Pathogen inactivation in cellular blood products by photodynamic treatment

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CHAPTER III

SELECTIVE PROTECTION OF RBCS AGAINST PHOTODYNAMIC DAMAGE BY THE BAND 3 LIGAND DIPYRIDAMOLE

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In memoriam to John van Steveninck

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ABSTRACT

BACKGROUND: All studied photosensitizers for virus inactivation impair RBCs. To reduce damage to the RBCs without affecting virucidal activity, selective protection of the RBCs is necessary. The ability of the band 3 ligand, dipyridamole (DIP), to react with singlet oxygen and to increase the selectivity of photodecontamination was investigated.

STUDY DESIGN AND METHODS: Solutions of DIP were illuminated in the presence of AlPcS4 and DMMB. Solutions of amino acids, RBCs, and vesicular stomatitis virus (VSV) in RBC suspensions were photodynamically treated in the presence or absence of DIP.

RESULTS: Illumination of a solution of DIP in the presence of AlPcS4 or DMMB resulted in changes in the optical spectrum of DIP. The photooxidation of DIP was inhibited by azide and augmented by deuterium oxide, which suggests the involvement of singlet oxygen. Photooxidation of amino acids and photodamage to RBCs was strongly reduced in the presence of DIP. In contrast, photoinactivation of VSV in RBC suspensions was only slightly affected by DIP.

CONCLUSION: DIP can improve the specificity of photodynamic decontamination of RBCC, thereby increasing the practical applicability of this photodecontamination method.

INTRODUCTION

The safety of blood transfusion components is a major issue in transfusion medicine practice. Improved donor screening and the implementation of diagnostic screening of blood samples have substantially reduced the risk of virus transmission by transfusion. However, mainly because of window-period donations, residual risk exists.[1] For plasma components, the risk has been reduced considerably by the application of virucidal treatments such as heating or solvent/detergent (SD) treatment.[2,3] These procedures, however, are not applicable to cellular blood components, because of the fragility of the cells. The use of light-activated agents, photosensitizers, is widely studied for the inactivation of viruses in cellular blood components, especially RBCC.[4-6] Upon activation of the photosensitizer with visible light, various reactive oxygen species are formed. Although photosensitizers can mediate both type I (charge transfer, radicals) and type II (energy transfer, singlet oxygen [$^1O_2$]) reactions, most photosensitizers are considered to act mainly via $^1O_2$. It is able to inactivate a broad range of enveloped viruses.[4] However, as $^1O_2$ is not selective and can be generated in all locations where sensitizer molecules are present, phototreatment can also damage RBCs. This means that photosensitizers with high affinity for virus particles must be used to protect the RBCs from photodynamic damage. Promising results have been obtained with phenothiazine dyes, such as MB and DMMB[6,10,11] , and with certain phthalocyanines.[12-14] However, all photosensitizers tested so far also induce damage to the RBCs, as detected by an increased permeability of the membrane to cations. To minimize the damage to RBCs induced by photodynamic pathogen inactivation, we searched for chemicals that bind preferentially to the RBC membrane and react readily with $^1O_2$. An obvious way to achieve effective binding of candidate scavengers is the use of band 3 ligands. Band 3 constitutes the anion transporter of the RBC, and, with about a million copies per cell, it is the most abundant protein on the RBC membrane. An additional argument for using scavengers that bind to band 3 is the proposed involvement of band 3 damage in photohemolysis.[15] Band 3 is widely studied and many ligands are known. A well-known ligand for band 3 is DIP (Fig. 1). DIP binds noncovalently, with high affinity (KD = 1.2 μM) to band 3 and with stoichiometry

of one molecule of DIP per band 3 dimer. The inhibition of anion transport is not due to binding of DIP to the anion-binding side, but to blocking of the anion channel.[16] Besides its use as ligand for band 3, DIP is widely used in the treatment of cardiovascular diseases because of its vasodilating activity and its ability to inhibit platelet aggregation.[17] It has previously been reported that DIP also exerts an inhibitory effect on lipid peroxidation[18,19] and possesses activity as a scavenger of superoxide anion, hydroxyl radical, and peroxyl radical.[18,20] These properties make DIP a promising candidate for selective protection of RBCs against photodynamic damage. The aim of the present study is to investigate whether DIP is able to react with photodynamically formed $^1O_2$, thereby protecting substrates against photodynamic damage. In addition, the possibility of using DIP for selective protection of RBCs against virucidal PDT was investigated. For this we used $AlPcS_4$ and DMMB, both of which are photosensitizers whose role in virus inactivation in RBC suspensions is well studied.

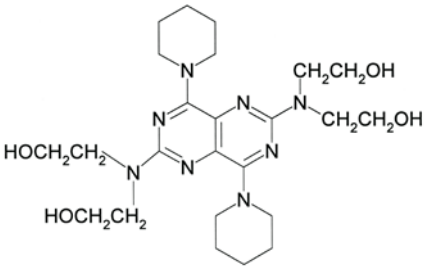


Figure 1. Structure of dipyridamole

MATERIALS AND METHODS

MATERIALS

DIP (2,6-bis(diethanolamino)-4,8-dipiperidino-[5,4-d]pyrimidine) was purchased (Sigma-Aldrich BV, Zwijndrecht, the Netherlands). Stock solutions (20 m*M*) were prepared in ethanol. $AlPcS_4$ was obtained from Porphyrin Products (Logan, UT). VSV (San Juan strain) and the host cells A549 were provided by the departments of Virology and Lung Diseases respectively (Leiden University Medical Center, Leiden, the Netherlands). A549 cells were cultured in medium (RPMI 1640)

supplemented with 10-percent fetal bovine serum (Gibco BRL, Breda, the Netherlands). All other chemicals were obtained from Sigma-Aldrich and were of the highest purity available. Heparinized human blood, provided by the regional Red Cross Blood Bank (Leiden-Haaglanden, the Netherlands), was centrifuged shortly after collection. The RBCs were washed three times with 150 m*M* NaCl and 20 m*M* HEPES, pH 7.6, and resuspended at 2-percent Hct in the same buffer.

PHOTODYNAMIC TREATMENT

For all illuminations, the light source used was a slide projector with a 150 W lamp (HLX 64640, Xenophot, Osram, Germany).[21] To avoid light absorption by Hb and DIP, a cutoff filter, transmitting only light with a wavelength above 590 nm, was used in all experiments. Both $AlPcS_4$ (maximum absorption at 675 nm) and DMMB (maximum absorption at 652 nm) strongly absorb in this region. The irradiance was 350 W per m^2, as measured with a photometer (IL1400A, International Light, Newburyport, MA) equipped with a detector (SELO33, International Light). All illuminations were performed at a constant temperature of 20°C under continuous mixing.

Solutions of DIP (10 μ*M*) were illuminated in the presence of 10 μ*M* $AlPcS_4$. Absorption spectra of DIP after different illumination times were recorded with a spectrophotometer (DU-64, Beckman, Fullerton, CA). Suspensions of RBCs (2% Hct) either with or without VSV (10^5 infectious particles/mL) were incubated with 100 μ*M* DIP at 20°C in the dark. After 5 minutes, 1 μ*M* $AlPcS_4$ or DMMB was added to the suspension. After another 5 minutes of incubation, the suspensions were illuminated for different periods.

OXYGEN CONSUMPTION

Oxygen consumption during illumination was measured with an oxygen monitor (YSI, Yellow Springs, OH) equipped with a Clark-type electrode. A 3-mL sample of histidine (2 m*M* in 150 m*M* NaCl/1% Triton-X100/20 m*M* HEPES, pH 7.6) was illuminated in the presence of 2.5 μ*M* $AlPcS_4$ or 0.5 μ*M* DMMB plus various amounts of DIP at a constant temperature of 20°C under continuous mixing. The rate of oxygen consumption was calculated from the initial slope of the curve.

POTASSIUM LEAKAGE AND DELAYED HEMOLYSIS

K^+ leakage from RBCs was determined immediately after illumination by means of a flame photometer (Clinical Flame Photometer 410C, Corning, Halstead, UK). To study delayed hemolysis, RBCs were treated with a light dose inducing minimal K^+ leakage. The light dose used with DMMB was 75 kJ per m^2, and that used with $AlPcS_4$ was 200 kJ per m^2. After treatment, the cells were centrifuged, resuspended in fresh buffer, and stored in the dark at room temperature. After different storage times, released Hb was determined by measuring the OD of the supernatant at 540 nm using a DU-64 spectrophotometer. Both K^+ and Hb release were expressed as a percentage of the total efflux evoked by lysis of the cells in distilled water.

VSV INFECTIVITY ASSAY

Assay of the infectivity of VSV by an endpoint dilution assay was performed as described previously.[14] In short, VSV samples were serially diluted (10 times), inoculated into A549 cell cultures in 96-well microtiter plates (Greiner, Alphen a/d Rijn, the Netherlands), and incubated for 72 hours. The cytopathology of the cells was scored in eight well replicates for each dilution. Quantification of the virus titer was performed according to the Spearman-Karber method.[22]

STATISTICS

All experiments were performed at least four times with blood from different donors. Values are expressed as mean ± standard error.

RESULTS

PHOTOOXIDATION OF DIP

It has previously been shown that oxidation of DIP with hydroxyl radicals results in a changed optical spectrum of DIP.[23] To investigate whether $^1O_2$ also can induce

oxidation of DIP, a solution of DIP (10 µ*M*) was illuminated in the presence of 10 µ*M* AlPcS4.

After application of different light doses, the optical spectrum of DIP was recorded. The characteristic yellow color of DIP (absorption maximum at 410 nm) disappeared gradually upon illumination, while at 270 nm a new absorption maximum appeared (Fig. 2). Illumination, also with white light, in the absence of $AlPcS_4$ had no effect on the optical spectrum of DIP, which indicates that DIP does not act as a photosensitizer. The photodynamically induced degradation of DIP was slowed by the addition of 1 m*M* sodium azide (Fig. 3). When the buffer was made in deuterium oxide (D_2O) instead of in H_2O, photodynamic degradation was strongly augmented (Fig.3). Comparable results were obtained with DMMB (2 µ*M*) as photosensitizer (results not shown).

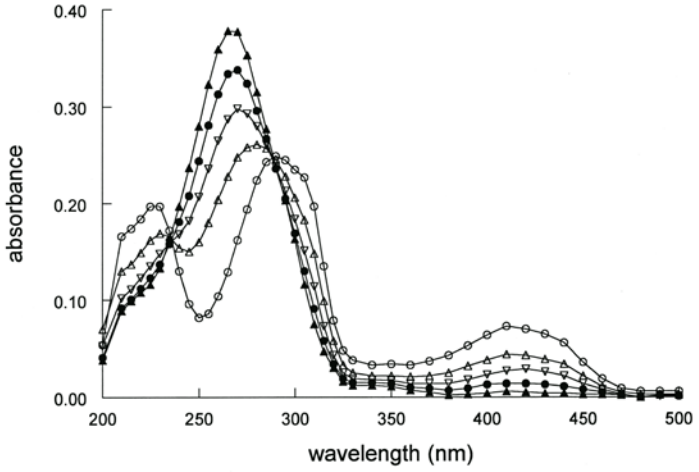


Figure 2. Effect of photooxidation on the spectral properties of DIP.
A solution of DIP (10 µ*M* in 150 m*M* NaCl/10 mM Hepes, pH 7.6) was illuminated in the presence of 10 µ*M* $AlPcS_4$. After 0 (○), 20 (△), 40 (▽), 60 (●) and 100 (▲) kJ/m^2, the optical spectrum of DIP was recorded using a Beckman DU64 spectrophotometer.

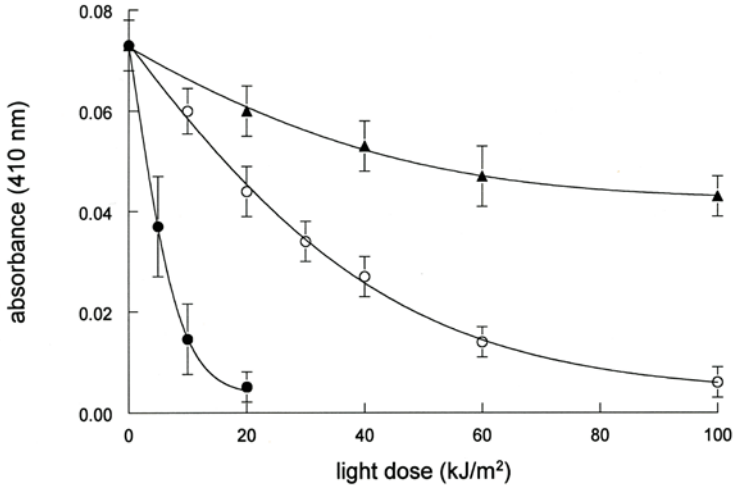


Figure 3. Effects of sodium azide and deuterium oxide on the photooxidation of dipyridamole.
A solution of DIP (10 µ*M* in 150 m*M* NaCl/10 m*M* Hepes, pH 7.6) was illuminated in the presence of 10 µ*M* $AlPcS_4$. (○): no extra additions, (▲): 1 m*M* sodium azide added, (●): H_2O replaced by D_2O.

EFFECT OF DIP ON THE PHOTOOXIDATION OF HISTIDINE

Illumination of a 2 m*M* solution of histidine in the presence of $AlPcS_4$ (2.5 µ*M*) or DMMB (0.5 µ*M*) resulted in a rapid decrease in the oxygen content of the buffer. The rate of oxygen consumption in the absence of DIP amounted to 48 ± 4 µ*M* of O_2 per minute for $AlPcS_4$ and to 56 ± 6 µ*M* of O_2 per minute for DMMB. The inclusion of increasing amounts of DIP in the illumination mixture resulted in a decreasing rate of photooxidation of histidine (Fig. 4). Comparable results were obtained when the amino acids tryptophan, methionine, and tyrosine or the DNA base guanosine was used as substrate. Illumination in the absence of substrate, in either the presence or absence of DIP, induced no significant oxygen consumption. In addition, no changes in the optical properties of DIP were

observed, which indicates that, under these conditions of low sensitizer concentration and high DIP concentration, DIP was not significantly oxidized.

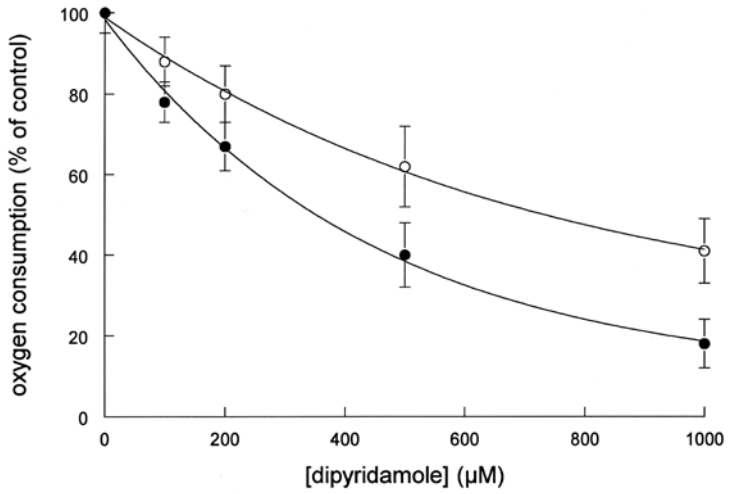


Figure 4. **Effect of dipyridamole on the photooxidation of histidine.**
A histidine solution (2 m*M* in 150 m*M* NaCl/10 m*M* Hepes/1% Triton-X100, pH 7.6) was illuminated in the presence of 2.5 µM $AlPcS_4$ (○) or 0.5 µ*M* DMMB (●) with increasing concentrations of DIP. The initial oxygen consumption during illumination was recorded using a Clark-type electrode. The oxygen consumption rates obtained in the absence of DIP were normalized to 100%.

EFFECT OF DIP ON PHOTODYNAMICALLY INDUCED DAMAGE TO RBCS

To investigate whether the presence of DIP can protect RBCs against photodynamic damage, RBC suspensions (2% Hct) were incubated in the dark for 5 minutes with DIP before photosensitizer was added. As can be seen from Fig. 5, both AlPcS4 and DMMB induced damage to the RBCs upon illumination, as reflected by the increased concentration of extracellular K^+. Neither illumination without photosensitizer nor incubation in the dark with photosensitizer induced any K^+ leakage. Incubation of the RBCs with DIP strongly reduced the

photodynamically induced K^+ leakage (Fig. 5). However, the magnitude of this reduction differed strongly with the photosensitizer used. The protection factor, defined as the ratio between the light doses needed to induce 50-percent K^+ leakage in the presence and in the absence of DIP, for $AlPcS_4$ amounted to 1.5, while that with DMMB was 4.5. The effect of DIP on delayed hemolysis induced by PDT is depicted in Fig. 6. In the absence of DIP, both PDTs induced complete hemolysis 10 hours after their use. When illumination was conducted in the presence of DIP, hemolysis was strongly delayed. With $AlPcS_4$ as sensitizer, complete hemolysis was obtained 24 hours after PDT. With DMMB, the protective effect of DIP was even more pronounced: 30 hours after PDT, hemolysis amounted to less than 10 percent. Because of its lipophilic nature, DIP can be inserted between phospholipids.[24] The insertion of molecules in the membrane results in membrane expansion, which can result in protection of the RBC against osmotic hemolysis.[25,26] To investigate the involvement of membrane expansion in the protection, DIP was added to the RBC suspension after the PDT. In this case, DIP had no effect on delayed hemolysis (not shown). These results imply that it is very likely that the protective effect of DIP is due to its scavenging properties.

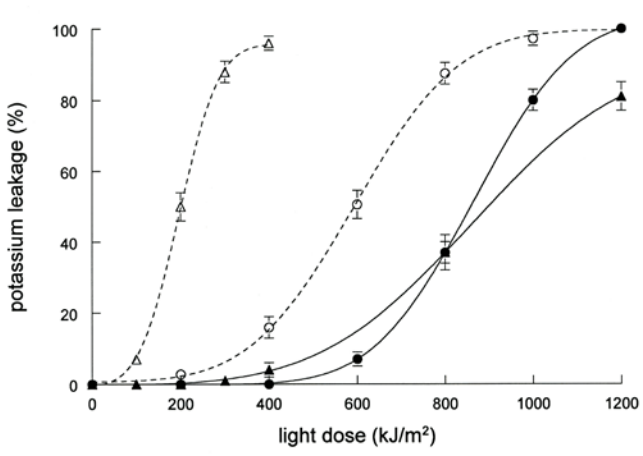


Figure 5. Effect of dipyridamole on photodynamically induced potassium leakage from RBCs.
Two percent-erythrocyte suspensions were incubated in the dark without (open symbols) or with 100 μ*M* DIP (closed symbols). After 5 min, the suspensions were exposed to light in the presence of 1 μ*M* $AlPcS_4$ (○,●) or 1 μ*M* DMMB (△,▲).

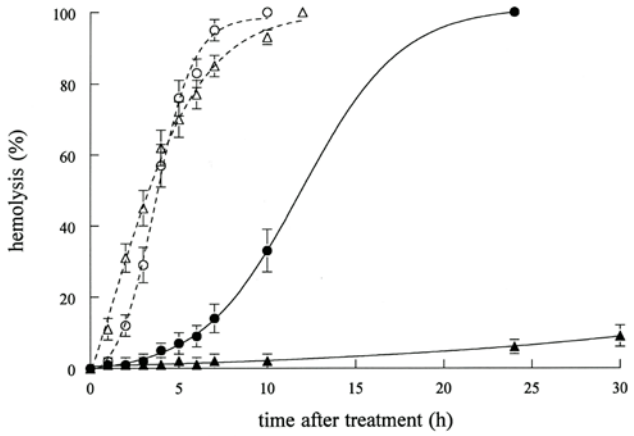


Figure 6. Effect of dipyridamole on photodynamically induced delayed hemolysis. Two percent-erythrocyte suspensions were incubated in the dark without (open symbols) or with 100 μ*M* dipyridamole (closed symbols). After 5 min, the suspensions were exposed to light in the presence of 1 μ*M* $AlPcS_4$ (○,●) or 1 μ*M* DMMB (△,▲). After a light dose of 200 kJ/m^2 or 75 kJ/m^2 respectively, the cells were washed, resuspended in fresh PBS (without dipyridamole or sensitizer) and stored at room temperature in the dark.

EFFECT OF DIP ON THE PHOTOINACTIVATION OF VSV IN RBC SUSPENSIONS

From the above, it is clear that DIP strongly protects RBCs against photodamage. To investigate whether DIP can increase the specificity of a photody-namic virucidal treatment, the effect of DIP on the photoinactivation of VSV was investigated. For this, VSV was spiked (10^5 infectious particles/mL) in 2 percent RBC suspensions. The suspensions were incubated in the dark for 5 minutes with DIP before the photosensitizer was added. After different light doses, samples were taken to assay for viral infectivity. As can be seen from Fig. **7**, both $AlPcS_4$- and DMMB-mediated photoinactivation of VSV was only slightly affected by DIP. When measured directly after the treatment, the light doses needed for 5 log inactivation of VSV induced only very limited leakage of potassium out of the RBCs; this indicated the potential use of these photosensitizers for photodecontamination of RBCC.

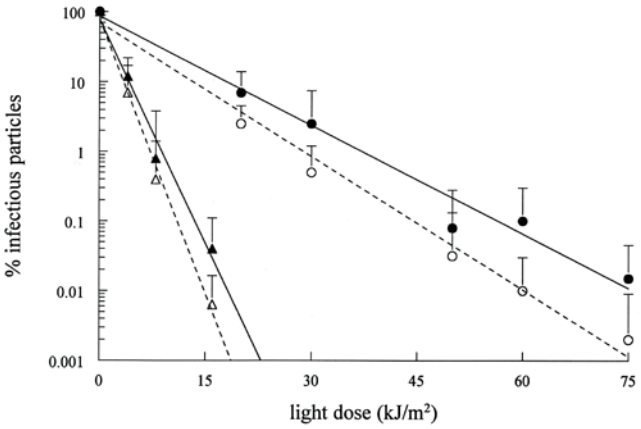
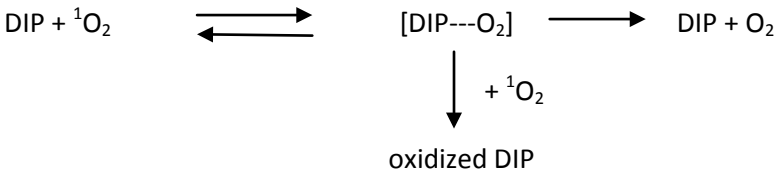


Figure 7. Effect of dipyri-damole on photodynamic inactivation of VSV.
VSV (10^5 infectious particles / mL) in 2 % erythrocyte suspensions, were incubated without (open symbols) or with 100 μ*M* DIP (closed symbols). After 5 min, the suspensions were exposed to light in the presence of 1 μ*M* $AlPcS_4$ (○,●) or 1 μ*M* DMMB (△,▲).

DISCUSSION

The cardiovascular drug DIP is known to be a scavenger of superoxide anions, hydroxyl radicals, and peroxyl radicals.[18,20] The present study is the first to show that DIP can also react with $^1O_2$, the most important reactive oxygen species formed upon PDT. With illumination of a solution of DIP (10 μ*M*) in the presence of $AlPcS_4$ (10 μ*M*) or DMMB (2 μ*M*) as photosensitizer, the optical spectrum of DIP changes (Fig. 2), and this indicates a chemical reaction between DIP and a photogenerated reactive species. The involvement of $^1O_2$ in this reaction was demonstrated by both the inhibitory effect of the $^1O_2$ scavenger azide and the faster oxidation in D_2O (Fig. 3). Because of the much longer lifetime of $^1O_2$ in D_2O than in H_2O, reactions involving $^1O_2$ are strongly accelerated in D_2O.[27] The changes in the optical spectrum of DIP differ from those obtained with DIP exposed to hydroxyl radicals, which indicates the formation of different reaction products.[23] The exact nature of the reaction product(s) and the possible intermediates remains to be elucidated. DIP is able to protect various substrates (histidine,

tryptophan, methionine tyrosine, and guanosine), which are known to be oxidized by $^1O_2$,[28,29] against photooxidation (Fig. 4). Under the conditions used in these experiments, no change in the optical spectrum of DIP could be observed. This suggests that, under certain circumstances; that is, a high concentration of DIP and a low concentration of $^1O_2$; DIP reacts with $^1O_2$ without being chemically changed. This physical interaction can most likely be attributed to the amine groups present in DIP, which are well known physical quenchers of $^1O_2$.[30] The following mechanism for the reactions of DIP with $^1O_2$ is suggested:



The present study proposed to investigate whether the cardiovascular drug DIP is able to selectively protect RBCs against damage induced by virucidal PDT. DIP binds with high affinity to the anion transport protein, band 3, the most abundant protein on the RBC membrane. This means that the concentration of $^1O_2$ scavengers in the vicinity of the RBC is higher than in the rest of the solution, which makes feasible the selective protection of RBCs against PDT. It was shown that DIP strongly protects RBCs against damage induced by PDT. The conditions used in these experiments are quite similar to those used in the photooxidation of histidine, which suggests that the protection of RBCs is due to the physical quenching of $^1O_2$ by DIP. The extent of RBC protection by DIP strongly depends on the sensitizer used to induce the RBC damage. With DMMB as photosensitizer, the protection by DIP was much stronger than with $AlPcS_4$. A feasible explanation for these differences in protection by DIP could be the localization of the photosensitizers. For effective scavenging, the activated photosensitizer and DIP should be in proximity to each other. DIP is known to bind in the hydrophobic interior of band 3.[16] Because of its hydrophilic nature it is very unlikely that $AlPcS_4$ will be located in proximity to DIP.

DMMB is much more hydrophobic and possibly is located in the same microenvironment as DIP, which makes effective $^1O_2$ scavenging feasible. To study the effect of DIP on the inactivation of viruses, we used the model virus, VSV, which was shown to be as sensitive to photoinactivation as HIV.[10] In contrast to the very strong protection found with RBCs, DIP caused hardly any decrease in the AlPcS4- or DMMB-mediated photoinactivation of VSV. Because it has been shown that DIP binds with low affinity (950-1150 M^{-1}) to lipid bilayers,[24] the binding of DIP to VSV cannot be excluded. But because DIP does not interfere with the photoinactivation of VSV, it must be concluded that the binding of DIP is not in the proximity of the critical target for virus photoinactivation. The present results were obtained by illumination of a model virus in a severely diluted RBC suspension (2% Hct). Further research is focused on the effect of DIP on the photoinactivation of other viruses, including HIV-1/2 and HCV, in a more concentrated RBC suspension. In addition, the effect of DIP on the shelf-life of photodynamically decontaminated RBCC must be investigated. Thus, the present study shows that DIP has the potency to become a very valuable tool to increase the specificity of the pathogen inactivation of RBCC, thereby increasing the practical applicability of this photodecontamination method.

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