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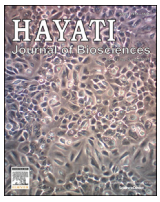

Original Research Article

Generation of Oxygen Free Radicals by Proflavine: Implication in Protein Degradation

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ABSTRACT

Proflavine, an acridine dye, is a known DNA intercalating agent. In the present study, we show that proflavine alone on photoillumination can generate reactive oxygen species (ROS). These proflavine-derived ROS cause damage to proteins, and this effect is enhanced when the divalent metal ion Cu (II) is included in the reaction. Bathocuproine, a specific Cu (I) sequestering agent, when present in the reaction mixture containing Cu (II), was found to inhibit the protein degradation, showing that Cu (I) is an essential intermediate in the reaction. The effect of several scavengers of ROS such as superoxide dismutase, sodium azide, potassium iodide, and thiourea were examined on the protein damaging reaction. Potassium iodide was found to be the most effective in inhibiting protein damage followed by sodium azide and thiourea. Our results indicate the involvement of superoxide, singlet oxygen, triplet oxygen, and hydroxyl radicals in proflavine-induced damage to proteins.

1. Introduction

Proflavine, an acridine dye with a flavine nucleus, can penetrate the epidermal and dermal structures in the *in vivo* stained cells and accumulate in the cell nuclei; only cells of the central nervous system did not absorb any proflavine. In human cell culture also, proflavine is known to be taken up by many kinds of cells and is concentrated in the nuclei (Mu and Liu, 2017). Proflavine is a strong DNA intercalating agent. The generation of reactive oxygen species (ROS) by photoexcited proflavine is reported in the presence of a macromolecule in the reaction (Schöneich, 2015). Proflavine when excited with visible light can induce DNA strand cleavage. Another study had shown the production of ROS from proflavine, that cause base modification and strand breaks in DNA (Gong, 2014). Normally replication of DNA at this stage leads to mutation or apoptosis.

Riboflavin (vitamin B_2) is structurally similar to proflavine; proflavine has an amino group while riboflavin has a ribityl group in the side chain structure. Earlier studies from a laboratory had shown that riboflavin generates superoxide anion in visible light and the rate of formation of superoxide anions is stimulated in the presence of double-stranded DNA (Chakraborty, 2013). This experiment had also shown that riboflavin causes breakage of calf thymus and supercoiled plasmid DNA and hemolysis of red blood cells. Photoilluminated riboflavin also causes protein degradation. This degradation is enhanced when a transition-metal ion, such as Cu (II), is present in the reaction with riboflavin (Stohs, 1995). This protein degradation is preceded by the binding of riboflavin to the protein at or around tryptophan residues.

It was, therefore, of interest to examine whether photoilluminated proflavine, like riboflavin, can lead to production of ROS in the absence of macromolecules. The effect of these ROS on bovine serum albumin (BSA) has also been examined.

2. Materials and Methods

2.1. Materials

Proflavine (hemisulfate), riboflavin, bathocuproine, superoxide dismutase, and catalase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). BSA (fraction V, which was used without further purification) and nitro blue tetrazolium (NBT) were

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purchased from SISCO Co. (India). All other chemicals were of analytical grade.

All solutions were prepared fresh. Proflavine was dissolved in distilled water to prepare 1 mM stock solution just before use.

2.2. Detection of superoxide anion ($O^{\cdot-}_2$)

Superoxide anion ($O^{\cdot-}_2$) was detected by the reduction of NBT (Radtsig, 1986).

A typical assay mixture, in a total volume of 3 mL, contained 50 mM sodium phosphate buffer, pH 8, 0.06% Triton X-100, 0.1 mM Ethylene diamine tetra acetic acid (EDTA), 0.033 mM NBT, and proflavine concentration ranging from 20 to 300 μM. The solutions were exposed to 800 Lux of cool white fluorescent light for various time intervals at room temperature. The progress of the reaction was followed by recording the increase in absorbance of formazan, the end product of NBT reduction, at 560 nm on a Beckman DU-40 Spectrophotometer against a reagent blank. Varying amount of free radical scavengers were included in some experiments.

2.3. Spectral studies

A 25 μM solution of proflavine, in 10 mM sodium phosphate buffer, pH 7.4, was exposed to 800 Lux of cool white fluorescent light for 4 hours at room temperature. The absorption spectra between 200 and 600 nm were then recorded on a Beckman DU-40 Spectrophotometer, National Technical Laboratories, USA. In some cases, the absorption spectra were recorded in the presence of various free radical scavengers.

2.4. Protein degradation and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

The standard reaction mixture, having a total volume of 0.1 mL, contained 2 mg/mL BSA and 50–200 mM proflavine in the presence or absence of 200 μM $CuCl_2$ in 10 mM sodium phosphate buffer, pH 7.4. After incubation for various times in fluorescent light, the reaction was terminated with 0.025 mL of sample dye containing 10% glycerol, 5% β-mercaptoethanol, 3% SDS, 1 mM EDTA, and 62.5 mM Tris–HCl, pH 6.8. The samples were heated at 100°C for 3 min and 10 μg protein was loaded in each well. Electrophoresis was performed on 10% (w/v) polyacrylamide gels containing 0.1% SDS prepared, and the protein bands were visualized by silver staining (Phillips, 2010).

In some experiments, 50 mM of free radical scavengers such as sodium azide, potassium iodide, and thiourea were used. Bathocuproine, a Cu (I) sequestering agent, was also used in some experiments containing Cu (II) at a molar ratio of 2:1 with Cu (II).

3. Results

3.1. Production of superoxide radicals by photoilluminated proflavine

Production of $O^{\cdot-}_2$ by photoilluminated proflavine was quantitated by NBT reduction. Photoillumination of proflavine by visible light leads to the production of $O^{\cdot-}_2$ in a time-dependent manner (Figure 1). The production of $O^{\cdot-}_2$ increased linearly up to 4 hours of illumination and then leveled off showing no further increase. In the presence of 100 μg/mL superoxide dismutase (SOD), there was 55% inhibition in $O^{\cdot-}_2$ production as measured by the reduction of NBT to formazan. This shows that $O^{\cdot-}_2$ is one of the ROS generated by photoilluminated proflavine. No $O^{\cdot-}_2$ was produced by proflavine in the absence of light.

3.2. Spectral studies of proflavine alone and in the presence of free radical scavengers

In aqueous solution, proflavine exhibited two characteristic peaks of absorption, at 260 and 443 nm. We have determined the spectral changes in proflavine as a function of time of irradiation

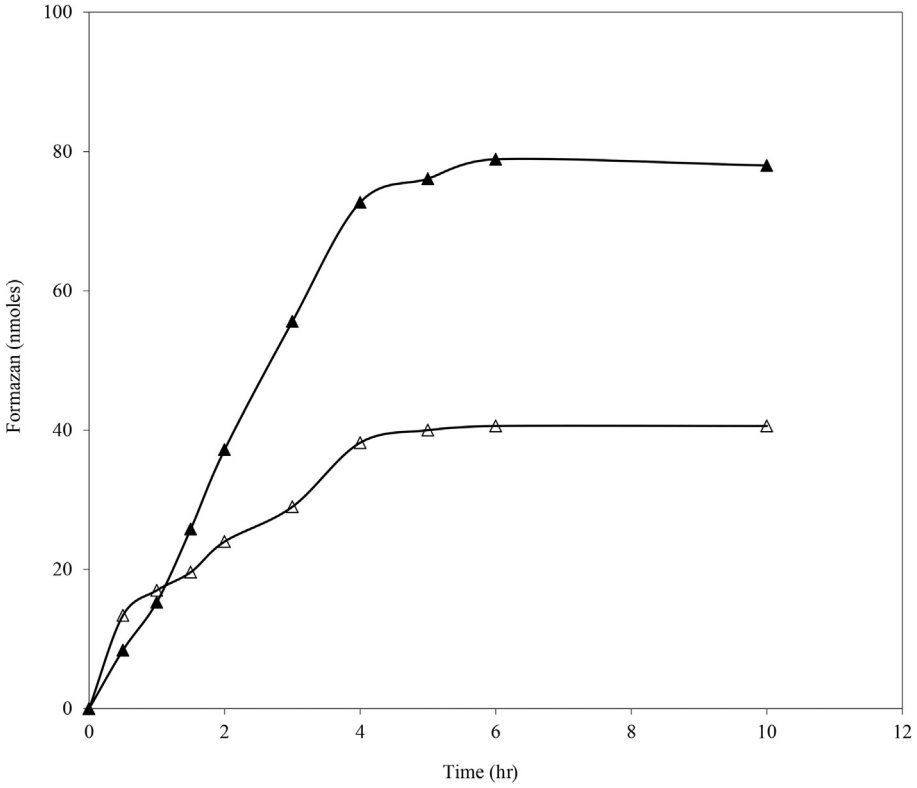

Figure 1. Production of $O^{\cdot-}_2$ by photoilluminated proflavine as a function of time. (▲) 25 μM proflavine alone; (Δ) proflavine with 100 μg/mL SOD. All reactions were carried out in 50 mM sodium phosphate buffer, pH 8 (see Method for details).

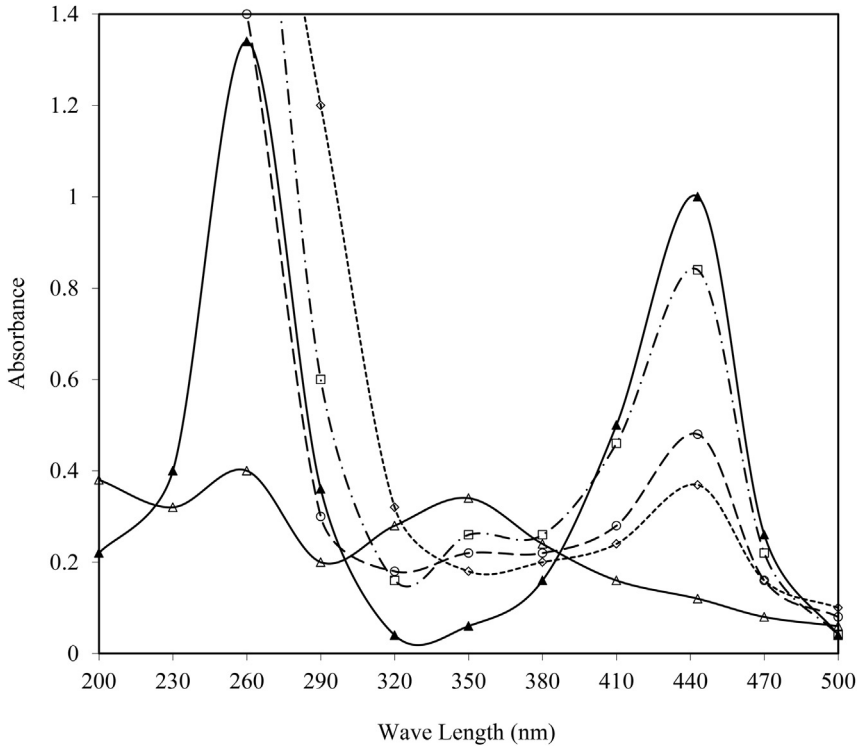

Figure 2. UV-visible absorption spectra of 25 μM proflavine in 10 mM sodium phosphate buffer, pH 7.5. (▲) Proflavine alone without photoillumination. Absorption spectra were recorded after 4 hours of photoillumination of (Δ) proflavine in fluorescent light alone and proflavine in the presence of 50 mM (○) sodium azide, (□) potassium iodide, or (◊) thiourea.

(Figure 2). On incubation in fluorescent light for 4 hours, both absorption peaks decreased significantly suggesting photodegradation of proflavine. The addition of free radical scavengers such as sodium azide, potassium iodide, and thiourea, which are quenchers of O^1_2, O^3_2, and ·OH, respectively, significantly inhibited this photodegradation of proflavine. Potassium iodide gave maximum inhibition of around 80% followed by sodium azide and thiourea that inhibit by 46% and 35%, respectively. This suggests that O^3_2 is the major ROS generated on photoillumination of proflavine in addition to O^1_2 and ·OH. Protein or metal ion (Cu [II]), when present in the reaction, did not have any effect on photodegradation of proflavine.

3.3. Modification of BSA by photoilluminated proflavine in the presence and absence of Cu (II)

To investigate the effect of ROS generated by photoilluminated proflavine on proteins, BSA was used as a target molecule. The commercial BSA used showed bands of higher molecular weight, in addition to the BSA monomer of 67 kD. These bands may represent aggregates of BSA and have been previously seen by other workers too (Basoah et al, 2005). BSA was incubated with 200 μM proflavine alone or with varying concentrations of Cu (II). The SDS-PAGE profile of BSA after exposure to photoilluminated proflavine showed significant degradation (Figure 3, lane b). The cleavage of the large aggregates, present in the commercial sample, that migrate near the top of the gels was decreased followed by the broadening of the band at monomer position after 5 hours of irradiation with fluorescent light. This is partially because of the overloading, the staining system and monomer fragmentation to slightly smaller peptides. Addition of Cu (II) resulted in enhanced protein degradation. At low concentration (50 μM), metal ion–induced degradation was not very pronounced (Figure 3, lane c). Increasing concentration of Cu (II), however, significantly enhanced degradation of protein (Figure 3, lanes d–f). Of the various other metal ions tested, only Fe^{+3} induced slight degradation. No degradation of BSA by proflavine was observed in the absence of light.

3.4. Effects of scavengers and bathocuproine on protein damage by proflavine and proflavine-Cu (II)

The effect of various free radical scavengers on protein degradation by proflavine was also studied (Figure 4). Potassium iodide, a typical O^3_2 scavenger, showed a significant inhibitory effect on BSA degradation. Sodium azide and thiourea, which are scavengers of singlet oxygen and ·OH, respectively, were also effective (Figure 4B). A similar inhibitory effect of these scavengers was seen when Cu (II) was also present in the reaction. Bathocuproine, a specific Cu (I) sequestering agent, when included in the reaction containing Cu (II), significantly inhibited the protein degradation (Figure 4B, lane g), demonstrating the involvement of Cu (I) in the reaction.

4. Discussion

Acridines dyes, especially proflavine, are photodynamic agents known to generate different types of ROS on illumination with visible light only when a macromolecule such as DNA is present in the reaction (Bonaca and Bilalbegović, 2011). These ROS are known to target DNA and other biomolecules (Chakraborty, 2011). Although the exact mechanism of damage to DNA by proflavine is not well characterized, there are indications that proflavine is a type 1 photosensitizer in which its electronically excited state reacts directly with DNA (Cuello and Lahora, 1993). Both single- and double-strand breaks of plasmid DNA have been observed (Dean, 1987).

Proteins are the key targets of ROS leading to their oxidation, which may sometimes play controlling roles in cellular remodeling and growth (Dean et al, 1984). We, therefore, studied the effect

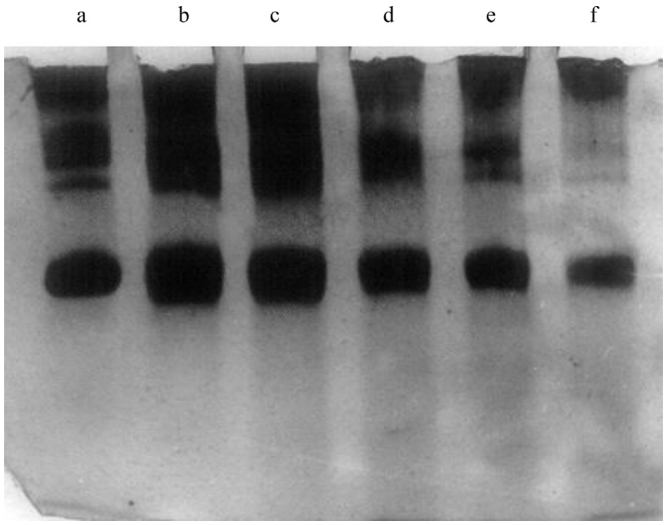

Figure 3. SDS-PAGE of BSA (2 mg/mL) incubated with 200 μM proflavine or with proflavine and increasing concentrations of Cu (II). Reactions were incubated for 6 hours in fluorescent light at room temperature and 10 μg protein was analyzed by SDS-PAGE. The gels were stained with silver nitrate. (lane a) BSA alone; (lanes b–f) BSA incubated with proflavine and 0, 50, 100, 150, and 200 μM Cu (II). BSA = bovine serum albumin.

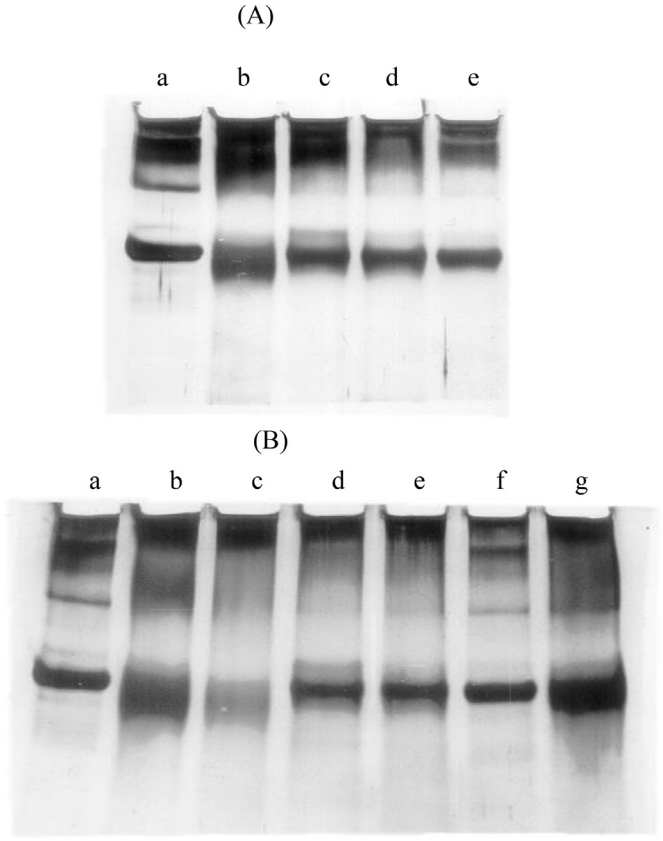

Figure 4. Effect of free radical scavengers on degradation of BSA. (A) SDS-PAGE of BSA incubated with 200 μM proflavine. (lane a) BSA alone; (lane b) BSA incubated with proflavine; (lanes c–e) BSA, proflavine, and 50 mM of sodium azide, potassium iodide, and thiourea, respectively. (B) SDS-PAGE of BSA incubated with 200 μM proflavine and 200 μM Cu(II). (lane a) BSA alone; (lane b) BSA with proflavine; (lane c) BSA with proflavine and Cu(II); (lanes d–f) 50 mM of sodium azide, potassium iodide, and thiourea, respectively, with BSA, proflavine, and Cu(II) system; (lane g) represents bathocuproine with a molar ratio to Cu(II) of 2:1. In both (A) and (B), the reactions were incubated for 6 hours in fluorescent light at room temperature before electrophoresis. BSA = bovine serum albumin.

of ROS generated on photoillumination of proflavine on BSA. Proflavine alone on photoillumination caused pronounced degradation of BSA. The addition of Cu (II) in the reaction significantly enhanced this degradation of BSA target molecule. Spin-trapping technique provided evidence for the production of $OH^{\cdot}$ during proflavine-mediated degradation of DNA (Du and Gebicki, 2002). We found that $^{\cdot}OH$ are involved in degradation of BSA by photoilluminated proflavine because the addition of thiourea (a scavenger of $^{\cdot}OH$) to either proflavine or proflavine-Cu (II) significantly inhibited this degradation. However, there is evidence for the formation of $O^1{}_2$, $O^3{}_2$ also as sodium azide and potassium iodide both inhibited protein degradation reaction to a significant extent. The inhibition was also evident when bathocuproine, a Cu (I) sequestering agent, was used in the reaction containing Cu (II) indicating the involvement of Cu (I). Thus, it appears that binding of proflavine leads to the formation of proflavine-protein-Cu(II) complex, which on photoexcitation via charge transfer is converted into proflavine-protein-Cu(I), resulting in the generation of ROS, which then mediate protein fragmentation.

The spectral studies indicate that on photoillumination of proflavine undergoes photodegradation and in the process ROS are generated. Significantly, these ROS are generated even in the absence of a macromolecule. This is in contrast to the observations of some workers, who found that the ROS are generated on photoillumination of proflavine, but only when a macromolecule like DNA is also present in the reaction (Gerlock et al, 1995). The addition of sodium azide, potassium iodide, and thiourea significantly inhibited the photodegradation of proflavine. The effect is most pronounced with potassium iodide indicating the $O^3{}_2$ is the major species generated in the reaction. The formation of $^{\cdot}OOH$, $^{\cdot}OH$ in the DNA strand breakage has been proposed (Giulivi and Cadenas, 1998). According to their proposed mechanism, proflavine may also give rise to $O^{\cdot-}{}_2$ either by direct reaction between the electron ejected by excited proflavine and molecular oxygen or by the decomposition of $^{\cdot}OOH$. We have shown that around 55% inhibition of NBT reduction reaction occurs when SOD is included confirming the formation of $O^{\cdot-}{}_2$ in addition to other ROS in the reaction.

We, therefore, propose the following probable mechanism for the generation of various ROS from photoexcited proflavine (Figure 5). Proflavine on photoillumination is excited to singlet state, which gives rise to triplet state through intersystem crossing. When H_2O and O_2 are present in the reaction, photoexcited proflavine can then give rise to $^3O_2$ and $^1O_2$ through direct energy transfer (pathway [I]). These $^3O_2$ and $^1O_2$ can participate in the protein degradation reaction. Through an alternative pathway (II), the photoexcited proflavine can accept electron from molecular oxygen and give rise to cationic radical, which further reacts with molecular oxygen and give peroxide radical. This peroxide radical in the presence of H_2O can give $^{\cdot}OH$ or $^{\cdot}OOH$; and in this process, Cu (II) may be reduced to Cu (I) if present in the reaction, and proflavine then returns to the ground state. The $^{\cdot}OH$ and $^{\cdot}OOH$, in addition to $^3O_2$ and $^1O_2$, are also available in the reaction to attack the target molecule and cause further damage.

The above study gains significance because proflavine is structurally similar to tacrine and quinacrine, drugs approved for the treatment of Alzheimer's disease and malaria, respectively (Kumar et al, 2012). Studies using human hepatocytes with tacrine and proflavine show subcellular changes and mitochondrial dysfunction (Bonaca and Bilalbegović, 2011). 7-Hydroxy tacrine and proflavine have been implicated as potential precursors of reactive metabolites (Du and Gebicki, 2002). A toxic role of oxidized proteins, rather than oxidized lipid, has been recently proposed in the etiology of Alzheimer's disease (Marković et al, 1984). As tacrine is known to be converted to 7-hydroxy tacrine, the risk of protein

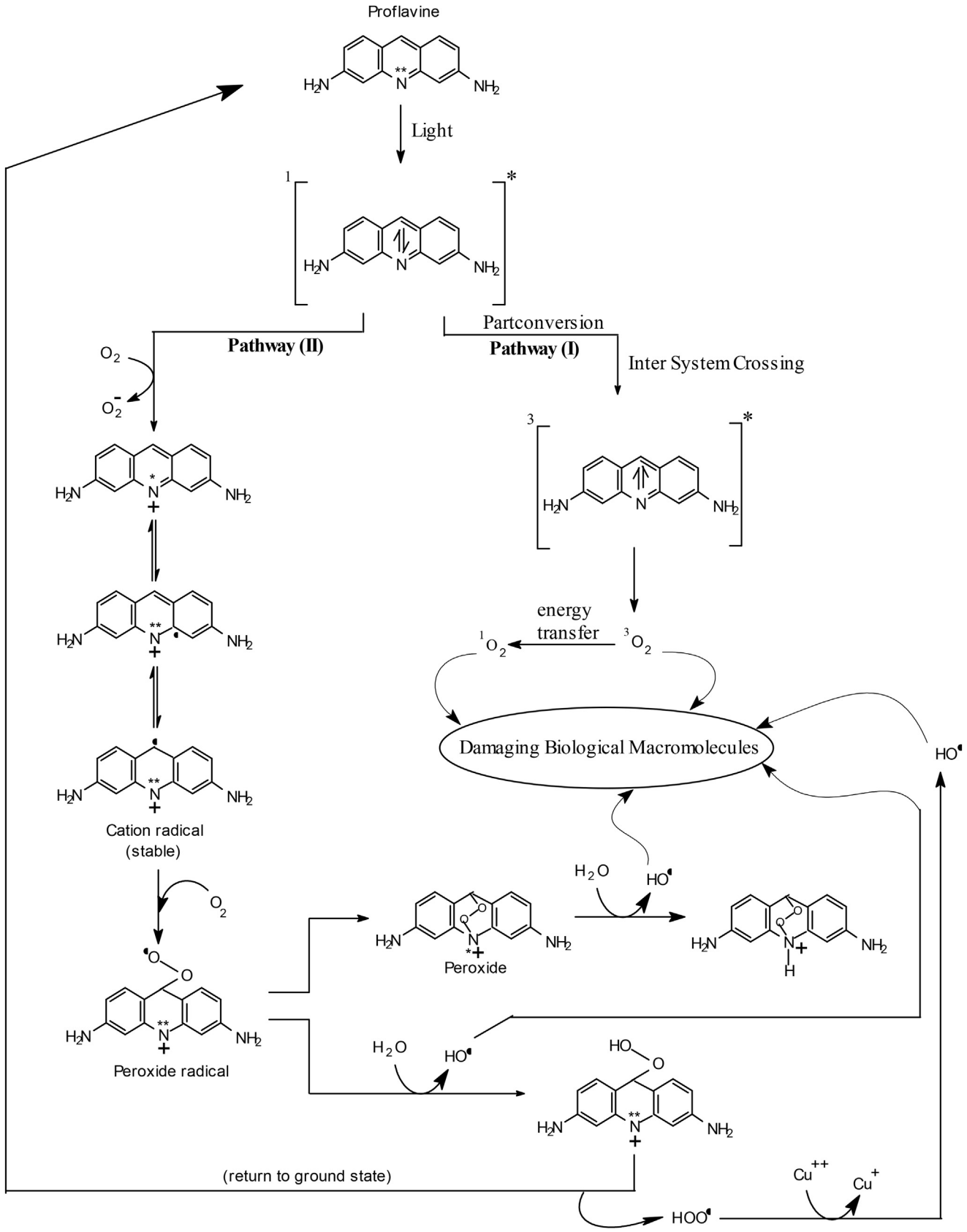

Figure 5. Scheme for photoexcitation of proflavine and generation of ROS. ROS = reactive oxygen species.

oxidation leading to precipitation of disease should probably increase.

Conflict of interest

Authors declare that there is no conflict of interest.

Acknowledgements

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