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The photochemistry of dipyridamole

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Abstract

The photochemical reactions of dipyridamole (DIP), a healing agent, was studied to predict the photobiological implications of its use which will be the subject of a future article. Its photolysis product under UV-A and aerobic conditions was isolated and identified. DIP was shown to be photostable under inert atmosphere (argon). On the other hand, under the same conditions a process of electron transfer was detected in the presence of nitro blue tetrazolium (NBT) which can be inhibited by oxygen. The photodegradation of DIP occurs probably via a type II mechanism involving irreversible trapping of self-photogenerated reactive oxygen species (ROS). This fact could be indicative of its antioxidant activity. The formation of singlet oxygen and superoxide during the DIP photodegradation makes one suspicious of a possibility that DIP could also be involved in oxidative stress in biological systems. Further studies on biological systems will contribute to elucidate this probable dual behavior.

Keywords: Dipyridamole; Photodegradation; Singlet oxygen; Superoxide radical; Antioxidant

1. Introduction

Dipyridamole (DIP, 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimino-[5,4-*d*]pyrimidine, Fig. 1) is a well-known coronary vasodilator and antiplatelet agent [1] widely used in medicine. DIP, a yellow crystalline powder with a yellowish-blue fluorescence in solutions, has also been reported to possess antioxidant activity. Studies have demonstrated that the drug inhibits lipid peroxidation and scavenges superoxide and hydroxyl radicals. The affinity of DIP for the lipid phase has been confirmed by the fact that it acts as an inhibitor of membrane peroxidation [2–5]. Furthermore, photosterilization of cellular blood products, upon activation by a photosensitizer with visible light is being developed. In this method active oxygen species are formed of which singlet oxygen is the most important [6,7]. DIP seems to give a selective protection to the red cells against damage induced by photosensitizers in the process of viral inactivation [8].

In this context, not much is known as yet about the relationship between the photochemistry, photosensitizing properties and phototoxicity of this drug in biological systems. We examined the photolysis of DIP under a variety of conditions, with the purpose of establishing the role of oxygen (especially singlet oxygen and superoxide) in its photodegradation as well as the mechanism of the reaction. Furthermore, we have an additional interest on its application as a protective agent for red blood cells in cellular blood and plasma photosterilization processes.

2. Experimental details

2.1. Chemicals

DIP was extracted from Persantin® (Boehringer Ingelheim Laboratories) with dichloromethane and purified by TLC-preparative, alternatively it was also obtained from Sigma (St. Louis, MO) as a pure compound. Nitro blue tetrazolium (NBT) and triamterene, were purchased from Sigma, as well, Rose bengal, histidine, furfuryl alcohol (FFA) and 2,5-dimethylfuran (DMF) were purchased

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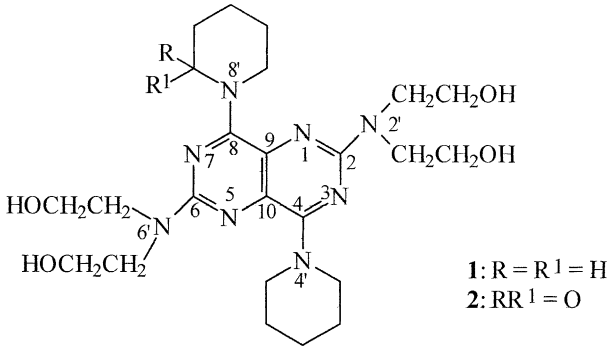

Fig. 1. Structure of the drug DIP (**1**) and its photoproduct (**2**).

from Aldrich (Steinheim, Germany). All analytical or high-performance liquid chromatography (HPLC) grade solvents were obtained from Merck (Darmstadt, Germany). Sodium 1,3-cyclohexadiene-1,4-diethanoate was synthesized according to a known procedure [9].

2.2. Photochemical reactions

Solutions of DIP in PBS, MeOH and also in presence of human serum albumin (HSA) were irradiated under aerobic conditions at room temperature, with an Osram HQL 250 W medium pressure Hg lamp placed inside a Pyrex immersion-well photoreactor (Applied Photophysics parts no. 3230 + 3307) for UV-A irradiation (output spectral 320–400 nm) with a maximum at 370 nm with a total irradiance of 17 mW cm^{-2} as measured with a model of UVX Digital Radiometer after 1 h of continued illumination. The distance between the light source and the test aliquots was 10 cm. The temperatures detected in the cuvette during a standard 1 h irradiation were no higher than 27 °C. Irradiation was also carried out under the same conditions with a nitrogen laser with spectral output 337 nm (GL-3300 Photon Technology International, NJ) and peak power at 5 Hz of 2.4 MW and 1.45 mJ of energy per pulse. The course of the reaction was followed by UV–Vis spectrophotometry (for 10^{-4} M solutions) using a Milton-Roy Spectronic 3000 array instrument (Milton Roy Company, USA).

All preparative irradiations (1.6×10^{-2} M) were monitored by (HPLC, Waters Delta Prep 4000) equipped with an analytic and a preparative C18-Bondapak column using a MeOH/H_2O gradient as mobile phase at a flow rate of 0.4 ml min^{-1}, with monitoring at 225 nm. When irradiation was completed, the PBS solution was extracted with CH_2Cl_2 and the organic phase evaporated at reduced pressure (14 Torr) at room temperature. The residue was purified by preparative HPLC. The isolated product was analyzed by ^{1}H NMR and ^{13}C NMR spectroscopy (Bruker Aspect 3000, 300 MHz), FT-IR (Nicolet DX V 5.07) and MS (Carlo Erba/Kratos MS25RFA). The fluorescence spectra were registered with a Shimadzu RF 1501 spectrofluorophotometer.

Degradation product (**2**) melting $point_{obs}$ = 152–154 °C (ethanol), green crystals, r.f. = 0.45 (CH_2Cl_2/MeOH, 5:1), UV (ethanol): λ_{max} = 237 and 266 nm, fluorescence: λ_{ex} = 436 nm, λ_{emit} = 507 nm with a Φ_F = 0.11, IR (KBr): ν = 1710, 1540, 1440, 1430, 1350, 1210, 860, and 750 cm^{-1}. ^{1}H NMR (CD_3OD, 300 MHz): δ = 4.37 (t, J = 5.4 Hz, 8H, –NCH_2CH_2OH), 3.93 (t, J = 5.4 Hz, 8H, –NCH_2CH_2OH), 2.76 (m, 2H, –CH_2–N–CO-piperidino), 2.70 (m, 2H, –CH_2–CO–N-piperidino), 2.60 (m, 4H, –CH_2–N-piperidino), 2.02 (br. s, 4H, –OH), 1.75 (m, 10H, –CH_2CH_2-piperidino). ^{13}C NMR (CD_3OD, 100 MHz): δ = 175.1 (s, C-4), 172.7 (s, C-8), 170.3 (s, C=O), 162.6 (s, C-2), 158.0 (s, C-6), 125.4 (s, C-10), 124.8 (s, C-9), 62.3 (t, N–CH_2–CH_2–OH), 49.0 (t, N(4′)-CH_2), 48.0 (t, N–CH_2–CH_2–OH), 46.0 (t, N(8′)–CH_2), 35.1 (t, N(8′)–CO–CH_2), 25.7 (t, N(4′)–CH_2–CH_2), 25.5 (t, CH_2–CH_2–CH_2-(ring 8′)), 24.0 (t, CH_2–CH_2–CH_2-(ring 4′)), 22.0 (t, CH_2–CH_2–CH_2-(ring 8′)). MS: *m/e* (%) = 519 (5, M^+), 449 (30, $M^+ - 70$), 435 (50, $M^+ - 84$), 415 (15, $M^+ - 104$), 365 (100), 345 (14), 311 (20), 261 (45), 70 (20).

The reaction in the presence of methanol produces also traces of a secondary product with incorporation of OCH_3 group to photoproduct **2** (analyzed by gas chromatography (GC)–mass spectrometry (MS)).

2.3. Quantum yields

Quantum yields were determined both for DIP fluorescence and for product formation. The relative quantum yields of fluorescence at room temperature were determined either by comparing the corrected fluorescence intensity of the DIP in ethanol with that of rhodamine B (at a concentration of 1×10^{-6} M in ethanol; fluorescence quantum yield, 0.69) or with that of quinine bisulfate in 0.05 M H_2SO_4 (fluorescence quantum yield, 0.55) [10].

In the product quantum yields determination the photolysis was allowed to proceed to less than 10% product formation to minimize light absorption by the photoproducts and additional products from side reactions. The photon flux incident on 3 ml of solution in quartz cuvettes of 1 cm optical path was measured by means of a ferric oxalate actinometer and it was of the order of $10^{15}$to 10^{16} quanta s^{-1}.

2.4. Singlet oxygen trapping

In separate experiments analogous irradiations to those described above were carried out under the same experimental conditions in the presence of 2,5-DMF (2.00 mM) which is used as a trap for singlet oxygen ($^1O_2$) [11]. This process was followed by GC and by MS. Rose bengal, a well-known $^1O_2$ sensitizer, was used as a standard for comparison with DIP for $^1O_2$ formation, under identical conditions of photolysis. On the other hand, when the irradiation of DIP was carried out in the presence of Rose bengal using a potassium chromate solution (150 mg l^{-1}) as a filter allowing $\lambda > 450$ nm and maintaining all other conditions equal

photodegradation of the compound under investigation was observed.

Another trap method has been successfully used to detect $^1O_2$ generated in a variety of samples [12,13]. This method consists in following the consumption of a chemical trap FFA that reacts with singlet oxygen. The consumption of FFA was followed by HPLC using a 90:10 H_2O/CH_3CN mobile phase composition. The detection wavelength used for monitoring FFA consumption was $\lambda = 222$ nm.

A new method for the determination of the quantum yield of singlet oxygen formation was also used with more precision and with smaller ambiguity than the usual chemical trapping processes. A water solution of sodium 1,3-cyclohexadiene-1,4-diethanoate (10^{-2} M) and DIP = 0.03 M) was irradiated under oxygen atmosphere under the same conditions previously described and the determination of the $^1O_2$ quantum yield was carried out by HPLC following a procedure described by Nardello et al. [14].

Indirectly, photosensitized degradation of histidine [15] was measured in the presence of 0.25, 0.50, 1.0 and 1.5 × 10^{-5} M solution of DIP. These solutions were mixed with an equal quantity of L-histidine solution at 0.60–0.74 mM in phosphate buffer 0.01, pH 7.4. Samples of this mixture were irradiated with an Osram HQL 250 W medium pressure Hg lamp through a filter Pyrex (radiation dose 4.5 J cm^{-2}) at time intervals from 60 to 180 min and the respective controls were maintained protected from light. The histidine was determined by a colorimetric reaction using phosphate buffer, sulfanilic acid, sodium nitrite, sodium carbonate and ethanol as reagents. The optical density was read on a spectrophotometer at 530 nm against a blank reagent. Also a sensitive spectrophotometric method for the detection of $^1O_2$ based on secondary bleaching of *p*-nitrosodimethylaniline (RNO) as induced by the reaction of singlet oxygen with histidine was carried out. DIP (1.0×10^{-5} M) was exposed to light in the presence of histidine (10 mM) and RNO (50 mM) in PBS. The formation of singlet oxygen was monitored spectrophotometrically at 440 nm (bleaching of RNO by the transannular peroxide intermediate formed as a result of the reaction between $^1O_2$ and histidine) [16,17]. Rose bengal, a well-known $^1O_2$ sensitizer, was used as a standard for comparison with **1** as far as $^1O_2$ formation, under identical conditions of photolysis.

2.5. Electron transfer mechanism detection by reduction of NBT

Under the same conditions of the photololysis of DIP (1.0×10^{-5} M), the photoreduction of NBT ([NBT] = 4.0×10^{-5} M) was followed in presence of NBT (1:1) under oxygen atmosphere, argon and air, as a function of the irradiation time by determining the increase in absorbance at 560 nm due to the formation of diformazan product [18,19]. The same experiment was carried out in the presence of superoxide dismutase (SOD) (1.0×10^{-6} M).

2.6. Statistical treatment of results

At least three independent experiments were performed except where indicated. Results are presented as the mean value ±S.E.M., $n = 3$. Statistical analyses were performed using *t*-test and one-way ANOVA. A probability value of <0.05 was considered significant.

3. Results

The drug DIP, **1** is photolabile under aerobic conditions in methanolic and also in buffered aqueous medium (pH 7.4). The photolysis of **1** was followed by monitoring the disappearance of the 420 nm bands at 15 min intervals. An increment of the bands at 210–235 nm was also observed. The quantum yield for DIP decomposition was $\Phi = 0.02$ with UV-A light. When the reactions were carried out upon irradiation with a nitrogen laser case obtained the same process of photodegradation that them effected with the Osram HQL lamp. The results are shown for a methanolic solution (1.0×10^{-5} M) of **1** in Fig. 2.

The relative quantum yields of fluorescence of DIP at room temperature was $\Phi_F = 0.23$ ($\lambda_{ex} = 305$, $\lambda_{emit} = 483$ nm). In anaerobic conditions (argon atmosphere) no photodegradation of the drug was detected after 48 h irradiation.

Only one photoproduct of the reaction in aerobic conditions was detected and identified as the main product, (structure **2** (95480-83-4), Fig. 1, previously isolated by Kigasawa et al. [20]).

Furthermore, degradation of DIP was observed when irradiation was carried out in the presence of Rose bengal, using a potassium chromate solution (100 mg l^{-1}) as a filter (which allows $\lambda > 400$ nm) and maintaining all other conditions the same. Therefore, an interaction or quenching of singlet oxygen with DIP is possible.

DIP was capable to produce singlet oxygen through photosensitization via type II mechanism. This process was detected by trapping it with 2,5-DMF during the photolysis of DIP in oxygenated media. Notably ca. 60% of DIP was auto-oxidized by $^1O_2$ produced by itself. Trapping of singlet oxygen in this manner leads to the formation of hexene-2,5-dione (24%), *cis*- and *trans*-3-oxo-1-butenyl acetate (16 and 30%, respectively), and 2-methoxy-5-hydroperoxide-DMF (28%) as detected by GC–MS [11]. The yields were calculated on the base of 54% conversion of DMF. In a control experiment performed in the presence of DMF under argon atmosphere no formation of the corresponding products was detected. The formation of $^1O_2$ was confirmed also by trapping it with FFA (determined by HPLC [12]) and a histidine test [15–17]. The histidine model should be regarded simply as a test for oxygen dependent photosensitized damage to cellular protein. DIP at several concentrations was efficient for photooxidation of histidine which is susceptible to singlet oxygen attack.

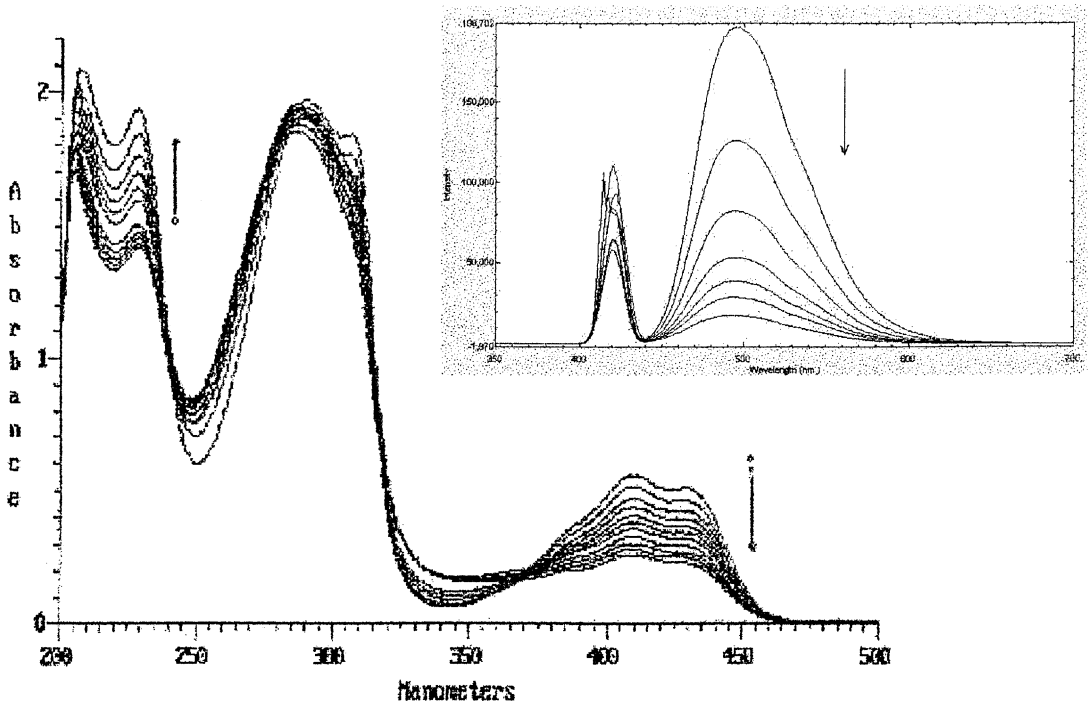

Fig. 2. UV and fluorescence emission monitoring of the photolysis of DIP under oxygen atmosphere.

Due to the ambiguity about the specificity of the 2,5-DMF trapping method which is good as a preliminary indication of the presence of $^1O_2$ an additional, more precise method, than that of FFA and histidine [14] was used. Another measurement with 1,3-cyclohexadiene-1,4-diethanoate (a specific chemical $^1O_2$ trapping) was determined in water giving as a result a value of $\Phi(^1O_2) = 18\%$ for singlet oxygen formation.

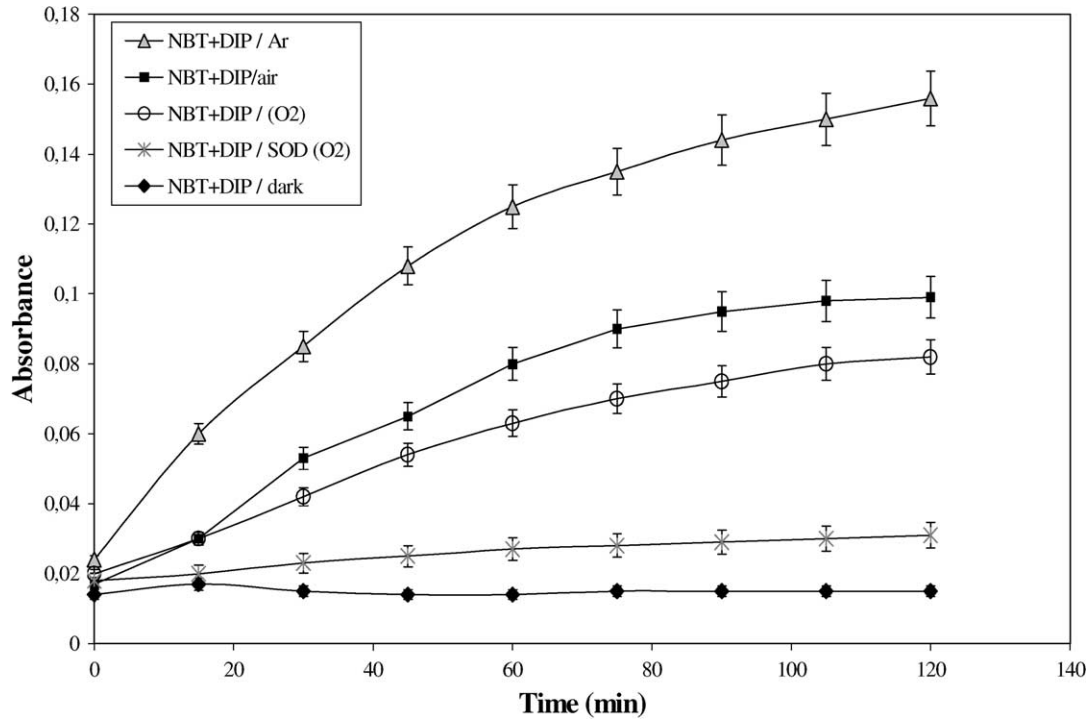

Fig. 3. Photoreduction of NBT (4.0×10^{-5} M) by DIP ([DIP] $= 1.0 \times 10^{-5}$ M) in PBS solution, under UV-A light in anaerobic and aerobic. [SOD] $= 1.0 \times 10^{-6}$ M. Values are mean ± S.E.M. of three experiments with three samples each. S.E.M. was always less than 3%.

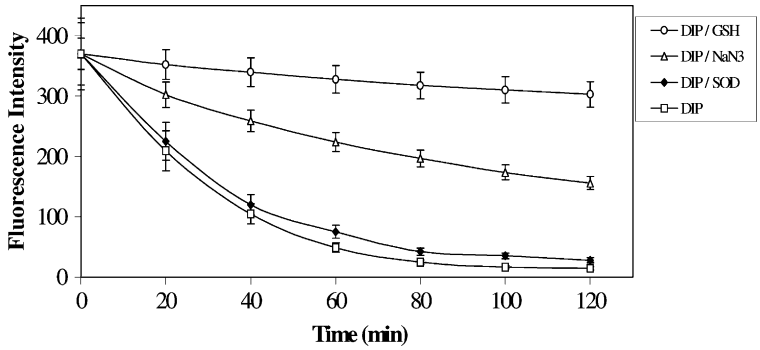

Fig. 4. Photodegradation of DIP ([DIP] = 1.0×10^{-5} M) under UV-A light in aerobic conditions and different additives followed by fluorescence spectrometry. Values are mean ± S.E.M. of three experiments with three samples each. S.E.M. was always less than 4%.

DIP was also capable to produce a singlet electron transfer reaction in anaerobic condition during its photolysis via type I mechanism. This was determined using NBT as a sonde during the photolysis of DIP under argon atmosphere. So that the reduction of NBT to diformazan was detected by this procedure (with spectrometric reading at 560 nm) as previously described [21,22]. Control experiments indicated that reduction of NBT under argon or oxygen atmosphere in the presence of DIP in the dark were not detectable. The same results were observed in absence of DIP.

In the presence of air or saturated oxygen solutions this reaction was quenched and the NBT reduction to diformazan was not detectable (Fig. 3). Thus, suppression of the reduction by SOD, used frequently as a diagnostic for the participation of oxygen superoxide, was also significant. These experiments confirm that oxygen maybe involved as an electron carrier in the form of the superoxide anion radical. Thus, it appears that reduction of NBT photosensitized by DIP can be a direct reaction from the excited state of DIP to NBT but strongly subjected to interference by molecular oxygen. In this study it was found that sodium azide demonstrated an inhibiting effect on the photodegradation of DIP (Fig. 4, reaction (1)).

With the purpose of studying the antioxidant character of the DIP against the phototoxic drug triamterene (photosensitized damage to erythrocytes) it was necessary to establish unambiguously the interaction between them by means of absorbance and fluorescence spectrophotometry. The results obtained could explain the antioxidant character of the DIP at low concentrations in the photosensitized photohemolysis test.

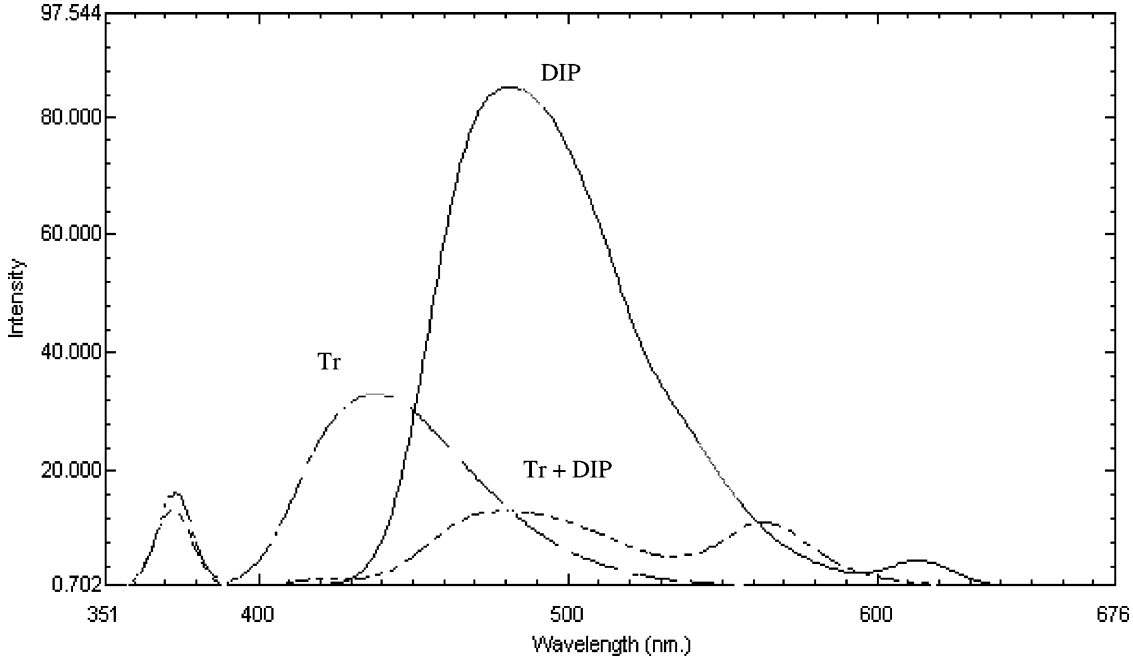

Fig. 5. Emission spectra of triamterene (Tr) and DIP alone and in an equimolar solution.

If the absorption spectrum of a molecule (DIP, λ_{max} = 415 nm, broad band from 370 to 460 nm) overlaps the fluorescence emission spectrum of a second molecule (triamterene, $\lambda_{max\text{-}emit}$ = 437 nm, broad band from 390 to 560 nm), quenching of fluorescence may occur by dipole–dipole interaction. The efficiency of the fluorescence quenching, E_q, can be calculated using the formula $E_q = (1 - I_{TD}/I_T)$ [23,24], where I_{TD} is the fluorescence intensity from a cell sample labeled with both triamterene and DIP (λ_{ex} triamterene + DIP = 370 nm, λ_{emit} = 479 nm) = 13.159, and I_T is the fluorescence intensity from a cell sample labeled with triamterene alone (λ_{ex} triamterene = 370 nm, λ_{emit} = 436 nm) = 33.070, indicating the efficiency of the fluorescence quenching of the DIP on the triamterene in ethanol was E_q = 0.602. From the reciprocity of fluence rate and irradiation time for phototoxicity of triamterene and the efficiency of the fluorescence quenching of DIP, the photoprotection factor (P) is 2.51 ($P = 1/(1 - E_q)$). This experiment is illustrated in Fig. 5. These photobiological implications will be discussed in detail and reported at a later date.

4. Discussion

Three important processes describe the photochemical behavior of DIP: (a) it is photostable under inert atmosphere (argon), (b) under the same conditions it is capable of acting as a sensitizer via a single electron transfer (SET) (type I mechanism) which is inhibited in the presence of oxygen and (c) under aerobic conditions photodegradation occurs probably via a type II mechanism to yield product **2** and oxygen excited species. Evidence for a SET mechanism (b) was supported by the used of NBT as a sonde during the photolysis of DIP under argon atmosphere. The reduction of NBT to diformazan was detected by means of spectrophotometric measurements as described above [18,19]. Control experiments either under argon or oxygen in the dark did not give evidence of the reduction reaction. The same results were obtained in the absence of DIP. Suppression of the reduction reaction by SOD was noted; it is presumed that oxygen maybe involved as an electron carrier in the form of superoxide anion radical. Perhaps the reduction of NBT photosensitized by DIP can be a direct reaction from the excited state of DIP to NBT but strongly subjected to interference by molecular oxygen.

In reference to (c), the photodegradation itself, can be illustrated by the following reaction:

$$\text{DIP} + \text{O}_2 \xrightarrow{h\nu} {}^1\text{O}_2 + \text{O}_2^{\bullet -} + \text{photoproduct } \mathbf{2} \quad (1)$$

ESR studies of peroxy radical and electrochemical-mediated oxidation shed some light on this photochemical reaction, concluding from the ESR spectrum of the DIP radical and the kinetics of inhibition of oxygen consumption that DIP is oxidized in a two-electron transfer reaction [4]. The formation of singlet oxygen and of superoxide ion radical has been well-substantiated by trapping reactions (2,5-DMF, 1,3-cyclohexadiene-1,4-diethanoate), photosensitized degradation of histidine, secondary bleaching of *p*-nitrosodimethylaniline, etc., and SOD suppression of reduction, respectively.

In the course of this investigation it was also found that sodium azide inhibits the photodegradation of DIP (Fig. 4, reaction (3)). Thus, sodium azide could also have an inhibiting effect on superoxide production which could be interpreted as being due to triplet quenching by azide indirectly, inhibiting the progress of reaction (2) by reducing the amount of singlet oxygen produced in reaction (3). It is possible, though, that superoxide could derive from an electron transfer reaction not involving singlet oxygen as illustrated by reaction (4) [25].

$$\text{DIP}^* + \text{O}_2 \rightarrow {}^1\text{O}_2^* + \text{DIP} \quad (2)$$

$${}^1\text{O}_2^* + \text{DIP} \rightarrow \text{O}_2^{\bullet -} + \text{DIP}^{\bullet +} \quad (3)$$

$$\text{DIP}^* + \text{O}_2 \rightarrow \text{O}_2^{\bullet -} + \text{DIP}^{\bullet +} \quad (4)$$

The fact that in spite of the formation of oxygen excited status DIP still exhibits an antioxidant character strongly supports the idea of the irreversible photooxidation triggered by self-photogenerated reactive oxygen species (ROS) as an adequate explanation for its capability of activating a protective mechanism of biological systems (red cells) exposed to damage induced by photosterilization induced by means of ${}^1\text{O}_2$ or $\text{O}_2^{\bullet -}$.

Another type of protecting mechanism exhibited by DIP consists of fluorescence energy transfer as evidenced by the overlapping of its absorption spectrum to the fluorescence emission spectrum of a molecule such as triamterene, a phototoxic drug which photosensitize damage to erythrocytes [26]. DIP maybe a selective protector of red cells against damage induced by excited oxygen species because it binds preferably to the lipid bilayer avoiding the interaction with the membrane protein [27]. It can also react with ${}^1\text{O}_2$ or $\text{O}_2^{\bullet -}$ whenever the appropriate concentration is used ($<3.0 \times 10^{-5}$ M) [28]. Although it does not seem a prominent factor in most studies, auto-oxidation of photosensitizer can increase cellular steady levels of oxidants rather than decrease them. Therefore, DIP is an ROS-trap, but its photo-auto-oxidation also generates ROS. These species as ${}^1\text{O}_2$ or superoxide, can generate structural modifications resulting from damage to lipids, protein, DNA and recently used as second messengers to propagate pro-inflammatory or growth-stimulatory signals [29,30]. To balance these antagonistic activities and tilt the balance to the beneficial effects it is necessary that a combination of ROS and adequate concentrations operate favorably.

In relation to the main photoproduct of the DIP irradiation, oxidation in a piperidine group, it is important to emphasize that from data obtained with DIP derivatives all nitrogen substituents are necessary for the antioxidant activity. In fact,

the absence of one piperidine group causes the loss of its antioxidant activity [31].

5. Conclusion

DIP is an interesting photoprotecting agent of red blood cells that may act through two photochemical mechanisms to exert its action: (a) irreversible trapping of self-photogenerated ROS and (b) fluorescence energy transfer from a phototoxic sensitizer to DIP acting as a quencher. This mechanism can be efficient at low concentrations of DIP. The formation of the photoproduct derived from its photodegradation proceeds by involving a two-electron transfer reaction which results in oxidation of one of the two piperidine groups present in the molecule. The lack of one of these two groups causes the loss of its antioxidant activity.

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