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Synthesis, pulse radiolysis, and in vitro radioprotection studies of melatoninolipoamide, a novel conjugate of melatonin and α-lipoic acid

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Abstract—A novel conjugate of melatonin **2** and α-lipoic acid **4** has been prepared using DCC mediated coupling. The conjugate named melatoninolipoamide has been assigned its structure **1** on the basis of spectral analysis (UV, IR, NMR, and EI-MS). Pulse radiolysis studies of the conjugate were carried out in aqueous solutions with both oxidizing and reducing radicals. The results indicate that the melatonin moiety of the conjugate reacts preferably with oxidizing radicals and the lipoic acid moiety exhibits preferential reaction with reducing radicals. The in vitro radioprotection ability of **1** was examined by γ-radiation induced lipid peroxidation in liposomes and hemolysis of erythrocytes, and compared the results with those of melatonin and α-lipoic acid. The studies suggest that the conjugate can be explored as a probable radioprotector.

1. Introduction

The importance of antioxidants in health and prophylaxis is now well established.[1] Melatonin is a pineal gland hormone which regulates circadian rhythms; critically controlling the sleep–wake cycle.[2] It has been identified as a potent antioxidant as well as radioprotectant.[3] Besides, this hormone has importance as a prophylactic and therapeutic agent. Thus, melatonin has a role in the prevention of cancer,[4] neurodegenerative diseases,[4] and diabetic complications,[5] and is a potential therapeutic agent for breast cancer[6] besides it possessing immunomodulating activity.[7] α-Lipoic acid is an essential coenzyme for the activity of several key enzyme complexes.[8] Like melatonin, it is also a well-known antioxidant and radioprotecting agent with implications in prophylaxis and therapy.[9] This coenzyme is reported to play a role in the prevention of cataract, HIV-activation, radiation injury, neurodegeneration, hypertension, and hyperglycemia.[10,11] α-Lipoic acid has also been identified as a drug for the treatment of diabetic polyneuropathy, age-related memory decline, and liver diseases.[12–14] The aforementioned bioactivities of both melatonin and α-lipoic acid prompted us to devise a rational strategy directed toward the synthesis of novel derivatives possessing enhanced activities compared to the parent molecules. This report presents our work on the synthesis, characterization, evaluation of antioxidant and radioprotection activities, and pulse radiolysis of a novel conjugate of melatonin and α-lipoic acid for which we have assigned the name melatoninolipoamide (**1**).

2. Results and discussion

2.1. Synthesis of melatoninolipoamide 1

The conjugate melatoninolipoamide **1** was prepared starting from melatonin **2** and α-lipoic acid **4** (±) following a route as shown in Scheme 1. Amide hydrolysis of **2** in presence of strong acid, followed by base treatment, afforded 5-methoxytryptamine **3**. The latter

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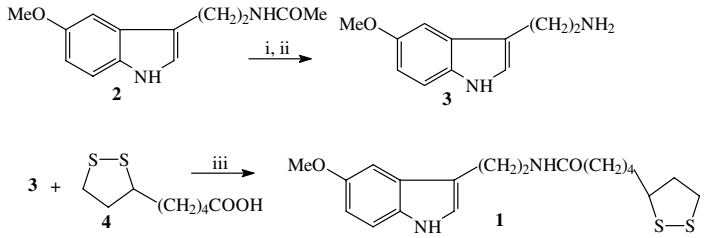


Scheme 1. Reagents and conditions: (i) 10% H_2SO_4, heat; (ii) 20% NaOH; (iii) DCC, $CHCl_3$/THF (8:1), rt.

on condensation with **4** in presence of DCC[15] afforded **1** in good yield. The product was purified by thin-layer chromatography (silica gel, 35% EtOAc in $CHCl_3$) and characterized by spectral analysis. Mass spectrum showed the molecular ion peak at m/e 378. The signal at 1648 cm^{-1} in its IR spectrum indicates the presence of amide functionality which was further confirmed from the signal at 178 in its ^{13}C NMR spectrum. There is a good resemblance of its UV spectrum with that of melatonin. Its ^{1}H- and ^{13}C-spectra gave evidence for aromatic and aliphatic signals attributed to its melatonin and lipoic acid segments, respectively (see Section 4).

2.2. Pulse radiolysis study of the conjugate 1

Reactions of radiolytically produced oxidizing and reducing radicals with **1** were studied and wherever required, the results were compared with those of melatonin and lipoic acid, and related compounds which have been published in the literature extensively.[16–20]

2.2.1. Oxidizing radicals. For these studies, hydroxyl radicals (·OH), $Br_2^{\cdot-}$ radicals, and $CCl_3O_2^{\cdot}$ radicals were employed. ·OH radical reaction with **1** at neutral pH produced a transient with absorption spectrum as given in Figure 1a. It shows broad absorption-maximum around 480–520 nm and a sharp band at 330 nm. ·OH radical is known to react with organic compounds by different reaction channels (abstraction, addition, and electron transfer). In order to differentiate various pathways of ·OH radical reaction, pulse radiolysis studies were carried out with $Br_2^{\cdot-}$ radical, which is a specific one-electron oxidant with $E^o(Br_2^{\cdot-}/2Br^-) = 1.7$ V).[21] The transient spectrum on oxidation by $Br_2^{\cdot-}$ radical is given in Figure 1b. Comparing the two spectra it can be seen that the transient formed by one-electron oxidation shows two distinct peaks one at 330 nm region and the other at 520–530 nm region., which mainly correspond to the one-electron oxidized species from the melatonin moiety, which is an indolyl-type radical.[18] The broad, featureless absorption in the 350–450 nm region in Figure 1a therefore may correspond to either hydroxyl radical adducts or the sulfur centered radicals formed by the oxidation of the α-lipoic acid moiety. The reaction with other specific one-electron oxidant, $CCl_3O_2^{\cdot}$, also produced similar type[22] of transient as seen by the transient spectrum in Figure 1c. The bimolecular rate constant for the reaction of the conjugate with $Br_2^{\cdot-}$ and $CCl_3O_2^{\cdot}$ radical was determined to be 1.3×10^{10} M^{-1} s^{-1} and 4.4×10^{8} M^{-1} s^{-1}, respectively.

In order to further confirm the nature of oxidation, the transient spectra were followed as a function of pH in the pH range from 2 to 7. Figure 2 gives the

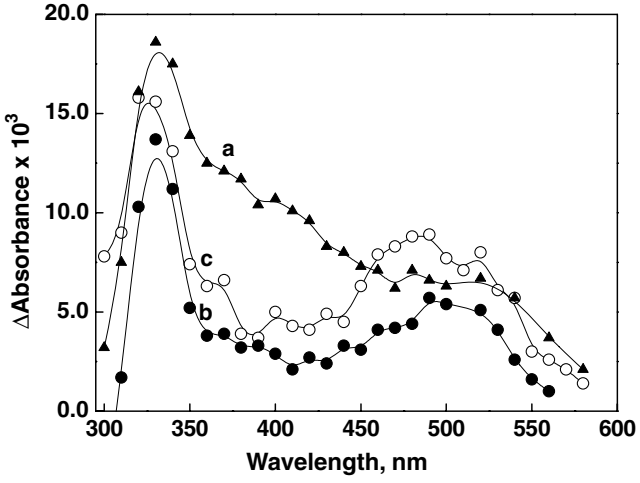


Figure 1. Transient absorption spectra of **1** (2.12×10^{-4} M) on pulse radiolysis of N_2O-saturated aqueous solution at pH 6.8 (a), in presence of 0.1 M Br^- (b). Spectrum (c) is transient absorption spectra of **1** (2.12×10^{-4} M) on pulse radiolysis of aerated aqueous solution of CCl_4 and isopropanol mixture.

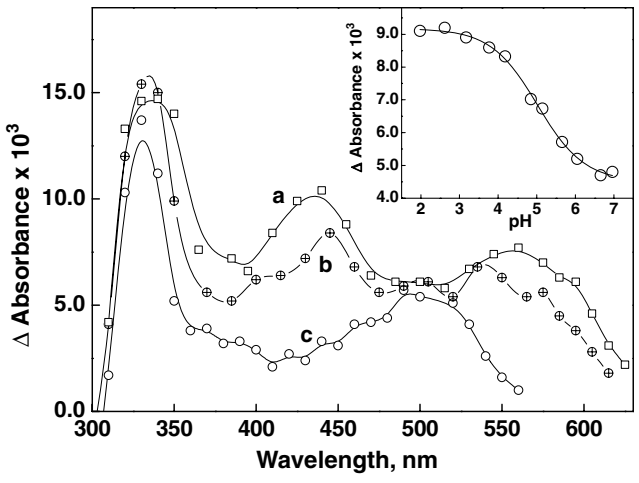


Figure 2. Transient absorption spectra of **1** (2.12×10^{-4} M) on pulse radiolysis of N_2O-saturated aqueous solution and in presence of 0.1 M Br^- at pH 3 (a), pH 4.7 (b), and pH 6.8 (c). Inset shows the variation of transient absorption of **1** (2.12×10^{-4} M) as a function of pH at 550 nm.

transient spectra at three different pH 3.0, 4.7, and 6.8, where the oxidation is induced by $Br_2^{\cdot -}$ radical reaction. As the spectra significantly differ with the pH, to evaluate the radical pK_a, the absorption changes (inset of Fig. 2) at 430 and 550 nm were followed as a function of pH from 2 to 7, which showed an inflection point due to the pK_a of the transient at 5.02. This pK_a matches well with that of the indolyl radical formed by the one-electron oxidation of melatonin and other indolic derivatives.[16–18]

The above results suggest that the reaction of oxidizing radicals preferentially takes place on the melatonin moiety of the conjugate. This was also confirmed by independently studying the reactions of $Br_2^{\cdot -}$ radicals with melatonin and α-lipoic acid. At pH 7, the one-electron oxidation potential of α-lipoic acid is 1.1 V versus NHE,[19] while that of melatonin is 0.95 V versus NHE,[16] therefore the melatonin moiety in the conjugate should undergo oxidation preferentially over the lipoic acid moiety. The indolyl radicals of melatonin show similar type of transient spectra as that of the complex, but α-lipoic acid shows altogether different transient spectra on reaction with $Br_2^{\cdot -}$ radical, having absorption maximum at ~425 nm.[19]

2.2.2. Reactions of reducing radicals. Pulse radiolysis studies of the conjugate **1** were carried out with hydrated electrons (e_{aq}^-) at neutral pH in the presence of *tert*-butanol, which scavenges ·OH radical produced by radiolysis. The transient spectrum having absorption maximum at 400 nm, obtained from the reaction of **1** with e_{aq}^-, is shown in Figure 3, which is very similar to the α-lipoic acid anion radical. A small shoulder at 330 nm may correspond to the anion radical of melatonin. The rate constant for the reaction of e_{aq}^- with α-lipoic acid is reported to be 1.5×10^{10} M^{-1} s^{-1}, while that of melatonin is 4.2×10^8 M^{-1} s^{-1}.[17,19] This difference in rate constants indicates that the hydrated electron preferentially attacks the lipoic acid part of **1**. It is reported in the literature that the hydrated electron reacts with

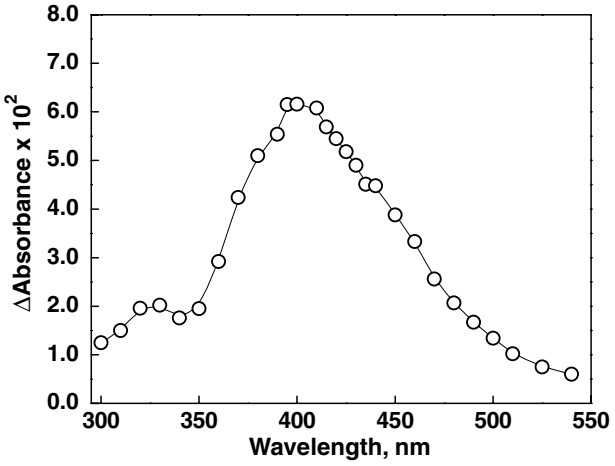
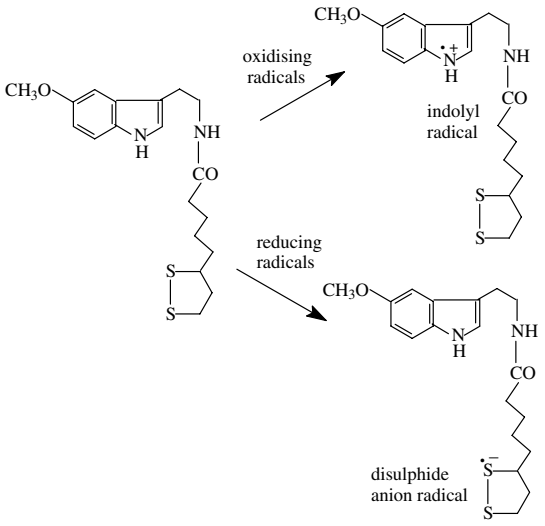


Figure 3. Transient absorption spectra of **1** (2.12×10^{-4} M) and on pulse radiolysis of N_2-saturated aqueous solution and in presence of 0.3 M *tert*-butanol at pH 6.8.



Scheme 2.

the disulfide bond of α-lipoic acid forming anion radical, while melatonin is less reactive toward hydrated electron.[16,19,20] Based on these oxidation and reduction studies, it can be inferred that **1** has two parts, of which one is reactive toward the oxidizing radicals, that is, the melatonin moiety, while the other part undergoes reaction with reducing radical, that is, lipoic acid moiety. The results are therefore encouraging to predict that **1** has the ability to react with both oxidizing and reducing free radicals. The radicals generated during oxidative stress are mostly oxidizing radicals.[23] However, some reducing type of radicals may attack the lipoic acid moiety forming dihydrolipoic acid, which is an efficient scavenger of superoxide radicals.[24] However, the relative reactivity by different moieties in the conjugate will be decided by the one-electron reduction potentials for the formation of radical anions of melatonin, α-lipoic acid, and the conjugate. The literature reported value for the reduction of α-lipoic acid of −0.29 V however corresponds to two-electron process,[25] while many free radicals react by one-electron process. Therefore, accurate estimation of one-electron reduction potentials under physiological conditions is necessary, our future experiments will be directed toward this. However based on these preliminary pulse radiolysis studies, it can be concluded that the conjugate **1** can be effective toward most of the free radicals produced. The overall reaction of oxidizing and reducing radicals with **1** is summarized in Scheme 2.

2.3. In vitro radioprotection studies with conjugate 1

2.3.1. Radiation induced hemolysis of erythrocytes. Melatonin has been reported to protect RBC against superoxide radical[26] and peroxynitrite mediated hemolysis in bovine erythrocytes.[27] This has been explained on the basis of its radical scavenging ability. Since ·OH radicals and other free radicals are implicated in radiation induced erythrocyte damage, we expect that the conjugate may also exhibit similar protection to RBC against radiation induced hemolysis. For this, the reduction in

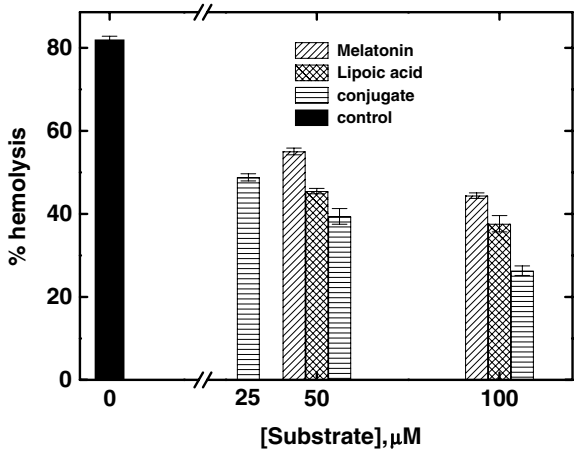


Figure 4. Percentage hemolysis in human erythrocytes after irradiating with γ-rays (75 Gy) in the absence and presence of different concentrations of melatonin, α-lipoic acid, and the conjugate. All the solutions contain 0.5% ethanol and control indicates percentage hemolysis after irradiation of erythrocytes containing 0.5% ethanol ($p < 0.001$ compared to control).

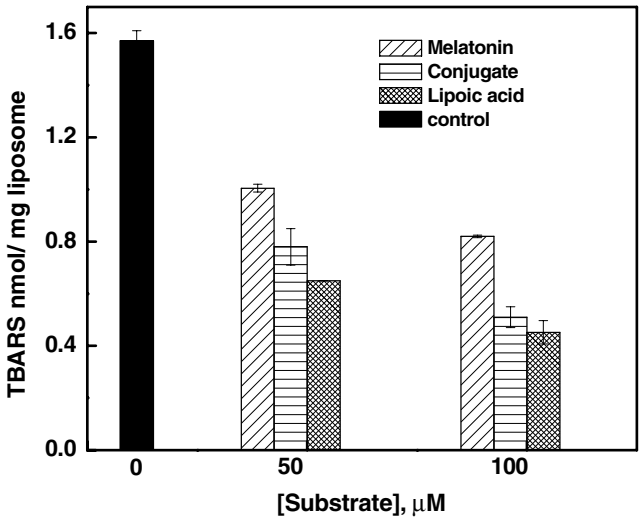


Figure 5. Inhibition of γ-radiation induced lipid peroxidation in liposomes (1 mg/ml) in presence and absence of melatonin, α-lipoic acid, and their conjugate **1**. (absorbed dose 280 Gy)

absorbance at 540 nm due to the released hemoglobin was monitored in presence of melatonin, α-lipoic acid, and **1** by independent experiments. Figure 4 gives percentage hemolysis in human erythrocytes in the absence and presence of the added substrates, **1** or melatonin or α-lipoic acid. In the same figure, percentage hemolysis on irradiation of erythrocytes in presence of 0.5% ethanol is given as control. Both melatonin and α-lipoic acid inhibited hemolysis, respectively, by 32.9% and 44.5% at 50 μM and 45.9%, and 54.1% at 100 μM concentration. Under similar conditions, **1** showed significant reduction in hemolysis of irradiated samples. It imparted up to 52% and 68% protections at 50 μM and 100 μM concentrations. If we compare the percentage protection at similar concentrations of **1**, or melatonin or α-lipoic acid, the protection imparted by the conjugate was much higher than either α-lipoic acid or melatonin alone. However, since the conjugate is a combination of two molecules, we compared the percentage protection of hemolysis at 25 μM of **1**, which is half the original concentration (i.e., 50 μM). It can be seen that at half the concentration, the protection rendered by the conjugate is in between that of melatonin and α-lipoic acid. This indicates that the conjugate can inhibit the hemolysis induced by radiation at similar molar concentration, which may be due to the fact that it may temporarily increase the resistance of erythrocyte membrane to free radical aggression, induced by γ-radiation. The conjugate therefore seems to be a promising candidate for use as a radioprotector and also as a protector against free radical mediated erythrocytic damage.

2.3.2. γ-Radiation induced lipid peroxidation in liposomes. The extent of γ-radiation induced lipid peroxidation in liposomes was monitored as TBARS[28,29] in the absence and presence of **1** and compared with that in presence of melatonin and α-lipoic acid individually. In such conditions, the radicals formed by radiolysis of water, mainly $^{\bullet}$OH radicals, induce peroxidation in the lipids, resulting in formation of malonaldehyde, which reacts with thiobarbituric acid to produce TBARS.[23,28] At a constant γ-radiation dose of 280 Gy, inhibition of peroxidation was followed at 50 μM and 100 μM. The TBARS formation was found to decrease with increasing concentration of **1** as shown in Figure 5. It protected the liposomes from peroxidation by 54.1% and 70.0% at 50 μM and 100 μM concentration, respectively. At the same concentrations, α-lipoic acid was found to inhibit the peroxidation by 61.8% and 73.4%, while melatonin protected the liposomes by 40.9% and 51.8% at 50 μM and 100 μM, respectively. The studies showed that **1** protects liposomes from peroxidation more than that by melatonin but its activity is less compared to α-lipoic acid.

3. Conclusions

A new conjugate of melatonin and α-lipoic acid was synthesized, purified, and characterized as **1**. Both melatonin and α-lipoic acid are known to exhibit good radioprotecting ability and are excellent free radical scavengers. It is therefore expected that the conjugate **1** formed by the amide linkage of the two parent residual moieties can show good radioprotecting ability. Pulse radiolysis induced one-electron oxidation and reduction of **1** showed that the melatonin moiety in the conjugate is more susceptible for the oxidation, while the lipoic acid moiety for the reduction. Thus, the conjugate can react with all types of free radicals formed during the radiation injury. It was found to be superior in protecting erythrocytes from radiation induced hemolysis and is a moderate inhibitor of radiation induced lipid peroxidation. These preliminary studies indicate that **1** can be regarded as a promising radioprotector. In view of recent finding that conjugates of L-Dopa and Dopamine and (*R*)-α-lipoic acid are multifunctional co-drugs with antioxidant properties,[30] the present work provides another example of lipoic acid conjugates possessing similar kind of biological potential.

4. Experimental

Chemicals used as starting materials are of Aldrich make and were used without further purification. All solvents used for extraction and chromatography were distilled twice at atmospheric pressure prior to use. Tetrahydrofuran was dried by heating over $LiAlH_4$. The IR spectra were recorded with a Perkin-Elmer spectrophotometer model 837. The ^1H and ^{13}C NMR spectra were scanned with a Brucker Ac-200 (200 MHz) instrument in $CDCl_3$. Electron impact (EI) mass spectrum was recorded on a ShimadzuQP5050A mass spectrometer. The organic extracts were desiccated over dry Na_2SO_4.

4.1. Synthesis of melatoninolipoamide 1

Melatonin **2** (464 mg, 2 mmol, Acros Organics, USA, 99%) was refluxed with 10% aq H_2SO_4 (25 ml) over a period of 8 h. The mixture was cooled, treated with 20% aq NaOH solution till basic, and then extracted with EtOAc. The combined organic extract was washed with water, brine and dried. Solvent removal under reduced pressure afforded a yellow solid residue. This was purified by column chromatography (neutral alumina, 0–5% MeOH in $CHCl_3$) to afford 5-methoxytryptamine **3** (265 mg, 69.7%).[31a]

A mixture of α-lipoic acid **4** (250 mg, 1.21 mmol, Aldrich, 98%, ±) and DCC (248 mg, 1.2 mmol, Aldrich, 99%) in a solvent mixture of CH_2Cl_2/THF (8:1, 50 ml) was stirred at ambient temperature for 4 h. To this, amine **3** (200 mg, 1.05 mmol) was added and stirring was continued overnight. Solvent was evaporated under reduced pressure to give a reddish mass which was purified by preparative thin-layer chromatography over silica gel plate (35:65 EtOAc in $CHCl_3$) to yield **1** as a pale yellowish semi-solid (178 mg, yield 45%).[31b] IR ($CHCl_3$) 3420, 3310, 1648 cm^{-1}; UV (λ_{max}): 300, 275 nm. ^1H NMR ($CDCl_3$): 1.3–1.7 (m, 6H), 1.84 (m, 1H), 2.07 (t, J = 7.2 Hz, 2H), 2.32 (m, 1H), 2.90 (t, 6.4 Hz, 2H), 3.0–3.1 (m, 2H), 3.40–3.56 (m, 3H), 3.80 (s, 3H), 5.91 (br s, 1H), 6.81 (d, J = 6 Hz, 1H), 6.93 (m, 2H), 7.19 (d, J = 6.8 Hz, 1H), 8.79 (s, 1H). ^{13}C NMR ($CDCl_3$): 25.09, 28.60, 34.29, 36.22, 38.20, 39.49, 39.92, 55.75, 56.22, 100.20, 100.80, 111.97, 122.95, 127.45, 131.45, 153.58, 172.87, 211.04. EI-MS: 378 (M^+), 173 (100%), 100 (30%), 145 (6%). Anal. Calcd for $C_{19}H_{26}O_2N_2S_2$: C, 60.28; H, 6.92; N, 7.40%. Found: C, 60.55; H, 6.68; N, 7.59%.

4.2. Pulse radiolysis studies

As the conjugate **1** is sparingly soluble in water, it was first dissolved in some organic solvent like acetonitrile. A known amount of this solution is taken in a flask and the solvent was evaporated on a rotary evaporator to form a thin film. For ·OH radical reaction, the film was incubated and sonicated with aqueous buffer solution and the saturated solution was used for the transient studies. For reactions with $Br_2^{\cdot-}$ radicals, a known amount of acetonitrile solution of **1** was taken and then diluted with buffer solution such that the acetonitrile content does not exceed 10%. For $CCl_3O_2^{\cdot}$ radical reactions, **1** was dissolved in the matrix, which is given below. The pH of the solution was adjusted by phosphate buffers. Nanopure water from Millipore system was used for preparing the solutions and freshly prepared solutions were used for each experiment. Electron pulses from 7 MeV linear electron accelerator were used for irradiation.[32] The absorbed dose was measured using thiocyanate dosimeter by monitoring the $(SCN)_2^{\cdot-}$ species at 500 nm.[33] Typical dose/pulse used for these studies varied from 15 to 16 Gy.

Radiolysis of water[34,35] produces e_{aq}^-, H· and ·OH radicals in addition to the molecular products H_2, H_2O_2, and H_3O^+.

$$H_2O \rightarrow e_{aq}^-, H^{\cdot}, {}^{\cdot}OH, H_2, H_2O_2, H_3O^+ \quad (1)$$

The reaction of ·OH radical was studied in N_2O-saturated condition where all the e_{aq}^- is quantitatively converted into ·OH radical.

$$e_{aq}^- + N_2O \xrightarrow{H_2O} N_2 + OH^- + {}^{\cdot}OH \quad (2)$$

More selective oxidizing radicals, $Br_2^{\cdot-}$ radicals, were generated by the reaction of ·OH radicals (in N_2O-saturated solutions) with KBr (0.1 M) according to the reactions and are briefly described below:

$$Br^- + {}^{\cdot}OH \rightarrow Br^{\cdot} + OH^- \quad (3)$$

$$Br^{\cdot} + Br^- \rightarrow Br_2^{\cdot-} \quad (4)$$

Chloroperoxyl radicals were generated by allowing e_{aq}^- to react with CCl_4 (4% by volume) in aerated or oxygen-saturated solution. 2-Propanol (48% by volume) reacts with ·OH and H· to give the reducing acetone ketyl radical, which in turn reduces CCl_4 compounds. $^{\cdot}CCl_3$ reacts with oxygen to form $CCl_3O_2^{\cdot}$.[22]

$$CCl_4 + e_{aq}^- \rightarrow {}^{\cdot}CCl_3 + Cl^- \quad (5)$$

$$(CH_3)_2CHOH + H^{\cdot}/{}^{\cdot}OH \rightarrow (CH_3)_2C\,{}^{\cdot}OH + H_2/\,H_2O \quad (6)$$

$$(CH_3)_2C\,{}^{\cdot}OH + CCl_4 \rightarrow {}^{\cdot}CCl_3 + (CH_3)_2CO + HCl \quad (7)$$

$$^{\cdot}CCl_3 + O_2 \rightarrow CCl_3OO^{\cdot} \quad (8)$$

e_{aq}^- reaction can be studied by scavenging ·OH radical by adding 0.3 mol dm^{-3} *tert*-butanol under N_2-saturated condition.

$$^{\cdot}OH + (CH_3)_3COH \rightarrow {}^{\cdot}CH_2\,(CH_3)_2COH + H_2O \quad (9)$$

4.3. Studies on hemolysis of erythrocytes

Human RBCs were procured from venous blood and washed five times with phosphate-buffered saline (PBS) solution (pH 7.4) at 4 °C. They were then irradiated in hypotonic saline to an absorbed dose of 75 Gy in the

absence and in the presence of different concentrations of melatonin, α-lipoic acid, and **1** (25, 50, and 100 μM). The compounds were dissolved in ethanol such that the final concentration of ethanol was less than 0.5% during irradiation with appropriate controls. The suspensions were centrifuged and the absorbance in the supernatant was measured at 540 nm to analyze the extent of hemolysis in each sample.[26] The statistical significance of the difference in the parameters between the drug containing samples and the appropriate controls was assessed by Student's '*t*' test and the errors correspond to the standard deviation.

4.4. Lipid peroxidation

Lipid peroxidation studies were carried out in phosphatidylcholine liposomes prepared according to the procedure given in reference.[36] Lamellar liposomes of 150 nm size suspended in pH 7.4, phosphate buffer were subjected to γ-radiation. γ-Radiolysis was carried out using ^{60}Co γ-source with a dose rate of 48 Gy min^{-1} as measured by standard Fricke dosimetry.[37] It was followed at different absorbed doses in N_2O/O_2-purged liposomal solution in absence and presence of the compounds (melatonin, α-lipoic acid, and **1**) at pH 7.4 (phosphate buffer). The detailed methodology used in the lipid peroxidation is given in earlier references.[28,29] The extent of lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS) using 15% w/v trichloroacetic acid, 0.375% w/v TBA, 0.25 N hydrochloric acid, and 0.05% w/v BHT as TBA reagent measuring the absorbance at 532 nm ($\varepsilon_{532} = 1.56 \times 10^5$ M^{-1} cm^{-1}).

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