the different protocol applied, i.e. the use of broad spectrum fluorescence bulbs to initiate the photochemical reaction, which might included UVB bands, has been shown to stimulate the expression and secretion of different MMPs.^{25,26} It was demonstrated that, when fibroblast cells were treated with ALA-PDT, no change in MMP-2 secretion was observed, while the levels of MMP-1 and MMP-3 proteins increased by up to threefold, suggesting that MMP-1 and MMP-3 are produced by fibroblasts in response to photodynamic therapy (PDT) and are considered to be involved in the antisclerotic effects of ALA-PDT.¹⁹ This effects was not obvious on keratinocytes, since both enzymes, MMP-1 and MMP-3, were not altered significantly.²⁷ Studies by Du et al.²⁸ have analysed the effect of hypericin-based photodynamic therapy, on MMP-1 expression in two nasopharyngeal cancer (NPC) cell lines and in an animal tumour model, and found that an increase in MMP-1 protein and mRNA expression resulted in well-differentiated HK1 and poorly differentiated CNE-2 NPC cells *in vitro*. Similarly, there was up-regulation of MMP1 mRNA expression in hypericin-PDT-treated NPC/HK1-tumours.²⁸

In this study, the levels of uPA and VEGF were reduced by invasive cell lines. This agrees with the findings of Uehra et al., who reported a decrease in VEGF expression 24 h after PDT, and attributed this reduction to tumour re-oxygenation.²⁶ It has been recently revealed that the healthy tissue surrounding rat brain tumour expresses higher VEGF following PDT.²⁷ Taken together, these findings suggest that further research is needed in order to elucidate the PDT therapeutic outcome. In conclusion, this

in vitro study found that photodynamic therapy using m-THPC as a photosensitive drug had suppressed the activity of the tumour promoting factors MMP-2, -9 and -13, uPA and VEGF, at least in some, oral cancer cell lines. These, and our continuing investigations, may predict an effect of PDT on the invasion and metastatic potential of tumour cells and determine the treatment outcomes in patients undergoing PDT. Importantly, the cell lines displayed different behaviours in response to PDT; therefore, additional investigations are needed in order to elucidate the molecular events behind the different effects of PDT on the invasive behaviour of oral cancer, as well as further experiments required to study the issue of cell migration and invasion post-PDT application.

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