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Electron-transfer mechanisms in photosensitization by the anti-inflammatory drug benzydamine

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Abstract

The novel anti-inflammatory drug benzydamine has been shown to photosensitize the reduction of Nitro Blue Tetrazolium, ferricytochrome *c* and copper (II) bathocuproinedisulphonate in aqueous solutions (pH 7.4, 30°C) when irradiated with UV light at its maximum absorption wavelength of 308 nm. The reduction reactions all proceed most efficiently when the solutions are deoxygenated, clearly indicating that direct electron transfer occurs from the excited state of the sensitizer to the substrate. In aerated solutions the reduction reactions are slower and are partially inhibited by superoxide dismutase, suggesting that superoxide anion could be involved as an intermediate when oxygen is present. Benzydamine also photosensitizes the oxidation of l-histidine and 2,5-dimethylfuran by the singlet oxygen pathway in aerated solutions. The ability of benzydamine to participate as sensitizer in several types of photochemical reaction is relevant to the observed clinical photosensitivity of the drug.

Keywords: Benzydamine; Anti-inflammatory drugs; Electron transfer; Photosensitization; Phototoxicity; Nitro Blue Tetrazolium; Cytochrome *c*; Bathocuproinedisulfonate

1. Introduction

In addition to the direct responses to UVA and UVB exposure, the human system can be subjected to sunlight-caused effects similar to an exaggerated sunburn but mediated through exogenous photosensitizers such as prescription medication. In general, the generation of an adverse photosensitivity response can be postulated to involve one or more of the pathways shown in Fig. 1.

The molecular mechanisms of photosensitization by drugs are being investigated in our laboratory by studying the properties of their photo-excited states, and free-radical and singlet oxygen mediated photo-oxidation reactions that ensue in model biological systems. The photo-oxidation of susceptible biological substrates by singlet oxygen or free-radical pathways is widely believed to lead to the initiation of the adverse responses. However, it is possible that, in conditions of low oxygen concentration, or when the sensitizer is located close to susceptible substrates, direct electron transfer from sensitizer to substrate may become a significant contributor. Oxygen may also be involved as an electron carrier in the form of the superoxide anion radical.

Nitro Blue Tetrazolium (NBT) and (ferri)cytochrome *c* (Cyt) are reagents which are frequently used to detect the occurrence of the superoxide anion radical in an enzymic or photosensitized reaction [1,2]. Superoxide can be formed by electron transfer from a donor to molecular oxygen, but it is quenched by NBT or Cyt which are thereby reduced to diformazan and ferrocytochrome *c*, respectively. The detection of superoxide is confirmed when addition of the enzyme superoxide dismutase (SOD) causes a decrease in production of diformazan from NBT. The possibility exists for direct electron transfer from the photosensitizer to NBT if its rate is competitive with that of superoxide formation. This is more likely when oxygen is in short supply.

Another compound which can be used for this test is the copper (II) complex with a bifunctional ligand such as bathocuproine disulphonic acid (BCDS), which shows SOD-like activity [3]. The electron transfer to the Cu(II)–BCDS complex resulting in reduction to the Cu(I) complex is detectable by a spectrophotometric change [4].

A number of photosensitizing drugs previously tested in this laboratory [5–7] have been shown to undergo photoionization and/or are active in free-radical generation. They may also participate in electron-transfer processes. We have already reported that 6-mercaptopurine reacts with NBT or *p*-nitroso-dimethylaniline when irradiated in an oxygen-free

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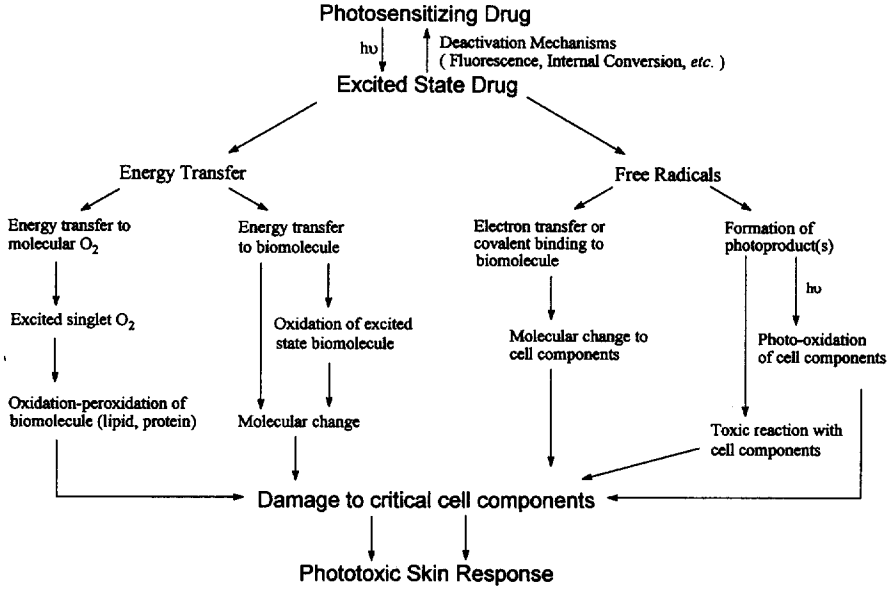

Fig. 1. General scheme of possible reaction processes by which a photosensitizing drug may give rise to adverse photobiological effects (phototoxicity).

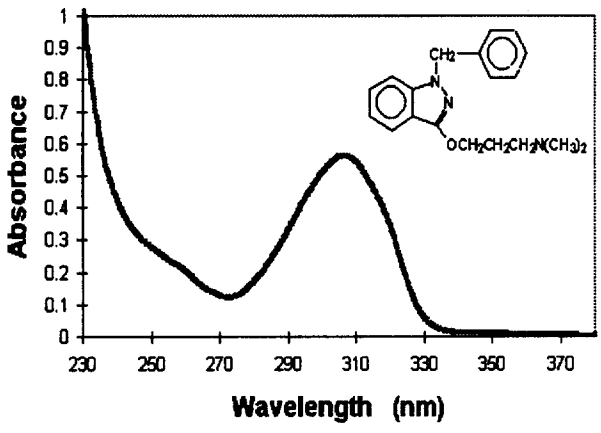

Fig. 2. Structure and absorption spectrum of benzydamine (1×10^{-4} M) in aqueous buffer solution.

solution [5]. The antibacterial drug sulfamethoxazole was also found to participate in photo-initiated electron transfer to NBT and Cyt [7]. In this paper the use of NBT, Cyt and BCDS is described for the investigation of electron-transfer mechanisms involving the drug benzydamine. This compound is a unique non-steroidal anti-inflammatory agent which also possesses local anaesthetic properties. However, many cases of photosensitivity reactions following topical application or oral ingestion of benzydamine have been reported [8–14]. Its chemical structure is given with the UV absorption spectrum in Fig. 2.

2. Methods

Benzydamine hydrochloride was kindly supplied as the pure substance by 3M Pharmaceuticals Pty Ltd, Sydney, and used as received. Buffered aqueous solutions (phosphate, 0.05 M, pH 7.4) containing Cyt (0.1 mg/ml) NBT (5.0×10^{-5} M) or BCDS (0.1 mM) with 50 μM benzydamine were flushed with gas (N_2 or O_2 as required) for 40 min before irradiating. The samples were contained in stoppered cylindrical quartz vessels of 10 mm pathlength and irradiated with a 400 W medium-pressure mercury arc (Applied Photophysics Ltd, UK) through a 2 mm Pyrex glass filter (Corning O-53). The arc source, filter and reaction vessel assembly was immersed in a thermostat maintained at 30.0°C. The photoreduction of NBT was followed as a function of the irradiation time by determining the increase in absorbance at 560 nm due to the diformazan product [1]. The extent of reduction of Cyt was determined by measuring, as a function of irradiation time, the differences in absorbance between the maximum at 550 nm and the minimum near 535 nm [2]. The reduction of Cu(II)–BCDS was monitored at 484 nm [4].

3. Results

3.1. Reduction of Nitro Blue Tetrazolium

When NBT in N_2- or O_2-flushed solution was irradiated in the absence of a photosensitizer, no detectable reaction occurred. With the addition of benzydamine in an N_2-flushed solution the reduction of NBT could be seen by the appearance of the diformazan at 560 nm. Fig. 3 shows the reaction photosensitized by benzydamine as a function of time. The reaction occurred to a lesser extent when an air-saturated solution was employed under otherwise identical conditions, and was completely inhibited when the solution was flushed with oxygen before irradiation. Thus it appears that the reduction of NBT photosensitized by benzydamine can be a direct reaction from the excited state of the sensitizer to NBT which molecular oxygen is able to inhibit.

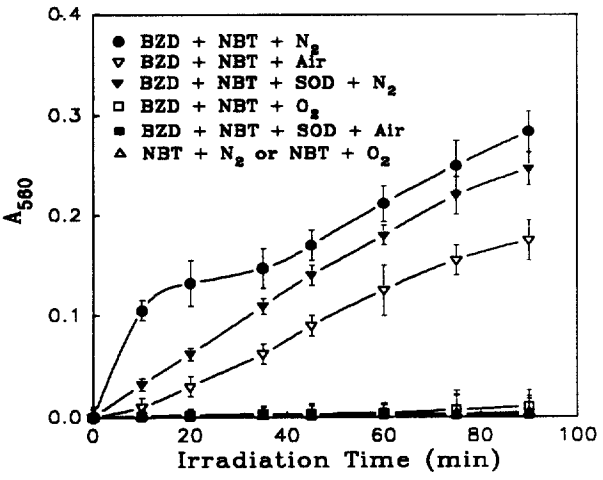

Fig. 3. Photoreduction of NBT (5.0×10^{-5} M) sensitized by benzydamine (5.0×10^{-5} M) in aqueous buffer solution at pH 7.0 and 30°C.

There are, however, a number of competing processes in which oxygen can be involved, such as singlet oxygen or superoxide formation. These were investigated as follows. Addition of SOD to the reaction mixture irradiated under air-saturated conditions completely inhibited the formation of diformazan, implying the involvement of superoxide in the reaction when some oxygen was present. The question arises as to why oxygen saturation inhibits the reaction completely when superoxide is diagnosed as being present under air-saturated conditions. It is suggested that the reaction is oxygen-concentration dependent. This behaviour is similar to that observed with 6-mercaptopurine [5], but different from that with sulfamethoxazole [7] or α-terthienyl [15]. However, all results are consistent with the report [16] that direct elevation of pO_2 will suppress the photochemical reduction of NBT to formazan by simple mass action, as follows:

$$S^* \rightarrow S + e^- \xrightarrow{O_2} O_2^{\cdot-}$$

$$NBT^{2+} + O_2^{\cdot-} \rightleftarrows NBT^{+} + O_2 \quad (1)$$

$$2NBT^{+} \xrightleftharpoons{H^+} MF^{+} + NBT^{2+} \quad (2)$$

The first step in the reduction of NBT^{2+} to the monoformazan MF^+ by $O_2^{\cdot-}$ is the production in (1) of the tetrazoinyl radical which then disproportionates by reaction (2). The monoformazan then reacts to the end-product diformazan by the same sequence of reactions. Addition of SOD shifts reaction (1) to the left by decreasing the steady-state concentration of $O_2^{\cdot-}$, thereby reducing the concentration of the tetrazoinyl radical and subsequently the monoformazan. Another way of affecting reaction (1) is by raising the pO_2 level. Since oxygen is both essential to the production of $O_2^{\cdot-}$ and is an inhibitor of the reduction of NBT^{2+}, there is a range of pO_2 that will be optimal for the photochemical reduction of NBT^{2+} to the formazan. The pO_2 value in air-equilibrated solutions may be near that optimum value, as suggested by irradiation of riboflavin, NBT^{2+} and tetramethylethylenediamine [16].

The reduction of NBT induced by irradiated benzydamine under anaerobic conditions appears biphasic with a short rapid initial stage superimposed on a slower reaction. SOD diminished the extent of reduction only in this initial stage with little influence on the latter part. Similar results were also reported for photoreduction of NBT by riboflavin and methionine [17] and 6-mercaptopurine [5]. This result suggests that under anaerobic conditions, two mechanisms may be considered: either direct electron transfer from excited sensitizer to NBT, or initial expulsion of one electron from the excited sensitizer to the medium, and subsequent reaction of the solvated electron with the reagent. In both cases, the possibility that the reactions were actually due to one or more photoproducts could not be excluded, since the UV spectrum of benzydamine has changed after this period of irradiation time. All the above results implicate electron transfer from the excited states of benzydamine.

3.2. Cytochrome c reduction

Ferricytochrome *c* is an electron acceptor which differs from the NBT reduction in that only one electron is required to reduce the fully oxidized 'ferri' form to the fully reduced 'ferro' form [18]. From the standard redox potentials (hydrogen scale) for $O_2^{\cdot-}$ [$E^\circ(O_2/O_2^{\cdot-}) = -330$ mV, $E'^\circ(O_2^{\cdot-}, H^+/H_2O_2) = 940$ mV] [19] and cytochrome *c* [$E^\circ(Fe^{III}$ cytochrome c/Fe^{II} cytochrome $c) = 260$ mV] [20], it is evident that, on thermodynamic considerations alone, $O_2^{\cdot-}$ could either oxidize or reduce ferricytochrome *c*. In the presence of $Na_2S_2O_4$ and O_2, $O_2^{\cdot-}$ reduces ferricytochrome *c* in 100% yield [21]. After capturing one electron, Cyt(III) will be reduced to Cyt(II), producing an absorption band at 550 nm. The absorption spectra of oxidized and reduced cytochrome *c* are very characteristic and distinct, and this spectral change provides a sensitive and convenient assay for an electron-transfer reaction. It has been used to detect the superoxide anion in aerated conditions [22,23] and electron release from a sensitizer in deaerated conditions. Such electron transfer has been demonstrated with benzo[*a*]pyrene [24], α-terthienyl [15], anthracene [25], 2-chloro-3,11-tridecadiene-5,7,9-triyn-1-ol [26] and sulfamethoxazole [7].

When oxygen-, air- or nitrogen-saturated samples containing benzydamine and cytochrome *c* were kept in the dark or when cytochrome *c* alone was irradiated, no spectral changes were observed. When the experiments were performed under continuous irradiation in an oxygen-saturated solution containing benzydamine 5.0×10^{-5} M and cytochrome *c* (0.1 mg/ml) in pH 7.4 phosphate buffer, there was an increase in absorbance at 550 nm. The difference in absorbance ($A_{550}-A_{535}$), plotted as a function of irradiation time in Fig. 4, shows a steady increase with time, reaching a plateau after about 10 min irradiation. However by adding sodium hydrosulfite to the irradiated medium, a further increase in the absorbance of the 550 nm peak was observed, with conversion of all the Cyt(III) to Cyt(II). This means that cyto-

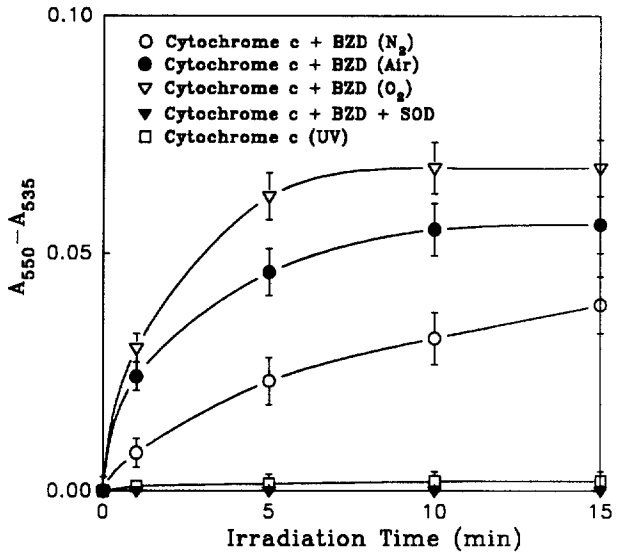

Fig. 4. Photoreduction of cytochrome *c* (0.1 mg/ml) sensitized by benzydamine (5.0×10^{-5} M) in aqueous buffer solution at pH 7.4 and 30°C. Each value shown is the average of three determinations.

chrome *c* was not the limiting reagent. There appear to be other factors inhibiting this reduction process. One possibility is that cytochrome *c* itself introduces an inner filter effect by light absorption at 365 nm, although this usually happens at cytochrome *c* concentrations higher than 0.1 mg/ml [15]. Upon addition of SOD (300 units/ml), which competes with Cyt(III) for reaction with superoxide anion, the reduction was totally suppressed, implying that photoreduction of cytochrome *c* induced by benzydamine is a photodynamic process, in which the superoxide anion radical was involved in the photoinduced transfer of an electron to cytochrome *c*. This result is similar to that described for α-terthienyl [15], in which the growth of the 550 nm band due to electron transfer induced by α-terthienyl to cytochrome *c* increased until it reached a constant value.

When the SOD experiment was carried out with benzydamine under air-saturated conditions, the extent of cytochrome *c* reduction decreased, but showed the same trend as observed under oxygen-saturated conditions. This suggests there may be intermediates involved in addition to superoxide anion. In fact, benzydamine can also participate in the photoreduction of cytochrome *c* by a mechanism that does not require oxygen. Under anaerobic conditions, cytochrome *c* photoreduction induced by benzydamine was demonstrated to take place at a rate faster than for cytochrome *c* alone, but slower than the photosensitized reduction under aerobic conditions, suggesting a direct reaction with cytochrome *c*. These results are similar to those reported with benzo[*a*]pyrene [24] and anthracene [25] in which the photoreduction was greater in the presence of oxygen than in its absence, and the photoreduction in the presence of oxygen was greatly diminished by addition of the enzyme SOD, indicating superoxide anion involvement.

The results obtained aerobically in the presence and absence of SOD prove that electronically excited benzydamine preferentially transfers electrons to cytochrome *c* using oxygen as an intermediate, even though the reduction can take place in its absence. This is probably because the dissolved oxygen concentration in air-saturated water is 235 μM at 30°C [27] representing a large molar excess over cytochrome *c* (8 μM) in the environment of each electronically excited molecule of benzydamine.

3.3. Reduction of copper(II) complex with SOD-like activity

The knowledge that the enzyme Cu/Zn-SOD controls superoxide via disproportionation of $O_2^{\bullet-}$ into O_2 and H_2O_2 suggests that some other compounds which are known as efficient catalysts of the dismutation process can be used to replace SOD to detect the involvement of electron transfer. For example, copper(II) is able to capture electrons in the presence of a complexing agent such as bathocuproinedisulphonic acid disodium salt hydrate (BCDS), which stabilizes the reduced copper as a copper(I) complex. The reaction course can be monitored by spectrophotometry at 484 nm, which is the maximum absorption of the Cu(I)–BCDS complex [4]. The SOD-like activity of the copper system with BCDS has been verified by an indirect method using cytochrome *c* assay. When $Cu(II)Cl_2$, BCDS and benzydamine were mixed with cytochrome *c* under oxygen-saturated conditions, the reduction of cytochrome *c* was completely suppressed.

When benzydamine 1.0×10^{-4} M in phosphate-buffered saline (PBS) solution was mixed with 0.1 mM BCDS and 30 mM copper sulphate, and irradiated both under aerobic and anaerobic conditions, an absorption change at 484 nm was observed. It was found that, under N_2, the reaction goes slightly faster than under O_2. The absorption increased with the time of irradiation until all the copper(II) was transformed to the copper(I) complex (Fig. 5).

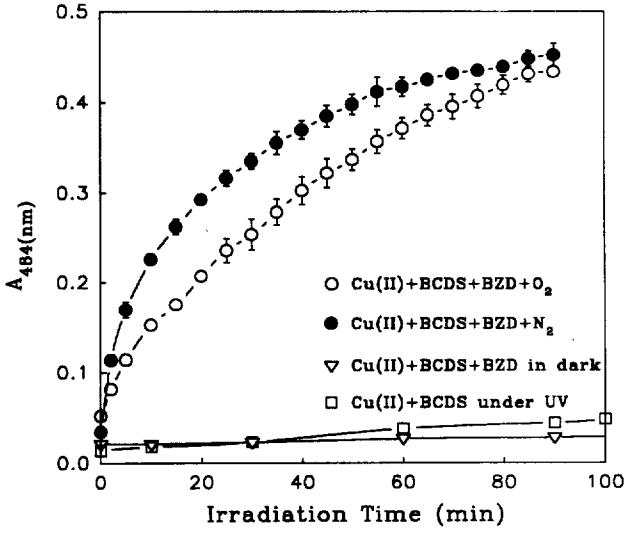

Fig. 5. Formation of copper(I)–BCDS complex induced by benzydamine (1×10^{-4} M) as a function of irradiation time at pH 7.4 and 30°C.

When Cu(II) and BCDS were irradiated without benzydamine or the solution of benzydamine and Cu(II) and BCDS was kept in the dark, no absorption at 484 nm was observed after 100 min. This result provided further confirmation that an electron-transfer reaction induced by benzydamine had occurred. The slower rate in the presence compared to the absence of oxygen could be due to oxygen quenching the excited state of benzydamine. This is consistent with the results obtained through experiments with NBT and cytochrome *c*.

3.4. Singlet oxygen mediated photo-oxidation

In separate experiments, benzydamine was found to photosensitize the oxidation of l-histidine or 2,5-dimethylfuran (DF) in air-saturated solutions. These compounds are substrates for singlet oxygen, and although they are not completely specific, a firm indication of the participation of singlet oxygen was found.

The photo-oxidation reaction was followed by measuring the depletion of oxygen with a Clarke-type oxygen electrode [28]. Fig. 6 shows the effect of increasing concentration of benzydamine on the oxygen uptake rate of pH 7 buffered solution in the presence and absence of substrates when irradiated by the medium-pressure mercury arc through a glass filter. Each result is the mean of triplicate measurements. In the presence of DF or histidine, increased oxygen uptake was observed. The rate of oxygen consumption increased linearly at low benzydamine concentrations, then the rate gradually approached a plateau value, implying that all the radiation has been absorbed, and the light intensity becomes rate determining. No oxygen uptake was detected when DF or histidine was irradiated in the absence of drug. When benzydamine was added into the solution and kept out of the light, there was no detectable dark reaction. Upon irradiation, benzydamine was itself oxidized, the extent increasing with increasing concentration. At each concentration, the oxygen uptake rate was linear with time, i.e., zero order, indicating that the absorption of light was the rate-limiting factor. The participation of singlet oxygen was also indicated by the fact that addition of 0.01 M azide ion, a $^1O_2$ quencher in aqueous solutions [29], reduced the photo-oxidation rate by 75%.

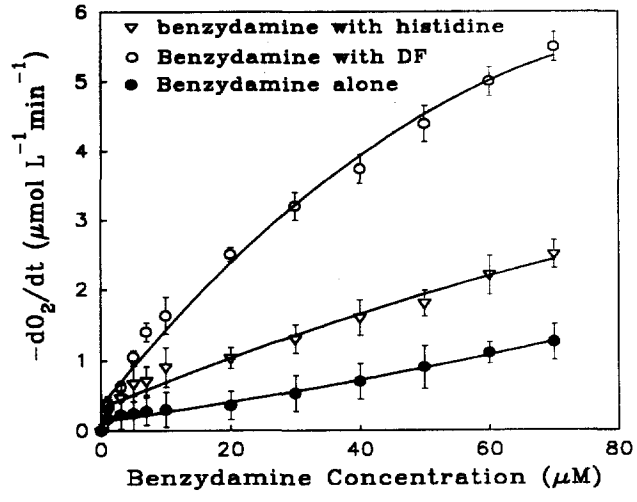

Fig. 6. Oxygen uptake rates as a function of benzydamine concentration at pH 7.0 and 30°C using 2,5-dimethylfuran (DF, 2 mM) and histidine (2 mM) as substrates.

Variation of pH of the solution showed that the cation form of benzydamine (pK_a 9.2) is three-fold more efficient as a photosensitizer than the neutral form. Although the site of ionization in benzydamine is the alkylamino group that is distant from the absorbing chromophore, the difference in oxygen-uptake capability between the base and its conjugate acid shows that the positive charge on the molecule is favourable for energy transfer to molecular oxygen. This is a similar pH dependence to that of the fluorescence yield of benzydamine and suggests that the free amino group quenches the excited state to some extent.

4. Conclusions

When benzydamine is irradiated in aerated solutions, competing pathways of photosensitization are possible, namely, singlet oxygen mediated oxidation, and electron-transfer mechanisms. Energy transfer to ground-state molecular oxygen occurs through the triplet state of the sensitizer, so the fact that oxygen interferes with the electron-transfer processes in all cases examined here strongly suggests that it is the triplet state of benzydamine from which the electron-transfer process originates. The relative importance of singlet oxygen mediated oxidation and electron-transfer processes in the overall mechanism of photosensitization would be dependent on the presence of the relevant substrates near to the photoexcited benzydamine molecule. The electron-transfer process is more likely to be an important factor in the photobiological activity of benzydamine in conditions of low oxygen concentrations.

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