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CHAPTER IV

POSITIVELY CHARGED PORPHYRINS: A NEW SERIES OF PHOTOSENSITIZERS FOR PATHOGEN INACTIVATION IN RED BLOOD CELL CONCENTRATES

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ABSTRACT

BACKGROUND: PDT could be a way to inactivate pathogens in RBCC. The objective of this study was to characterize the virucidal activity and RBC-damaging activity of a series of cationic porphyrins. Using the most efficacious photosensitizer, various in-vitro human RBC quality parameters and in-vivo RBC survival in Rhesus monkeys were evaluated.

STUDY DESIGN AND METHODS: RBCC, spiked with 5 log₁₀ of extracellular VSV, were treated with porphyrins (25 μM) and red light (100 W/m²) and assayed for virucidal activity. In-vitro RBC quality variables were assessed during 5 weeks of storage in various additive solutions. In-vivo survival was investigated with autologous RBCs in Rhesus monkeys.

RESULTS: Tri-P(4) was by far the best sensitizer of a series tested, giving the least hemolysis under conditions that resulted in 5 log₁₀ kill of extracellular VSV. Under our experimental conditions, the percentage hemolysis in treated cells was 5.1% ± 1.1% after five weeks of storage in SAG-M compared to 1.9% ± 1.1% in the untreated control. Storage in AS-3 resulted in hemolysis of 2.3% ± 1.9%. With the exception of IgG binding and potassium leakage, quality parameters of RBCs remained unchanged after photodynamic treatment. Addition of reduced glutathione (GSH) during treatment reduced IgG binding. The 24-hour recovery and the half-life span T₅₀ of treated RBCs in Rhesus monkeys were satisfactory.

CONCLUSION: Porphyrin Tri-P(4) may be a suitable photosensitizer for decontamination of RBCC. However, further exploration to optimize the method is necessary to reach clinically acceptable goals.

INTRODUCTION

Blood transfusions in developed countries are generally considered to be safe, however recent reports revealed that maximal vigilance is still needed and that considerable efforts are required to maintain optimal safety of blood transfusion practices.

The emergence of new viruses, such as the WNV in the North American continent or TTV in Asia,^{1,2} the potential risk of transmission of variant PrP^{Sc},^{3,4} the bacterial contamination of blood products responsible for sepsis,^{5,6} the reduction but not the elimination of the window-periods with increasing sensitivity of viral and bacterial testing,^{7,8} and in less developed countries, the lack of adequate and systematic viral and bacterial screening⁴ promote the development of new strategies to increase the safety of the blood products.⁹

Chemical and photodynamical pathogen inactivation technologies are the most recent developments for the reduction of pathogen transmission via blood transfusions. The chemical Pen 110^{10,11} and Frangible Anchor Linker Effector compounds S-303¹² are under investigation for the decontamination of RBCC. These compounds cause irreversible alkylation of nucleophilic groups in macromolecules, including nucleic acids, rendering pathogens noninfectious and inhibiting white blood cells (WBC) proliferation.^{13,14} However, both chemicals are toxic and must be removed before transfusion of the RBCC. PDT relies on the activation of a light-sensitive agent (photosensitizer) in an oxygen-rich environment, resulting in the generation of reactive oxygen species of which singlet oxygen is the most important. Singlet oxygen can react with proteins, lipids and nucleic acid. Because of its short lifetime, damage is localized at the binding site of the photosensitizer. The efficacy and selectivity of the photodynamic treatment depend on the physical and chemical properties of the photosensitizers used. Various photosensitizers, including phenothiazines DMMB and MB¹⁵⁻¹⁷, phthalocyanines¹⁸⁻²¹, merocyanines²⁰, riboflavin (vitamin B2)²², benzoporphyrin derivative monoacid ring A BPD-MA and, photofrin²³ have been tested for their virucidal and bactericidal activities and, their abilities to preserve the functional integrity of the therapeutic RBCs. All dyes tested so far induced considerable RBC damage under conditions inactivating 5 log₁₀ of enveloped viruses. To reduce RBC

damage, scavengers such as dipyridamol, and vitamin E derivatives (Trolox, vitamin E succinate),¹⁵ reduced glutathione (GSH), mannitol, or cremophore^{19,21} were added to the RBCC.

The aim of our study was to find a photosensitizer that efficiently inactivates viruses without RBC damage. We focused on a series of positively charged porphyrin derivatives which have been shown to have a strong photo-bactericidal potential.^{24,25} From this series, mono-phenyl-tri(N-methyl-4-pyridyl) porphyrin, Tri-P(4), gave the most promising results and was investigated in more detail. Various RBC variables were investigated after storage in various additive solutions. In addition, to evaluate the in-vivo survival of Tri-P(4)-treated RBCs, a limited study, not meant as a formal preclinical study, was performed using two Rhesus monkeys.

MATERIAL AND METHODS

STORAGE SOLUTIONS

AS-3 (Haemonetics Co, Braintree, MA) contains 70 mM NaCl, 61.1 mM glucose, 2.22 mM adenine, 2 mM citric acid, 20 mM Na₃citrate and 15.5 mM Na₂HPO₄. SAG-M (Baxter, Utrecht, the Netherlands) contains 150 mM NaCl, 1.25 mM adenine, 50 mM glucose and 29 mM mannitol. SAG-S and SAG-D have the same composition as SAG-M, except that mannitol is replaced by 29 mM sucrose or by 29 mM dextran-1000, respectively. These two storage solutions were experimental. The chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

OTHER CHEMICAL

Stock solution of 1 mM GSH (Boehringer-Mannheim, Germany) was made in PBS and stored at 2-6°C. Dipyridamol (DIP; 2,6-bis(diethanolamino)-4,8-dipiperidino-[5,4-*d*] pyrimidine) (Sigma-Aldrich, St. Louis, MO) was stored as a 200 mM stock solution in dimethylsulfoxide (DMSO) at 2-6°C. Radioactive disodium-⁵¹chromate (⁵¹Cr) was purchased from NEN, Boston, MA.

DISPOSABLES

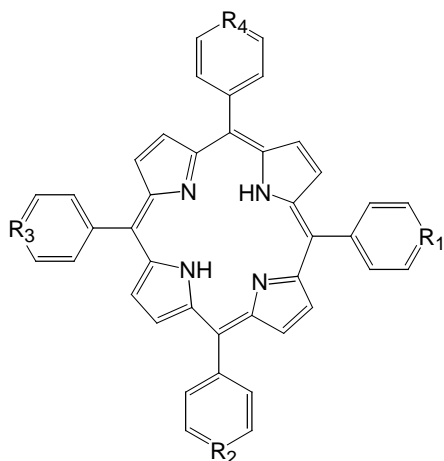
EDTA- and serum- tubes were purchased from Vacuette, Greiner Bio-One, Austria. Polystyrene culture dishes with a diameter of 9 cm and 11-mL sterile polystyrene test tubes were obtained from Greiner, Alphen a/d Rijn, the Netherlands.

MODEL VIRUS AND HOST CELLS

VSV (San Juan strain) was kindly provided by the Department of Virology at the Leiden University Medical Center (LUMC, Leiden, the Netherlands). A549 cells for the VSV infectivity assay were kindly provided by the department of Lung Diseases, LUMC.

SENSITIZERS

The following porphyrins (Fig. 1) (obtained from Mid-Century, Posen, IL, USA), were studied: meso-tetra-(N-methyl-4-pyridyl)-porphyrin chloride (Tetra-P(4)), trans-diphenyl-di-(N-methyl-4-pyridyl)-porphyrin chloride (Trans-P(4)), cis-diphenyl-di-(N-methyl-4-pyridyl)-porphyrin chloride (Cis-P(4)), triphenyl-mono-(N-methyl-4-pyridyl)-porphyrin chloride (Mono-P(4)), tetra-(N-2-hydroxy-ethyl-4-pyridyl)-porphyrin chloride (TNH-P(4)), tetra(4-N, N, N-trimethylanilinium)-porphyrin chloride (TAP). Photosensitizer mono-phenyl-tri-(N-methyl-4-pyridyl)-porphyrin chloride (Tri-P(4)) was obtained from Porphyrin Systems, Luebeck, Germany. Aluminum phthalocyanine tetrasulfonate (AlPcS₄) was purchased from Porphyrin Products (Logan, Utah, USA). Stock solutions (1 mM) were prepared either in 50 mM sodium-phosphate buffer (NaP) pH 7.4 (Tetra-P(4), Tri-P(4), TAP, TNH-P(4) and AlPcS₄) or in DMSO (Trans-P(4), Cis-P(4), Mono-P(4)). Solution in Na-phosphate buffer were sterilized by filtering through a 0.22 µm filter and stored at 4 °C. Sensitizer concentration, after a 10-fold dilution in absolute ethanol, was measured fluorimetrically with a Perkin Elmer LS50B Luminescence Spectrometer. The concentration was determined using a linear calibration curve.



Tetra-P(4): $R_1=R_2=R_3=R_4= N^+-CH_3$

Tri-P(4): $R_1=R_2=R_3= N^+-CH_3, R_4= CH$

Trans-P(4): $R_1=R_3= N^+-CH_3, R_2=R_4= CH$

Cis-P(4): $R_1=R_2= N^+-CH_3, R_3=R_4= CH$

Mono-P(4): $R_1= N^+-CH_3, R_2=R_3=R_4= CH$

TNH-P(4): $R_1=R_2=R_3=R_4= N^+-CH_2-CH_2-OH$

TAP: $R_1=R_2=R_3=R_4= C-N^+-(CH_3)_3$

Figure 1: Structures of the positively charged porphyrins used.

RED BLOOD CELL CONCENTRATES

After written consent blood from volunteer donors (500 ± 50 mL) was collected in 73 mL citrate-phosphate-dextrose adenine (CPDA) in quadruple polyvinylchloride bags (NPBI, Emmer-Compascuum, the Netherlands). After extraction of the buffy-coat, the Hct of the RBCC was adjusted to $60 \pm 4\%$ with the storage solution SAG-M.²⁶ In Europe, the allowed maximum shelf life of CPDA-RBCC in SAG-M is limited to 35 days.

PHOTODYNAMIC TREATMENT OF RBCC

Porphyrins were added to RBCC to give a final concentration of $25 \mu M$. Where indicated, DIP or GSH were added to RBCC before illumination. The RBC suspensions were thoroughly mixed. Samples of 6 mL were transferred to 9 cm-polystyrene culture dishes and agitated at room temperature on a horizontal reciprocal shaker (60 cycles/min, GFL, Burgwedel, Germany) for 5 min in the dark. The resulting thickness of the cell layer was 1 mm. The dishes were illuminated

from above with a 300 W halogen lamp (Philips, Eindhoven, the Netherlands). To avoid heating of the samples, the light passed through a 1-cm water filter. A cut-off filter (Kodak, wratten nr. 23A, Rochester, NY), only transmitting light > 600 nm, was used in all experiments. The irradiance at the cell layer was 100 W/m^2 , as measured with an IL1400A photometer equipped with a SEL033 detector (International Light, Newburyport, MA, USA).

Cells illuminated without sensitizer were used as control because illumination alone was shown to have no effect on RBC function or virus inactivation. Cells with sensitizer kept in the dark were used as dark control.

VSV INFECTIVITY ASSAY

Stock solutions of VSV were added to RBCC so that the volume of the spike was less than 10% of the total volume of RBCC. The number of extracellular virus particles was approximately $10^5/\text{mL}$ and was our maximum starting titer of inoculum. PDT was performed as described above.

Infectivity of VSV was assayed as previously described.²⁷ In short, RBC samples taken after various light doses were centrifuged for 5 min at $500 \times g$. The supernatants were serially diluted (10x) and added to A549 cell cultures in 96 well microtiter plates. After 72-hours incubation the cytopathology of the cells was scored in 8-well replicates for each dilution. Quantification of the virus titer was performed according to the Spearman-Kärber method,²⁸ expressing the quantity of virus infecting 50% of the cells (TCID_{50}). Results are expressed as percentage of the control.

RBC QUALITY VARIABLES

Hemolysis was determined by measuring the absorbance of free Hb at 540 nm. Cell supernatants were obtained by centrifugation of diluted (10x) RBCs (in PBS) at $12000 \times g$ for 3 min. Absorbance was measured using a DU-64 spectrophotometer (Beckman, Fullerton, CA). This method was shown to be as sensitive as the hemiglobincyanide method.²⁹

Potassium leakage from RBCs was measured using a flame photometer (Model 410C, Corning, Halstead, UK). Both K^+ leakage and hemolysis were expressed as a percentage of the total concentration.

ATP content of the cells was determined by adding firefly lantern extract after lysis of the cells in H_2O , and dilution with a glycine buffer (25 mM gly-gly, 20 mM $MgCl_2$, pH 7.5). The luminescence was measured using a Model 1251 luminometer (Bio-Orbit, Turku, Finland), as described before.²¹

Measurement of RBC lactate and glucose content of the medium were performed according to the manufacturer's instructions (Procedure 735 and 16-UV respectively, Sigma Diagnostics, St. Louis, MO).

Lipid peroxidation was assayed by measuring the generation of thiobarbituric acid reactive species as described by Miller et al.³⁰ Intracellular GSH was measured according to Sedlak and Lindsay.³¹ RBC deformability was measured as described previously.³²

Anion transport through band 3 was measured by the influx of $Na_2^{35}SO_4$ as described previously.³³ Briefly, after PDT, cells were centrifuged and resuspended (10% Hct) in PBS plus 1 mM Na_2SO_4 and 0.5 $\mu Ci/mL$ $Na_2^{35}SO_4$ at 37°C. After 0, 5, 10, 15, 20 and 30 min incubation at 37°C, samples were washed twice with ice-cold PBS to remove free $Na_2^{35}SO_4$. After precipitating the Hb with 5% trichloroacetic acid, radioactivity in the cells was determined by means of liquid scintillation counting (Tri-Carb 4000, Packard Instrument Co., Downers Grove, IL).

AGGLUTINATION AND IGG BINDING

The direct gel agglutination test (DAT) was performed on human RBCs and monkey RBCs using DiaMed-ID micro typing system LISS/Coombs (DiaMed SA, Cressier sur Morat, Zwitterland) according to manufacturer's instructions. The test includes antibodies against the four human IgG sub-classes and C3d.

IgG binding to human-RBCs was analyzed by flow cytometry. Briefly, RBCs were washed twice with PBS / 0.5% Bovine Serum Albumin (BSA) and resuspended to 5% Hct in the same buffer. A 50 μL sample was incubated with 50 μL of diluted (20x) FITC-conjugated $F(ab')_2$ fragment of rabbit anti-human IgG (gamma-chains) or FITC-conjugated $F(ab')_2$ fragment of rabbit Ig (negative control) (DAKO,

Glostrup, Denmark) for 30 min at 4°C. Labeled cells were washed with PBS/0.5% BSA before analysis on the Epics XL (Beckman Coulter, Mijdrecht, the Netherlands). A total of 20.000 events were analyzed, and mean fluorescence intensity (MFI) of the FITC signal (FL1) was determined. The indirect antiglobulin test (IAT) was performed using plasma-free RBCs (washed 6 times in SAG-M) and autologous plasma, which was photodynamically treated separately. IgG binding was analyzed macroscopically and by flow cytometry.

STORAGE OF HUMAN RBCC AFTER PHOTODYNAMIC TREATMENT

After illumination of RBCC, Hct 60% in SAG-M, 5-mL samples were transferred to 11-mL sterile polystyrene test tubes. After centrifugation at 500 x g for 10 min the supernatant was removed. Cells were resuspended to 60% Hct in SAG-M, SAG-S, SAG-D or AS-3. The RBC suspensions were stored in tubes in the dark at 2-6°C. These conditions were chosen for convenience when working with small samples. RBC quality was assayed after 1, 7, 21 and 35 days of storage.

STATISTICS

All in-vitro experiments were performed at least 4 times with blood from different donors. Values are expressed as mean \pm standard deviations (SD). The p values were calculated using student's t-tests. A p value less than 0.05 were defined as significant.

RHESUS MONKEY SURVIVAL STUDIES

All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee installed by Dutch law. In a pilot study, the effect of PDT on monkey RBCs was determined with respect to VSV kill, RBC quality (K⁺ leakage and hemolysis) and ⁵¹Cr uptake. In-vivo survival studies were carried out using two Rhesus monkeys (*Macaca mulatta*). Monkey RBCs were prepared from 20 mL of freshly drawn blood, collected after ketamine-anaesthesia in 2.5 mL CPDA. CPDA blood was centrifuged at 500 x g for 5 minutes, and the plasma and buffy-coat

were discarded. The RBCs were resuspended in SAG-M to a Hct of $60 \pm 4\%$ and photodynamically treated as described above.

In-vivo survival was investigated after autologous transfusion of either fresh or 35 days stored RBCC. (Table 1). RBCs were stored in AS-3 in polystyrene tubes at 4°C during 5 weeks. Twenty four hours before transfusion, RBCs were labeled with ^{51}Cr . Unbound radioactivity was removed by washing in excess volume with AS-3. A 1-mL sample was removed before transfusion and used to determine the labeling efficiency and the remaining Tri-P(4) concentration (excitation 424 nm, emission 659 nm). Labeling efficiency was shown to be $87 \pm 9\%$ in both untreated and PDT-treated RBCs. Freud et al.³⁴ have shown that until RBCs are committed to hemolysis, ^{51}Cr leakage does not occur during storage of PDT-treated RBCs. For parallel in-vitro analysis during storage, control and Tri-P(4)-treated monkey RBCs were washed and resuspended to 60% Hct in AS-3.

To determine the 24-hour recovery, blood samples in EDTA were drawn 30 min and 1, 2, 4, 8 and 24 hours after transfusion. To determine the life span of the transfused RBCs, samples were drawn at Day 2 and Day 3 after transfusion and thereafter twice a week until the radioactivity was decreased to less than 45% of the initial value. The Hb, Hct, Mean cell volume (MCV), RBC, WBC and platelet numbers, creatinin, total bilirubin, LDH and haptoglobin were measured before and after transfusion by standard procedures. Urine samples were recovered during 24 hours after transfusion. The 24-hour recovery and the RBC survival, expressed as T_{50} , were calculated according to the ICSH guidelines.³⁵

All radioactivity counts were corrected for Hct, decay and elution of ^{51}Cr , which was taken to be 1% per day.

Exp nr.	Monkey A	Monkey B	Storage time
1	RBC, non-treated (control)	RBC, PDT-treated	< 24 hrs
2	RBC, PDT-treated	RBC, non-treated (control)	< 24 hrs
3	RBC, non-treated (control)	RBC, PDT-treated	35 days
4	RBC, PDT-treated	RBC, non-treated (control)	35 days

Table 1: Experimental design of the in-vivo RBC survival studies

RESULTS

HUMAN RBC

INITIAL SCREENING OF PHOTSENSITIZERS

VSV inactivation. All sensitizers tested were capable of inactivating extracellular VSV. However, the light dose needed for 5- \log_{10} VSV kill varied strongly (Fig. 2A). Tri-P(4) appeared to be the most efficient sensitizer, requiring a light dose of 90 kJ/m² (15 min at 100 W/m²). The dose required for Mono-P(4) was fourfold higher. All other sensitizers required an intermediate light dose varying from 120 to 240 kJ/m².

RBC damage. A light dose needed to kill 5- \log_{10} VSV was used to study the effect of the different porphyrins on RBC integrity. Figure 2B shows the RBC hemolysis after storage of treated cells in SAG-M. Virucidal treatment with Tri-P(4) induced the least hemolysis, $5.1 \pm 1.1\%$ after 35 days of storage. Virucidal treatment with TAP, TNH and Tetra-P(4) induced intermediate levels of hemolysis (mean $16.13 \pm 0.81\%$), whereas Trans-P(4), Cis-P(4) and Mono-P(4) induced very high levels of hemolysis. Further experiments were restricted to Tri-P(4). RBCs were photodynamically treated with 25 μM Tri-P(4) and a light dose of 90 kJ/m² (15 min, 100 W/m²). RBCs were stored for 5 weeks at 4-6°C.

HEMOLYSIS AND POTASSIUM LEAKAGE: COMPARISON OF VARIOUS ADDITIVE SOLUTIONS

After 35 days of storage in tubes, hemolysis of control cells amounted to $1.9 \pm 1.1\%$ and was independent of the additive solution used. Storage of Tri-P(4)-treated RBCs in SAG-S or SAG-D resulted in lower hemolysis as compared to SAG-M (Fig. 3). Hemolysis was the lowest after storage in AS-3 ($2.3 \pm 1.9\%$). After storage in SAG-S, SAG-D and AS-3, there was no significant difference between treated cells and controls. When stored in SAG-M, SAG-S, and SAG-D, Tri-P(4) alone (dark control) resulted in a slight, but not significant, increase in hemolysis.

Potassium leakage during storage was substantially higher for Tri-P(4)-treated cells than for the controls and was independent of the storage solution (Fig. 4). We observed a rapid increase in extracellular K^+ 1 day after illumination, followed by a gradual increase to about 60% after 35 days of storage. In the controls, K^+ leakage was around 40% after 35 days. The presence of the scavengers GSH (32 mM) or DIP (125 μ M) during PDT had no effect on the extent of K^+ leakage (data not shown).

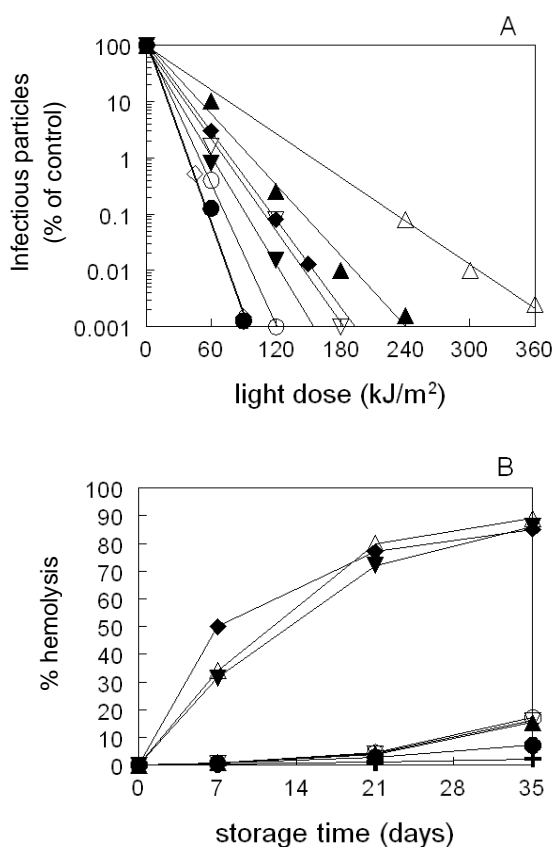


Figure 2: (A) Infectivity of VSV after PDT. RBCC spiked with 5-log_{10} extracellular VSV were illuminated with red light (light intensity: 100 W/m^2) in the presence of $25\text{ }\mu\text{M}$ Tetra-P(4) (▲), Tri-P(4) (●), TNH-P(4) (○), TAP (▽), Trans-P(4) (▼), Cis-P(4) (◆), Mono-P(4) (△). Results are expressed as percentage of un-treated control. **(B) RBC hemolysis after virucidal treatment.** RBCC with $25\text{ }\mu\text{M}$ porphyrins were illuminated with a light dose giving 5-log_{10} kill of VSV. After treatment, the cells were stored for 35 days at 4 to 6°C . Control (red light only; +), Tetra-P(4), 240 kJ/m^2 (▲), Tri-P(4), 90 kJ/m^2 (●), TNH-P(4), 120 kJ/m^2 (○), TAP, 180 kJ/m^2 (▽), Trans-P(4), 150 kJ/m^2 (▼), Cis-P(4), 190 kJ/m^2 (◆), Mono-P(4), 360 kJ/m^2 (△). Results are expressed as percentage of total Hb. Error bars were omitted for reason of clarity.

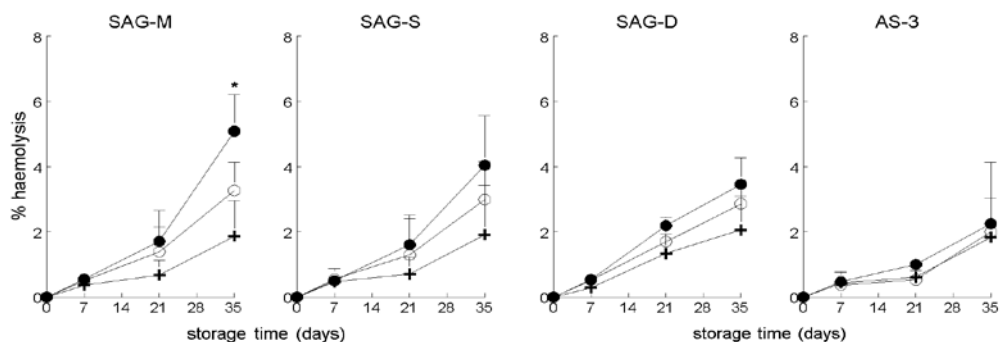


Figure 3. Role of the storage solution on RBC hemolysis.

RBCC, Hct 60% in SAG-M, were treated with red light only (+), 25µM Tri-P(4) only (○) or with 25µM Tri-P(4) plus red light (90 kJ/m², ●). After treatment, cells were resuspended in SAG-M, SAG-S, SAG-D or AS-3 and stored for 35 days at 2-6°C. Hemolysis is expressed as percentage of total Hb. Data presented are the average ± SD of 4 to 6 experiments. Significant differences are indicated with * (p value < 0.05).

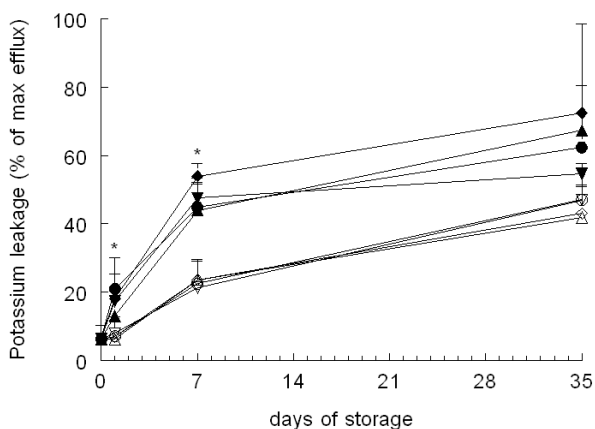


Figure 4: Potassium leakage after PDT with Tri-P(4).

RBCC, Hct 60% in SAG-M, were treated with red light only (90 kJ/m²; open symbols) or 25 µM Tri-P(4) plus red light (90 kJ/m², filled symbols). After treatment cells were resuspended in SAG-M (○;●), SAG-S (▽;▼), SAG-D (△;▲) or AS-3 (◇;◆) and stored for 35 days at 4 to 6°C. Data presented are average ± SD of four to six experiments. Significant differences are indicated with * (p value < 0.05).

OTHER RBC VARIABLES

RBC quality variables after storage in SAG-M are summarized in Table 2. After 35 days of storage, the cellular ATP content and extracellular glucose levels decreased and the lactate concentration increased to comparable levels in both control and Tri-P(4) treated cells. The GSH concentration in treated cells was slightly decreased after 35 days of storage. The photodynamic dose used in these experiments did not induce any detectable lipid peroxidation, changes in deformability of the cells or changes in anion transport through band 3. The use of different additive solutions had no effect on any of the mentioned variables (data not shown).

	Week	ATP ($\mu\text{mol/g Hb}$)	Glucose (mg/mL)	Lactate (mol/L)	GSH ($\mu\text{mol/g Hb}$)
Control	1	3.8 ± 0.4	4.1 ± 0.5	12.3 ± 2.2	1.5 ± 0.3
	5	1.8 ± 0.3	2.3 ± 0.5	23.0 ± 4.0	1.5 ± 0.2
Tri-P(4)	1	4.0 ± 0.5	3.5 ± 1.5	12.1 ± 3.9	1.6 ± 0.1
	5	1.6 ± 0.3	2.4 ± 0.5	23.9 ± 1.4	1.2 ± 0.3

Table 2: RBC quality after photodynamic treatment with Tri-P(4) and red light * and stored in SAG-M during 5 weeks (mean \pm sd).

* Light dose of 90 kJ/m^2 which corresponds to an illumination time of 15 min at 100 W/m^2

IgG BINDING AND AGGLUTINATION

Virucidal treatment of RBCC, Hct 60% in SAG-M with Tri-P(4) resulted in binding of IgG to RBCs. The extent of the binding was light dose dependent and increased with illumination time. IgG binding after treatment at 90 kJ/m^2 was very low, sometimes undetectable by DAT (Table 3) and flow cytometry (Fig. 5A). The MFI of untreated cells was 4.7 ± 0.6 , that of treated cells with 90 kJ/m^2 was 5.1 ± 0.7 and with 360 kJ/m^2 the MFI was 8.2 ± 2 (Fig. 5A). PDT with AIPcS₄ ($2.5 \mu\text{M}$, 360 kJ/m^2) was previously described to induce IgG binding¹⁹ and was used as a positive control. Both Tri-P(4)-induced IgG binding measured by flow cytometry and

agglutination in the DAT could be prevented by the presence of GSH (32 mM) during the illumination. Addition of GSH after PDT did not reduce or prevent IgG binding or agglutination (Fig. 5B and Table 3). The presence of the singlet oxygen scavenger DIP (125 μM) during illumination did not prevent IgG binding or agglutination.

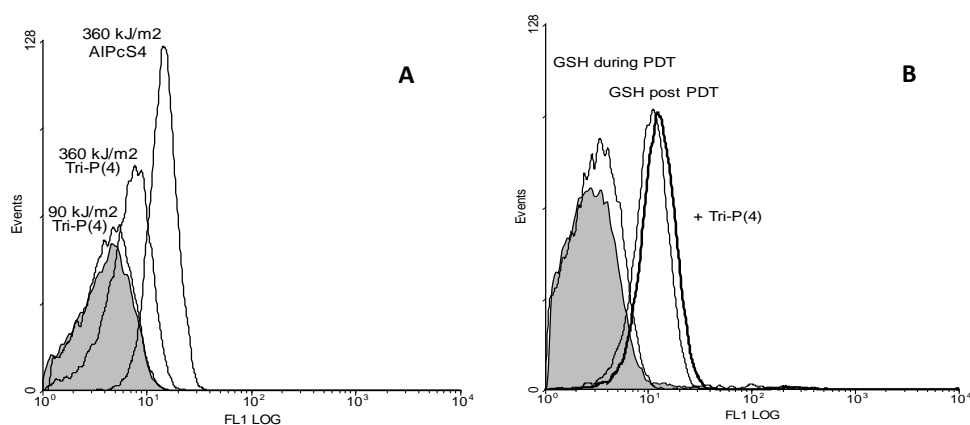


Figure 5: (A) Binding of IgG to RBCs after PDT with Tri-P(4). RBCs were treated in the presence of 25 μM Tri-P(4) with a light dose of 90 kJ/m^2 or 360 kJ/m^2 . RBCs treated with AIPcS₄ (2.5 μM , 360 kJ/m^2) served as positive control. One representative experiment of a total of seven experiments is shown. **(B) Effect of GSH on the Tri-P(4)-induced binding of IgG to RBCs.** RBCs were treated with 25 μM Tri-P(4) with a light dose of 360 kJ/m^2 . GSH (32 mM) was added to the RBCs either before or after the illumination. After treatment cells were analyzed for IgG binding by flow-cytometry, as described in Material and Methods. One representative experiment of a total of three experiments is shown. The untreated sample is shown in solid gray.

To unravel the process of the photodynamically induced IgG binding, plasma and plasma-free RBCs were treated with Tri-P(4) (light dose 360 kJ/m^2) separately. No IgG binding was detectable after phototreatment of the plasma-free RBCs. Using a cross-match reaction (IAT), i.e. plasma added to RBCs, it was shown that both Tri-P(4)-treated plasma and Tri-P(4)-treated plasma-free-RBCs were required for IgG binding and to induce agglutination. The IAT remained negative when a single treated component, RBCs or plasma, was added to an untreated one (Table 3).

DAT	Untreated	90 kJ/m ²	360 kJ/m ²	+ GSH* during PDT	+ GSH* after PDT
RBCC[†]	0 (n = 7)	0 / + (n = 4 / 3)	4+ (n = 7)	0 (n = 3)	4+ (n = 3)
IAT	Washed RBCs‡				
	Untreated	PDT (360 kJ/m ²)		PDT (360 kJ/m ²) + GSH*	
Plasma					
Untreated	0	0		0	
PDT (360 kJ/m ²)	0	2+		0	
PDT (360 kJ/m ²) + GSH*	0	0		0	
* [GSH] = 32 mM					
†RBCC, Hct 60% in SAG-M					
‡ Washed 6 times in SAG-M (= plasma-free RBCs)					

Table 3: DAT and IAT score after PDT with Tri-P(4) and red light

MONKEY RBCC

IN VITRO QUALITY

Tri-P(4) treatment of monkey RBCC spiked with VSV, using the same conditions as for human RBCC, resulted also in a 5-log_{10} kill of VSV. RBC hemolysis was assayed after 35 days of storage in AS-3. AS-3 was selected because it was shown to maintain human RBC integrity better than SAG-M (Fig. 3). After 35 days of storage, the hemolysis of untreated monkey RBCC was 2.7%, 1.5-fold higher than with human RBCC. After PDT with Tri-P(4), hemolysis amounted to 4.2% after 35 days of storage. This was twofold higher than observed with human RBCC in AS-3. After 1 week of storage, the level of photodynamically induced K^+ leakage was as high as in human-RBCC after 35 days of storage. After PDT with Tri-P(4) at 90 kJ/m², no IgG binding was found on monkey RBCs in the DAT using anti-Hu-IgG and C3.

IN VIVO SURVIVAL

The 24-hr recovery of autologous RBCs is shown in Table 4. After PDT, the 24-hr recovery of fresh RBCs was in the same range as for non-treated cells (85%). After 5 weeks of storage, the 24-hr recovery of treated RBCs was approximately 11% lower than that of untreated stored cells. The values for T_{50} showed a large variation, which was independent of the treatment. The residual Tri-P(4) concentration in the transfusion products was about 1-2 μM . No clinical symptoms suggesting transfusion-related adverse effects were observed.

Blood samples taken 30 min, 1 hour and 24 hours after transfusion were analysed for chemical and hematological values. Values for number of WBC, RBC and platelets, serum creatinin, total bilirubin and LDH were all in the normal range and there were no differences between PDT-treated and untreated transfusions (data not shown). Haptoglobin concentration was increased in some samples 24 hours after transfusion, but this was not associated with the treatment method of RBCs. Twenty-four hours after transfusion, urine analysis (^{51}Cr , bilirubin, protein) showed no change with the values obtained before the RBC infusions (data not shown).

Transfusion product	Fresh RBCC		Stored RBCC	
	Monkey A	Monkey B	Monkey A	Monkey B
24-hr recovery (%)				
Untreated	82	87	81	73
PDT	90	80	62	68
T_{50} (days)				
Untreated	16	18	23	26
PDT	22	19	15	20

Table 4: 24-hour recovery and T_{50} of Tri-P(4)-treated monkey RBCs.

DISCUSSION

Positively charged porphyrins are known to inactivate bacteria.^{24,36} Our study has shown that they are also able to inactivate extracellular VSV. VSV is a lipid enveloped, single stranded RNA virus which has been found to be as sensitive to PDT with Pc4 and red light as HIV and is therefore a useful virus to study PDT.³⁷ All positively charged porphyrins tested were able to inactivate extracellular VSV spiked in RBCC (Fig. 2a), however the compounds differed greatly by their potential to preserve RBC integrity during storage. Under conditions that induced a 5- \log_{10} inactivation of extracellular VSV, the hydrophobic compounds Trans-P(4), Cis-P(4) and Mono-P(4) caused a very high percentage of cell lysis after 35 days of storage. Because of their hydrophobic nature, these compounds most likely penetrated the bilayer of the RBCs. The hydrophilic compounds Tetra-P(4), TAP and TNH-P(4) showed a moderate percentage of lysed cells. The amphiphilic Tri-P(4) showed the least hemolysis and could therefore be a suitable photosensitizer for pathogen inactivation in of RBCC.

Tetra-, Tri- and Di-P(4)'s have been shown to intercalate in isolated calf thymus DNA.^{38,39} Although this property may contribute to the selective killing of viruses, the exact mechanism by which the porphyrins inactivate viruses still remains to be explored.

RBC integrity after Tri-P(4)-mediated phototreatment was influenced by the composition of the storage solution. Storage of treated cells in SAG-S, SAG-D or AS-3 resulted in much less hemolysis as compared to storage in SAG-M. Comparable results were recently obtained by Besselink et al.⁴⁰ using the photosensitizer DMMB. The presence of high concentrations of impermeable macromolecules in SAG-S (29 mM sucrose), SAG-D (29 mM dextran 1000) and AS-3 (22 mM citrate) compensate for the osmotic activity of intracellular Hb.⁴¹⁻⁴²

RBC metabolic function and deformability remained intact after PDT with Tri-P(4), whereas K^+ leakage during storage was increased. We have previously shown that K^+ leakage induced by PDT with DMMB was related to damage to band 3 and that both band 3 damage and K^+ leakage could be reduced by scavenger DIP.⁴³ Tri-P(4) seems to have a different mechanism, since K^+ leakage is induced without affecting band 3 activity, and the addition of DIP did not reduce K^+ leakage. The

mechanism underlying K^+ leakage induced by Tri-P(4) phototreatment remains unclear. Further investigations focusing on channels involved in K^+ concentration regulation such as the Na^+-K^+ pump, G α dos channel and the K^+Cl^- co-transporter might help to unravel the mechanism behind the K^+ leakage.

IgG binding was induced by PDT with Tri-P(4). However the extent of IgG binding at virucidal light dose (90 kJ/m^2) was very low and was sometimes not detectable (Table 3). IgG binding induced by PDT is a known phenomenon which can be prevented by the addition of the scavenger GSH during illumination. GSH can prevent IgG binding by prevention formation of disulfide bridges.¹⁹ In agreement with previous findings of Rywkin et al.¹⁹ the addition of GSH *after* the phototreatment was unable to reverse IgG binding or agglutination. Addition of DIP did not prevent IgG binding. To ensure cell survival and to avoid problems with compatibility testing, it may be necessary to add GSH or cysteine during the PDT with Tri-P(4). Both GSH and cysteine are natural constituents of the blood and therefore might not have to be removed before transfusion.

The cross-match IAT showed agglutination only when both plasma-free RBCs and plasma were photo-treated. This suggests that both the RBC membrane and plasma proteins were modified by PDT. Remarkably, there was no agglutination when Tri-P(4)-treated plasma-free RBCs were incubated with untreated plasma. This indicates that Tri-P(4) does not induce formation of neoantigens reacting with naturally occurring auto-antibodies. This however does not preclude that phototreated RBC membrane alterations may cause new antibody formation upon transfusion.

Based on the promising in-vitro results on one side and the considerable efforts to translate our experimental set-up for application in humans on the other, we evaluated the in-vivo survival of Tri-P(4) treated RBCs in 2 Rhesus monkeys. The number of animals was kept to a minimum by performing a cross-over study. The study was not meant to obtain statistic relevance but to evaluate whether proceeding with Tri-P(4) was worthwhile. Both the 24-hour recovery and the long term survival were satisfactory after transfusion of phototreated fresh RBCs. In accordance with previous reports,⁴⁴ we found monkey RBCs to be more fragile than human RBCs. Storage of monkey RBCC resulted in higher hemolysis as compared to human RBCC after the same storage interval. Moreover, the

experimental conditions storing RBCC in tubes were suboptimal compared to the Blood Bank conditions in which RBCC are stored in bags. After 5 weeks of storage, the 24-hour recovery was lower compared to fresh RBCs and recovery was approximately 10% reduced by Tri-P(4)-mediated PDT (Table 4). The T_{50} however, varied in our study from 15 to 26 days, and was not clearly associated with either the storage or the PDT. The true impact of PDT on in-vivo survival after storage is difficult to interpret as suboptimal storage condition, using tubes instead of bags, were used.

Recent publication by Vitex and Cerus^{45,46} have reported the formation of autoantibodies in patients receiving Pen-110- or S-303-treated RBCs and Phase III clinical trials have been abandoned. These results were unexpected and not foreseen despite extensive animal studies. Our study showed that treatment with Tri-P(4) preserved the structure and functionality of RBCs but resulted in a slight elevation of hemolysis after 5 weeks of storage, an acceleration of K^+ leakage, and a weak induction of IgG binding, which could be prevented by GSH. Although it seems that neoantigens reacting with naturally occurring IgG antibodies are not formed after Tri-P(4)-mediated PDT, to predict the immunogenicity of Tri-P(4)-treated RBCs in human trials in-vivo, further research is required to elucidate the cause of this event.

The results obtained in this study form an acceptable basis for further investigations on the use of Tri-P(4) for pathogen inactivation in RBCC. Further research will focus on the inactivation of viruses more relevant to transfusion medicine, such as HIV and hepatitis viruses. Also the inactivation of intracellular viruses has to be studied. It might well be possible that for inactivation of other more resistant viruses, a more stringent photoinactivation is needed, which might result in more RBC damage. Therefore, more insight in both the mechanism of pathogen inactivation and RBC damage after phototreatment with Tri-P(4) is of utmost importance.

In conclusion, although we realize that the step from “bench” experiments to a functioning pathogen inactivation system in a blood-bank setting is a big one, Tri-P(4)-mediated phototreatment might become a suitable method to inactivate pathogens in cellular blood products.

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