Control of cell death levels using TH9402-based PDT treatment on fresh PBMC, and potential application to patients with cGvHD.

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I – INTRODUCTION

Celmed Biosciences Inc. developed photoactive rhodamine derivatives as photosensitizers, among which 4,5-dibromorhodamine-123 (called TH9402) was selected because of its photophysical properties, low toxicity, and stability. The bromination of the original non-phototoxic rhodamine-123 increased the quantum yield of the molecule and generated a highly potent photosensitizer. Rhodamine is a dye capable of entering all cells, and is extruded through an active transport mediated by plasma membrane P-glycoprotein pumps (Pgp). Pgp, the product of the gene *mdr1*, is expressed not only in normal stem cells, but also in T lymphocytes. Interestingly, it has been proposed that activation of T-cells may lead to the inactivation of Pgp and the subsequent cell retention of rhodamine. Like rhodamine-123, its structural analog TH9402 enters lymphocytes and accumulates preferentially in activated lymphocytes. Its phototoxicity is triggered upon exposure to radiation with a wavelength in the visible spectra at 514 nm, emitted by Theralux™ device, and mediated primarily by the production of singlet oxygen species resulting in oxidative damage within the mitochondria as a result of drug localization. This procedure, also termed PDT, is a process that has already been evaluated in clinical studies for the purge of various malignancies including chronic myelogenous leukemia (CML) and B-lineage non-hodgkins lymphoma (NHL) from autologous grafts of hematopoietic stem cells.

Based on results obtained from clinical studies using 8-MOP/UVA based extracorporeal phototherapy (ECP) we believe that the TH9402/Theralux™ based process (PDT) could be beneficial to patients with cGvHD, with minimal long-term carcinogen risks, since the TH9402 cytotoxic effect is not based on DNA damage and no UV source is used. As our PDT process triggers an apoptotic mechanism originating from mitochondria, it is believed that once re-infused to the patient with cGvHD, the dying mononuclear cells will expose phosphatidylserines on the cell surface. Their subsequent capture and phagocytosis by macrophages and dendritic cells would trigger the secretion of specific cytokines. Consequently, some immunomodulatory activity leading to tolerance is expected, as described previously for cGvHD patients treated by ECP.

The results presented here show the effect of PDT on PBMC isolated from healthy volunteers and cGvHD patients, as well as the possible mechanism by which it may induce apoptosis of the treated cells.

II – METHODS

For the experiments described below, samples of whole blood or apheresis were collected from healthy donors or cGvHD patients as volunteers. Red blood cells were removed from blood by Ficoll gradient and from apheresis by using the ACK lysing buffer. After wash, PBMC were resuspended at $5x10^6$ cells/ml in coloration media containing various concentrations of TH9402 and incubated at 37°C, 5% CO_2, for various periods of time, as mentioned in figure legends. After coloration, cells were exposed to visible light on the Theralux™ device for the photoactivation of TH9402. The usual light intensity delivered to the cells was $5J/cm^2$, excepted for the dose-effect study in which cells were exposed to 0, 1, 2 or $5J/cm^2$.

During the coloration period, cellular incorporation of TH9402 was monitored by measuring the cellular mean fluorescence intensity (MFI) by flow cytometry at different time-points.

After treatment, cells were washed and incubated at 37°C, 5% CO_2, in order to analyze PDT-induced cell death, which was also studied using proliferating Jurkat T cells and PBMC stimulated with phytohemagglutinin (PHA). Thus, the exposure of phosphatidylserines was measured by AnnexinV/PI staining, the mitochondrial membrane depolarization was analyzed with the fluorescent dye JC1, and cells with DNA fragmentation were quantified by TUNEL assay. The analysis of DNA ladder formation was performed by the electrophoresis of purified nucleic acids in a 2% agarose gel and DNA was visualized with SYBR Green. Cell viability was assessed by counting cells in presence of the cell-impermeable dye trypan-blue.

The role of caspases in PDT-induced cell death was assessed with the use of the pan-specific caspase inhibitor Z-VAD-fmk. As a positive control for inhibition of apoptosis by Z-VAD-fmk, we used the well known system of Jurkat cells incubated with the caspase inhibitor and treated with the DNA-damaging agent camptothecin.

IV – CONCLUSION

We developed a simplified TH9402/Theralux™-based PDT process for the treatment of peripheral blood cells. The kinetics of TH9402 incorporation are quite consistent, with very low intra- and inter-donor variation. There is an obvious correlation between the concentration of TH9402 in the coloration media, the proliferating state of the cells, and the incorporation of TH9402 within the cells. In addition, there is a clear dose-response relationship between TH9402 concentrations in coloration media and percentage of cell death post-treatment. And there is no significant difference between PBMC isolated from cGvHD patients and healthy donors in respect to this dose-response.

Studies aimed to depict the mechanism of cell death suggest that photoactivation of TH9402 triggers various mechanisms of cell death originating in mitochondria such as caspase-dependent apoptosis, caspase-independent apoptosis, or a mix of apoptosis/necrosis, depending on the dose of TH9402 as well as on the activation state of treated cells. Moreover, the sensitivity of activated lymphocytes to PDT is significantly higher than the sensitivity of resting lymphocytes.

Overall, our results provide evidence that TH9402/Theralux™-based PDT could be beneficial to patients with cGvHD, inducing the preferential killing of alloreactive and autoreactive lymphocytes.

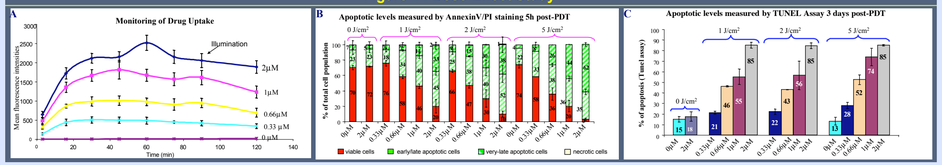
III – RESULTS

1 – Development of PDT conditions

1.A. – Levels of cell death directly correlates with TH9402 concentration and light intensity

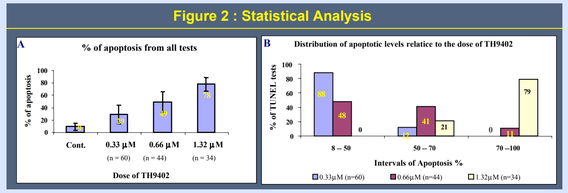
We analyzed the correlation between the concentration of TH9402 and the energy of illumination with the level of PDT-induced apoptosis, using PBMC isolated from 2 healthy volunteers. AnnexinV/PI and TUNEL assays were done in duplicate. The monitoring of cell MFI showed that the level of drug uptake by cells was directly proportional to the concentration of TH9402 (Fig 1A). Cells were illuminated after 90min of coloration. The levels of early apoptosis measured by AnnexinV/PI staining 5 hours post-PDT were proportional to both of TH9402 concentration and energy of illumination (Fig 1B). The correlation of late apoptosis with the energy of illumination was attenuated in cells fixed 3 days post-PDT, whereas the levels of apoptosis were still directly proportional to the dose of TH9402 (Fig 1C), probably because a portion of cells detected as viable by AnnexinV/PI staining became apoptotic later. When illuminated with $5J/cm^2$, the final apoptotic percentages 3 days post-PDT were on average 28%, 52%, 74% and 85% when cells were incubated with 0.33, 0.66, 1 and 2µM of TH9402, respectively (Fig 1C). TH9402 (2µM) and illumination ($5J/cm^2$) alone were both inoffensive.

Figure 1 : Dose-Effect Study



The data obtained from samples isolated from 44 subjects, n being the number of treatments done at each condition, were compiled to determine the most predictable results that could be obtained with different doses of TH9402. Incubating cells with 0.33, 0.66 or 1.32µM of drug gave 29±15%, 49±17% and 78±11% of apoptosis, respectively, with a background of 9±6% for the controls (Fig 2A). 88% of the treatments with the low dose (0.33µM/5J) gave less than 50% of apoptosis whereas 100% of the treatments with the high dose (1.32µM/5J) gave more than 50% of apoptosis, with 79% of them leading to 70-100% of apoptosis (Fig 2B).

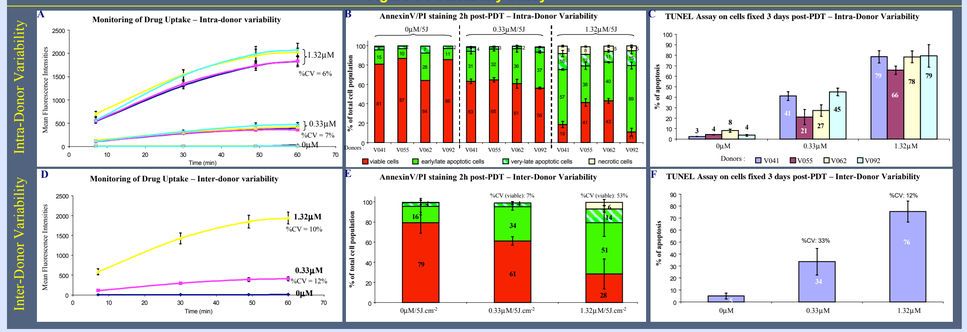
Figure 2 : Statistical Analysis



1.B. – TH9402-based PDT shows low variability in PBMC isolated from healthy donors

The intra- and inter-donor variabilities in drug uptake and apoptosis were evaluated using PBMC isolated from 4 healthy volunteers subjected to PDT in triplicate for each condition. The cellular uptake of TH9402 was consistently proportional to the dose of TH9402, although with some variation between donors (Fig 3A,3D). With 0.33µM of TH9402, intra- and inter-donor variation (CV) for MFI values were respectively 7% and 12%, and 6% and 10% with 1.32µM of TH9402. Cells were illuminated after 45 minutes of coloration. The levels of early and late apoptosis induced by the 2 doses of TH9402 showed limited intra-donor variability (Fig 3B, 3C). At the inter-donor level, the percentages of late apoptosis were on average 34±10% and 76±10% when cells were treated with 0.33 and 1.32µM of TH9402, respectively (Fig 3F). The difference in sensitivity to 0.33µM TH9402 between donors was reflected by a CV of 33% , which was reduced to 12% with 1.32µM of TH9402.

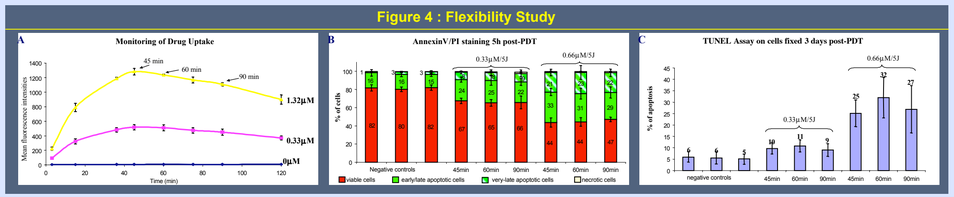
Figure 3 : Variability Study



1.C. – TH9402-PDT coloration time appears robust in healthy donors

The efficacy of PDT to induce apoptosis was tested on PBMC isolated from 3 healthy volunteers incubated with TH9402 for 45, 60 or 90 minutes. The drug uptake was monitored by FACS during coloration (Fig 4A) and the levels of apoptosis were assessed by AnnexinV/PI staining 5h post-PDT (Fig 4B) and TUNEL assay 3 days post-PDT (Fig 4C). Final levels of apoptosis were on average 10% and 28% for cells incubated with 0.33 and 0.66µM of TH9402, respectively (Fig 4C). Interestingly, incubating cells for 45, 60 and 90 minutes with the drug had no significant impact on the levels of early (Fig 4B) and late (Fig 4C) apoptosis.

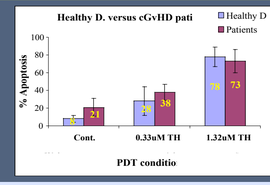
Figure 4 : Flexibility Study



2 – Application to Cells Isolated from cGvHD Patients

The comparison of the data obtained from the analysis of apoptotic levels in cells fixed 3 days post-PDT revealed similar sensitivities to the treatment between cells isolated from healthy volunteers (35 subjects) and cGvHD patients (10 subjects) (Fig 5). When treated with 0.33µM of TH9402, cells isolated from cGvHD patients or from healthy donors showed no significant difference with 38±9% and 28±16% of apoptosis 3 days post-PDT, respectively. Similarly, there was also no significant difference between those cells treated with 1.32µM of TH9402, with 73±13% and 78±11% of apoptosis, respectively.

Figure 5 : PDT on cGvHD Patient Samples

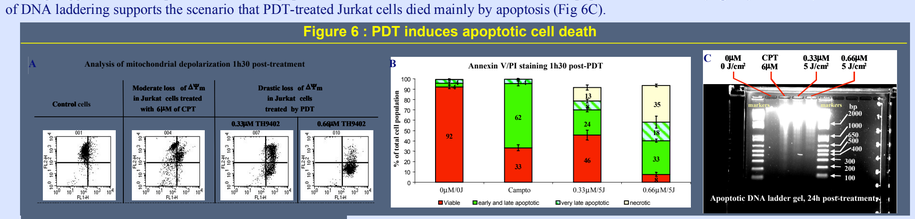


3 – Mechanistic Studies

3.A. – PDT induces apoptotic cell death

The effect of the PDT-treatment on the Jurkat T-cell line was compared to the effect of the well-known apoptosis inducer camptothecin (6µM, 4h). The mitochondrial depolarization was moderate in cells treated with camptothecin, as expected with DNA damaging agents (Fig 6A). In PDT-treated cells, the mitochondrial depolarization was higher and proportional to the dose of TH9402, reflecting the mitochondrial localization of the dye. The AnnexinV/PI staining suggested that camptothecin induced apoptosis whereas PDT seemed to induce a mix of apoptosis and necrosis (Fig 6B). However, the visualization of DNA laddering supports the scenario that PDT-treated Jurkat cells died mainly by apoptosis (Fig 6C).

Figure 6 : PDT induces apoptotic cell death

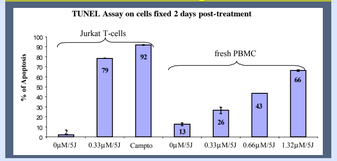


As suggested by the profile of mitochondrial depolarization (Fig 6A), PDT may induce the release of the mitochondrial matrix content, including oxygen reactive species (mROS). In turn, released mROS may induce an oxidative stress with the peroxidation of the plasma membrane compounds, altering its permeability. Then cells may become positive to PI (Fig 6B) although they die by apoptosis (Fig 6C). Thus, TH9402-based PDT may induce a necrotic-like apoptosis. Finally, as compared to resting PBMC treated in previous experiments, proliferating Jurkat cells seemed to be much more sensitive to PDT treatment since they showed 76% and 66% of apoptosis 24h post-PDT with 0.33 and 0.66µM of TH9402, respectively (Fig 6D). This difference may reflect the higher mitochondrial activity in proliferating cells.

3.B. – Proliferating Jurkat cells are more sensitive to PDT than resting PBMC

In fact, when proliferating Jurkat cells and resting PBMC were treated in parallel, 79% of Jurkat T-cells were apoptotic 2 days post-treatment when treated with 0.33µM of TH9402, whereas only 26%, 43% and 66% of PBMC were apoptotic when treated with 0.33µM, 0.66µM and 1.32µM of TH9402, respectively (Fig 7).

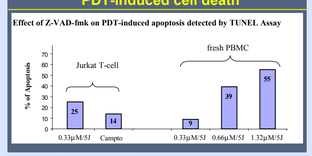
Figure 7 : Jurkat cells are more sensitive to PDT than resting PBMC



3.C. – PDT-induced apoptosis may be either caspase-dependent or caspase-independent

In parallel to the treatments discussed in 3.B., cells were treated in the presence of Z-VAD-fmk. The caspase-inhibitor induced a significant decrease of apoptosis in both cell types when treated with 0.33µM of TH9402 (Fig 7,8). However, the inhibition of apoptosis of PBMC treated with 0.66µM or 1.32µM of TH9402 was negligible (Fig 7,8), suggesting that TH9402 may trigger a caspase-dependent apoptosis in PBMC treated with 0.33µM and a caspase-independent apoptosis above that dose.
(Similar results were obtained with PBMC from 4 donors).

Figure 8 : Effect of Z-VAD-fmk on PDT-induced cell death



4 – Activated PBMC are more sensitive to PDT than resting cells

PBMC isolated from 3 donors were stimulated with PHA for 3 days prior to PDT (Fig 9A). In PHA-stimulated PBMC, activated lymphocytes incorporated significantly more TH9402 than non-activated lymphocytes (Fig 9B). The results obtained by TUNEL assay from stimulated and non-stimulated cells showed: i) a strong linear correlation between apoptotic levels and concentrations of TH9402, and ii) that PHA-stimulated cells were significantly more sensitive than non-stimulated cells to PDT ($\alpha_{Stim} > \alpha_{NS}$) (Fig 9C).

Figure 9 : Comparison between activated and resting PBMC sensitivity to PDT treatment