4,6,4'-trimethylangelicin shows high anti-proliferative activity on DU145 cells under both UVA and blue light

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Abstract

Objectives: Furocoumarins (psoralens and angelicins) have been already used under ultraviolet A light (UVA) for the treatment of skin diseases and cutaneous T-cell lymphoma. Besides their high anti-proliferative activity, some severe long-term side effects have been observed, for example genotoxicity and mutagenicity, likely strictly related to the formation of crosslinks. It has been demonstrated that blue light (BL) activation of 8-methoxypsoralen, an FDA-approved drug, leads to less mutagenic monoadducts in the DNA. So far, in this work the less toxic and more penetrating BL is proposed to activate 4,6,4'-trimethylangelicin (TMA), an already known UVA photoactivatable compound.

Materials and methods: Photocleavage, crosslink formation and oxidative damage were detected in pBR322 plasmid DNA treated with 300.0 $\mu\text{mol/L}$ TMA activated with various exposures of BL. Anti-proliferative activity, reactive oxygen species (ROS) formation and activation status of some signalling pathways involved in cell growth and apoptosis were verified on DU145 cells treated with 5.0 $\mu\text{mol/L}$ TMA plus 2.0 J/cm^2 of BL.

Results: Under BL-TMA, no mutagenic crosslinks, no photocleavage and neither photooxidative lesions were detected on isolated plasmid DNA. TMA showed high anti-proliferative activity on DU145 cells through induction of apoptosis. Besides ROS generation, the proapoptotic effect seemed to be related to activation of p38 and inhibition of p44/42 phosphorylation. Interestingly, the decrease in nuclear β -catenin was coupled with a significant dropping of CD44-positive cells.

Conclusion: Overall, our results indicate that TMA can be activated by BL and may be considered for targeted phototherapy of prostate cancer lesions.

1 | INTRODUCTION

Prostate cancer (PCa) is one of the most frequent disease among male subjects. Conventional therapies may lead to recurrence and progression of disease,¹ being necessary the development of new approaches. Among these, photodynamic therapy (PDT) is a promising strategy in delivering focal treatment in PCa.² PDT involves the use of a photosensitizer, quite harmless in the dark, that generates cytotoxic reactive oxygen species (ROS) upon visible light irradiation. Besides porphyrin derivatives in PDT, furocoumarins (ie, 8-methoxypsoralen, 8-MOP) are

employed with ultraviolet A light (UVA) in PUVA (psoralen plus UVA) therapy to treat several skin diseases for patients with moderate-to-severe psoriasis, eczema, vitiligo, mycosis fungoides³ and some autoimmune disorders such as cutaneous T-cell lymphomas, graft-vs-host disease, systemic sclerosis and Crohn's disease through an extracorporeal therapy called photopheresis.⁴

Furocoumarins comprise natural and synthetic compounds: linear molecules, so called psoralens, and the angular ones, the angelicins. The photobiological effects of furocoumarins plus UVA are mainly related to their capacity to bind DNA and form monoadducts (MAs) and

interstrand crosslinks (XLS), mainly with pyrimidine bases. Furthermore, furocoumarins produce ROS that impair cellular functions through lipid peroxidation, oxidation of guanine and strand breaks in nucleic acids, oxidation of proteins and inactivation of enzymes.⁵ It is known that the combination of 8-MOP and UVA radiation causes apoptosis of treated leucocytes and may cause preferential apoptosis of activated or abnormal T cells. Moreover, these apoptotic cells may promote immune tolerance, production of antigen-specific regulatory lymphocytes (CD4/8 T, B) and rebalance of immune system.⁶

Even though furocoumarins possess high chemotherapeutic potency under UVA and lack toxicity in the dark, genotoxicity, mutagenicity and skin phototoxicity have been observed.^{7,8}

In the attempt to improve the activity of psoralens and avoid severe side effects thought to be mainly related to the formation of XLS, a variety of derivatives, hopefully monofunctionals, have been synthesized. Although angelicins, due to their angular geometry, do not generally form XLS, some of them, that is 4,6,4'-trimethylangelicin (TMA), are reported to crosslink certain types of folded DNA upon UVA irradiation.⁹ 8-MOP was found to photoreact under blue light (BL) leading to less mutagenic lesions in the DNA, that is preferentially MAs over XLS.¹⁰ Recently, we have demonstrated that 8-MOP, upon BL, exerts anti-proliferative effects in DU145 PCa cells.¹¹ Furthermore, cells treated with 419 nm light resumed normal growth rates faster than cells which received the same UVA dose.¹²

2 | MATERIALS AND METHODS

2.1 | Irradiation apparatus

UVA irradiation was carried out using 1 Philips HPW 125 lamp, mainly emitting at 365 nm. BL was delivered to the samples by the Waldmann UV436HF apparatus, mainly emitting at around 417 nm. The total energy hitting the sample was monitored by means of a radiometer (Variocontrol; Waldmann, Villingen-Schwenningen, Germany), equipped with a Variocontrol UV Sensor (Waldmann). The radiant power emitted by the UVA and BL lamp was about 8 and 20 mW/cm², respectively. All samples (isolated DNA and cell cultures) were maintained at room temperature during irradiation.

2.2 | Detection of interaction between TMA and isolated DNA

Strand breaks, photooxidative damage and XL formation were verified on pBR322 plasmid DNA as described previously.⁹ DNA was irradiated with various BL and UVA radiant exposures (up to 15.0 J/cm²) with 300.0 µmol/L TMA (Figure S1), kindly provided by Professor Adriana Chilin (University of Padova). Samples kept in the dark or only irradiated were used as controls.

2.3 | Cell cultures

Human PCa cell line DU145 (Cell Lines Service, GmbH, Eppelheim, Germany) was cultured as described previously.⁹ Cells (2.6×10^4 /cm²)

were seeded into 24-well plates. After 24 hours, medium was removed and replaced with MEM w/o phenol red (Sigma-Aldrich) containing TMA concentrations up to 10.0 µmol/L. After 1 hour of incubation in the dark, cultures were irradiated with UVA light or BL (2.0 J/cm²). Then, media were removed and replaced with fresh medium. Sphere formation, ROS generation, CD44 expression and apoptosis rate were determined as in.⁹ Briefly, to evaluate sphere formation, immediately after irradiation, cells were detached, seeded (3.3×10^3 /cm²) into each well of low-attachment 24-well plates and cultured for 13 days with sphere formation medium composed of DMEM/F-12 (3:1, v/v) (Gibco, Waltham, MA, USA) supplemented with 0.4% BSA (Sigma-Aldrich), 0.4% B27 (Gibco), 1% L-glutamine and 1% antibiotics. Then, cultures were observed using phase-contrast microscope Nikon T-s (Shinagawa, Tokyo, Japan) and photographed at 40x magnification. Spheres diameters and number were determined using ImageJ tools.

2.4 | Western blot

At 24 hours, total proteins were isolated by lysis buffer, whereas nuclear proteins by Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA). Protein separation, electroblotting and detection of target proteins were carried out as in⁹ with primary polyclonal rabbit anti-human GAPDH, phospho(Thr180/Tyr182)-p38, phospho(Thr202/Tyr204)-p44/42 (Cell Signaling Technologies, Danvers MA, USA), β-catenin and laminB1 (Santa Cruz, Dallas, Texas, USA) (1:500, v/v) antibodies. Data were reported as ratio within target protein and relative housekeeping protein expression.

2.5 | Statistical analysis

Data, obtained from at least 3 experiments, were expressed as mean ± the standard error of the mean. The difference between groups was evaluated using analysis of variance (ANOVA) and Student's *t* test.

3 | RESULTS

3.1 | Lesions induced by TMA on DNA under BL and UVA irradiation

Thin-layer chromatography followed by [H]NMR demonstrated that, although at a lower yield than under UVA, TMA plus BL was able to form MAs in salmon testes DNA (data not shown). To verify the ability of TMA to form XLS, alkali denatured linearized pBR322 DNA was loaded on denaturing agarose gel (Figure 1A). When XLS were formed, double-strand (ds) DNA was unable to denature and ran slower than the 2 single strands (ss). XLS formed even at the lowest UVA exposure (2.0 J/cm²) along with the ss-DNA; their formation seemed complete upon 12.0 and 15.0 J/cm². The slower migration of crosslinked DNA was a function of the light dose reflecting the increasing number of XLS and MAs per molecule. On the contrary, at the highest doses of BL, TMA behaved monofunctionally without any trace of undenatured crosslinked DNA. Moreover, the 2 ss-DNA bands appeared to run faster compared to the controls as the

FIGURE 1 (A) Photosensitization of linearized pBR322 DNA (L) with 300.0 μmol/L TMA under UVA light and BL. The samples were treated with 0.2 mol/L NaOH, boiled for 10 min, placed quickly in ice and loaded in alkaline 1% agarose. L-DNA: undenatured DNA. L-DNA+UVA+NaOH and L-DNA+BL+NaOH: denatured and irradiated (15.0 J/cm²) samples. ds: double strand. ss: single strand. (B) Relaxation assay in supercoiled pBR322 DNA. pBR322 DNA treated with 300.0 μmol/L TMA was irradiated with UVA and BL. In the first 2 wells, dark (super coiled, SC) and irradiated pBR322 DNA samples (UVA and BL, 15.0 J/cm²) were loaded. OC: open circular. (C) Photooxidative DNA damage. DNA base modifications photoinduced by TMA under UVA light and BL. SC: untreated SC plasmid pBR322 DNA; SC+ ENDO: pBR322 DNA digested with Endo III in the dark (0) and upon irradiation (2.0, 7.5 J/cm²); TMA + ENDO: pBR322 DNA with 300.0 μmol/L TMA in the dark (0) and upon irradiation after treatment with the enzyme Endo III

light increased, likely due to the presence of TMA-MAs containing furohydroxycinnamic derivatives resulting from the breakage of the pyrone ring by the alkali (Figure S2). Therefore, 2 more negative charges per TMA molecule bound to the DNA increased the migration of the separate strands towards the anode. These negative charges were surely present in the TMA crosslinked ds-DNA that contains also MAs, but probably did not increase its migration being masked inside the folded DNA.

When irradiated with BL, TMA did not induce any detectable cleavage on DNA and only the supercoiled (SC) form was present in all the samples (Figure 1B). On the contrary, under UVA, TMA relaxed the DNA with an increasing efficacy as the exposures augmented. Fragmentation of the strands was also visible as a smearing attesting the presence of multiforms of DNA differently modified by a varied number of photoadducts. It is noteworthy that as long as the SC DNA migrated slower as the UVA irradiation increased, under BL it happened the opposite. This

FIGURE 2 Sphere-forming assay. Cultures were irradiated with 2.0 J/cm² of UVA light (A) or BL (B). Student's *t* test, **P* < .05 vs untreated cultures taken as control

behaviour is likely due to the different types of photoadducts formed under the 2 lights and the resulting structure acquired by the plasmid.

The detection of DNA oxidative damage was achieved by the base excision enzyme Endo III, which recognizes 5,6-dihydropyrimidines induced by hydroxyl radicals and removes the oxidized base, leaving abasic sites. Notably, TMA in combination with BL induced less oxidation than BL alone (Figure 1C). However, both BL and UVA light

alone were able to induce a remarkable oxidative damage, higher than in the presence of TMA. Indeed, TMA molecules by absorbing the incoming radiation likely protected the macromolecule from photooxidation.

The quantification by densitometric analysis of each band of the gels shown in Figure 1 is reported in Figure S3 of the Supporting Information.

FIGURE 3 Effects on cell apoptosis, ROS formation and CD44 expression. Cultures were irradiated with 2.0 J/cm² of UVA light or BL. (A, B) Cell apoptosis. PI: propidium iodide; ANX: annexin V. (C) ROS generation. TBHP: tert-butyl hydrogen peroxide. (D) Cytofluorimetric data are reported as percentage of CD44-positive cells. Student's *t* test, **P* < .05 vs untreated cultures taken as control. Representative micrographs of untreated cell cultures (E) and cultures exposed to 5 μmol/L TMA plus BL (F). Immunoreactivity towards CD44-stained green, whereas nuclei were labelled with DAPI (blue). Bars: 20 μm

3.2 | Effects on DU145 PCa cells

To evaluate the anti-proliferative activity of TMA, DU145 cells were incubated with TMA (0.01–10.0 $\mu\text{mol/L}$) and irradiated with 2.0 J/cm^2 of UVA or BL. Then, detached cells were seeded to carry out the sphere-forming assay. TMA was more effective after UVA irradiation than under BL one (Figure 2). Lack of sphere formation was observed with 0.05 $\mu\text{mol/L}$ TMA upon UVA ($\text{IC}_{50} = 0.017 \mu\text{mol/L}$), whereas 100-fold higher concentration (5.0 $\mu\text{mol/L}$) was needed to achieve the same effect after BL ($\text{IC}_{50} = 2.47 \mu\text{mol/L}$). No significant variation was detected with samples treated with light alone or TMA in the dark. Further experiments were performed by treating monolayer cultures with 0.05 and 5.0 $\mu\text{mol/L}$ TMA activated by UVA light and BL, respectively.

Reactive oxygen species generation, determined immediately after cell treatments, was significantly enhanced by photoactivated TMA. TMA plus UVA induced higher levels of ROS than plus BL (Figure 3C).

Small but significant increases were noted in cultures treated with the compound without light. ROS generation was unaffected by UVA light or BL alone.

At 48 hours, TMA with either UVA light and BL significantly enhanced the percentages of apoptotic annexin V-positive cells and, at lower yield, those of necrotic propidium iodide-positive cells compared to non-treated cultures (Figure 3A,B). No significant variations were detected in cultures treated with light alone or TMA in the dark. Nevertheless, the

presence of large vacuoles inside the treated cells suggests that also autophagy may contribute to the anti-proliferative effects of TMA.¹³

DU145 cell cultures comprised a high percentage (about 98%) of cells expressing CD44 (Figure 3D). Significant decreases in CD44-positive cells were detected at 48 hours from treatment with the TMA under both lights, being BL more effective (about 12%) than UVA light (about 35%). Although to a lesser extent than upon TMA photoactivation, 5.0 $\mu\text{mol/L}$ TMA in the dark and BL alone lowered CD44 expression.

The expression levels of phosphorylated p38 were significantly enhanced in cultures treated with TMA plus UVA light or BL compared to non-treated cells (Figure 4, Figure S4). On the contrary, the amount of phosphorylated p44/42 was decreased by photoactivated TMA. Notably, also cultures treated with TMA alone presented lower expression of ERK $\frac{1}{2}$ than controls. Nuclear β -catenin expression was significantly lowered by TMA plus UVA light or BL.

DISCUSSION

Our data demonstrated that TMA activated by either UVA light or BL exerts strong anti-proliferative effects on DU145 cells through induction of apoptosis. Although inhibition of sphere formation under BL was achieved at concentration 100-fold higher than under UVA, different kinds of lesions were induced. Indeed, MAs were formed in *salmon testes* DNA under both lights, but XLs were detected in

FIGURE 4 WB analysis. Cultures were treated with 0.05 $\mu\text{mol/L}$ TMA plus 2.0 J/cm^2 UVA light or 5.0 $\mu\text{mol/L}$ plus BL. The quantification of protein expression levels was performed by densitometric analysis of the bands using ImageJ processing software. Data are reported as ratio between target protein and relative GAPDH expression. * $P < .05$ vs untreated cultures taken as control

pBR322 DNA only under UVA light. Moreover, both TMA-induced DNA photocleavages and photooxidations under UVA were not detected under BL. Accordingly, the production of ROS measured in the TMA-treated cells was less efficient under BL compared to UVA light. It is known that the oxidative lesions are as important as strand breaks for cellular function and survival.¹⁴

The results obtained under BL are very satisfactory assuming that the formation of less mutagenic lesions could be negligible and even absent in the DU145 cells when TMA activation is achieved through less noxious and more penetrating wavelengths. However, a significant amount of DNA lesions, that is MAs, can justify the effects on cell growth. In previous experiments with 8-MOP on the same cells, we assumed that XLs likely play a minor role in the anti-proliferative effects.⁹

Our findings indicate that the strong TMA photocytotoxicity can be related to the kind and number of DNA lesions; BL, compared to UVA light, can activate TMA as well, although at higher concentrations, but without the potential risk of mutagenic lesions.¹⁵

TMA activated by both UVA light and BL induced cell death by apoptosis enhancing the phosphorylation of p38 whose pathway modulates cell cycle checkpoints, cell apoptosis and autophagy.¹⁶ Furthermore, as in photopheresis, apoptotic bodies induced by TMA treatment with BL and UVA light, and the following antigen presentation by dendritic cells (DCs), could modulate the immune system, increasing the photoantiproliferative effect. It must be also noted that, despite UVA light, BL irradiation (2.5 J/cm²) does not impair the in vitro differentiation and maturation of human monocyte-derived DCs and reduces the production of pro-inflammatory cytokines.¹⁷

On the contrary, cultures treated with photoactivated TMA showed decreases in phosphorylation of p44/42 MAPK (ERK½), usually activated by growth factors and mitogens leading to anti-apoptotic effects.¹⁸ Taken together, our data agree to the observation that p38 signalling can indirectly regulate ERK½.¹⁹

Decreases in p44/42 phosphorylation were detected also in cultures treated with the compound in the dark, suggesting that TMA could interact with molecular targets without photoactivation. It has been demonstrated that in the dark some angelicins are able to inhibit NF-kB/DNA interactions demonstrating anti-inflammatory profile²⁰ and TMA has shown to be an effective enhancer of cystic fibrosis transmembrane conductance regulator.²¹

Photoactivation of TMA by either UVA light or BL affected the canonical Wnt signalling pathway as the nuclear accumulation of β -catenin decreased. The binding of Wnt ligands to Frizzled (Fz) receptors or low-density lipoprotein receptor-related protein 5/6 (LRP5/6) co-receptors inhibits degradation of cytoplasmic β -catenin that translocates into the nucleus leading to transcription of genes. Dysregulation of Wnt signalling has been associated with several types of cancers, including PCa.²²

Notably, the decrease in nuclear β -catenin was coupled to significant lowering of CD44-positive cells, being TMA plus BL more effective than TMA plus UVA light. The reduction in CD44-positive cells detected also in cultures treated with the compound alone may be related to the lowering of p44/42 MAPK phosphorylation, as pERK regulates CD44 expression.²³

Several evidences have shown that CD44 is overexpressed in several cancers as marker of cancer stem cells (CSCs). Thus, in the last years, the therapeutically targeting of CD44 has been attempted through various approaches.²⁴ In PCa, CD44 expression levels positively correlate to the cancer stage and can predict the therapeutic response to the treatment.²⁵ Our findings suggest a relation between CD44 and Wnt pathway. CD44 is not only a Wnt target gene but also a positive regulator of Wnt pathway through interaction with LRP6.²⁶ Indeed, nuclear translocation of β -catenin is inhibited by silencing CD44 in several cancer cells, including PCa cell lines.²⁷

Overall, our results indicate, for the first time, that TMA can be activated by BL, although at higher concentrations than UVA light, decreasing the risk of mutagenic lesions on treated cells. Thus, the toxicity of both light and compound itself on neighbouring healthy cells could be overcome with a BL-TMA-targeted treatment in the site of the cancer lesions. Besides ROS generation, the proapoptotic effects on DU145 cells seem to be related to activation of p38 and inhibition of p44/42 phosphorylation. Interestingly, photoactivated TMA was able to affect Wnt signalling and CD44 expression, both involved in CSC growth and renewal. To investigate the interaction between these 2 hallmarks of CSCs after treatment with BL-TMA, molecular docking studies will be carried out to verify whether TMA can bind CD44 and expression levels of Wnt target genes as well as glycogen synthase kinase-3b, involved in β -catenin degradation, will be determined.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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