CHAPTER 8

MOLECULAR MECHANISM OF FREE RADICAL MODE OF GENERATION BY MPT QUINOLINE: IMPLICATES BIOREDUCTIVE AND REOXIDATIVE CYCLING PROPERTY

8.1 INTRODUCTION

Different cellular factors pertaining to phototoxicity and photokilling in MCF-7 cells with different sensitizers have been studied in the previous chapter. Among the sensitizers investigated, MPTQ is found to be the most efficient. To understand whether this efficient mode of photokilling of tumor cells follow either the reported intercalating property or light induced enhanced generation of ROS, or by combining both factors, an in depth EPR study on MPTQ is attempted. This attempt shall elucidate the vital role of light in the molecular mode mechanism of free radical generation from MPTQ.

8.2 EXPERIMENTAL

8.2.1 Materials and Methods

DMPO, DETAPAC were from Aldrich Chemicals Co. (Milwaukee, WI) NADH, NADPH, SOD and catalase were purchased from Sigma (St. Louis). N, N-Dimethyl formamide (DMF) and sodium azide (Na$_2$N$_3$) from Fisher Chemical Company. DMPO was purified before use as described
in chapter 2. The concentration of the spin trap was determined spectrophotometrically at 227 nm (Kalyanaraman et al 1982).

EPR spectra were recorded using a variant–400 EPR Spectrometer, operating at 9 GHz with 100 KHz field modulation fitted to the instrument. Photoinduced EPR spectra were obtained from samples introduced into the flat quartz cell and illuminated directly inside the microwave cavity of the instrument by visible light using light from a projector lamp (300 W) or outside the EPR cavity such that reaction mixture was exposed to different doses of light (as given in 2.9.1 of 2nd chapter) and subsequently withdrawn in teflan capillary tube and inserted in microwave cavity of EPR for measurement. The light from light source was made to pass through a filter band with the maximum transmission around 480 nm which corresponds to the maximum absorption of Quinoline. The incident fluence rate was 10 W/m² as measured 1 cm from the front wall of the microwave cavity (Yellow spring, OH- Radiometer).

EPR spectrum of the stable nitroxide radical TEMPO of known concentration was recorded using the same spectrometer settings as in the photochemical experiments. Both peak to peak line width \( \Delta H \), and the amplitude \( A \) were measured for signals. The concentration of the spin in the standard sample (the nitroxide) can be approximated by the equation.

\[
[\text{Standard spin}] = m \times A_s \times (\Delta H_s)^2 \tag{8.1}
\]

And an analogue equation may be expressed for the radicals from MPTQuinoline as

\[
[Q^-] = m \times A_Q \times (\Delta H_Q)^2 \tag{8.2}
\]

Thus, these equations are permitting the determination of \([Q^-]\) in the steady state.
Thermal denaturation of the superoxide dismutase was accomplished by heating the enzyme solution (1 mg SOD / 1 ml phosphate buffer, pH 7.4) in a closed vial at 90° for 30 min.

8.3 RESULTS

8.3.1 Reactions in Deoxygenated Solution

Illumination of MPTQ and NADH in deaerated DMF / buffer (pH 7.4) mixture, with the given light inside or outside the microwave cavity generates reduced semiquinoline radical Q− of the sensitizer and its EPR spectrum is shown in Figure 8.1a. Generation of this spectrum requires the presence of the sensitizer sample, MPTQ, NADH and light. Photoirradiation of the sample with NADH omitted does not generate the EPR spectrum. However, short illumination (30-60 s) of such a sample with light of higher fluence rate (~150 W/m², by illumination outside the microwave cavity) also produces the same spectrum although of much lower intensity. The spectrum of sample (Figure 8.1a) is composed of 15 clearly distinguishable components. A simulated EPR spectrum corresponds well with the experimental spectrum (Figure 8.1b). The hyperfine coupling constants used for the simulation are comparable with those reported earlier by Schreiber et al (1989) and Lown and Chen (1981) and Lown (1989), for the radicals in DMSO/H₂O mixture and H₂O respectively (Table 8.1). When an aerated sample alone is permitted to undergo reaction no EPR spectrum is generated on omitting either light or sensitizer. These results are substantiating the observations of earlier works on other radicals study of like adriamycin, daunorubicin (Bonadona et al 1969; Carmichael et al 1983), anthracyclines (Lenaz and Page 1976; Bachur et al 1977, 1982; Lown et al 1982; Kalyanaraman et al 1984).
Note: Spectrometer settings: Microwave power 2 mW; Modulation amplitude, 0.54 G; gain $6.3 \times 10^5$; time constant 0.5 s; scan rate 1000 sec.
Table 8.1 The hfcc’s (In Gauss) for the similar quinone radicals in EPR spectrum (DMF/buffer, pH 7.4, 1:1 v/v)

<table>
<thead>
<tr>
<th>Carbon Position</th>
<th>DMF/H₂O</th>
<th>H₂O(^a)</th>
<th>DMSO/H₂O(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.8</td>
<td>0.8</td>
<td>0.88</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>0.8</td>
<td>1.01</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>1.5</td>
<td>1.45</td>
</tr>
<tr>
<td>4</td>
<td>0.65</td>
<td>-</td>
<td>0.52</td>
</tr>
<tr>
<td>5</td>
<td>0.75</td>
<td>-</td>
<td>0.63</td>
</tr>
<tr>
<td>6</td>
<td>2.15</td>
<td>2.85</td>
<td>2.04</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>2.25</td>
<td>1.57</td>
</tr>
<tr>
<td>8</td>
<td>2.15</td>
<td>2.25</td>
<td>2.22</td>
</tr>
</tbody>
</table>

\(^a\) Hyperfine coupling constants as reported by Lown and Chen (1981b).
\(^b\) Hyperfine coupling constants as reported by Schreiber et al (1989).

Higher modulation amplitude, 5.4G and 20mW microwave power are also employed in these experiments. The semireduced form of the sensitizer, MPTQ\(^-\) shows relatively high stability in the solvent mixture used and accumulates in readily detectable quantities. The kinetics of the photoinduced generation of radicals and their recombination in the dark have been also measured. They are shown in Figure 8.2. Figure 8.2a is for [Q] = 0.67 mM and [NADH] = 1.6 mM with 5.4G modulation and 20 mW microwave power.
Figure 8.2 Kinetics of the photoinduced generation of the EPR spectrum of MPTQ* and its decay in the dark (a) in DMF/buffer, (b) in 100% aqueous buffer

Note: Solutions were deaerated by purging in N2 gas before illumination. Different fluence scale and the abscissas are given in (a) and (b) Microwave power 20 mW, modulation amplitude 5.4G
The generation of the radicals starts immediately upon illumination. The lower initial rate may be ascribed due to the presence of traces of oxygen. This effect disappears when the same sample undergoes repeated illumination. A plateau corresponding to steady-state conditions is reached after about 8 min of exposure. Both the rate of radical formation and the steady-state concentration \( Q^- \) depend upon the concentration of NADH in a manner as shown in Figure 8.3. In the dark, the intensity of the EPR signal decreases (Figure 8.2a). The initial portion of the decay curve may be described by second order kinetics as explained elsewhere (Figure 8.4a). The kinetics of the photoinduced generation of the radicals \( Q^- \) and their decay in the dark is also studied in 100% aqueous buffer (pH 7.4). The EPR signal of the MPTQ semiquinoline in water is of much lower intensity and it does not possess hyperfine structure. Besides, its stability is greatly reduced in water medium. Figure 8.4b is showing the kinetics of generation and dark decay of the sensitizer MPTQ radicals. Again, second order kinetics is accounted for only the early stages of the decay curve (Figure 8.4b). Similar corroborating results have been documented by various workers (Goodman and Hochstein 1977; Bachur et al 1982; Lown et al 1982, 1986; Carmichael and Roesz 1985).

![Figure 8.3](image)

**Figure 8.3** Photoinduced production of semiquinoline radical of MPTQ dependence on NADH and optical dose

**Note:** Deaerated solutions DMF/buffer (pH 7.4) 1:1 v/v contained Q (0.67 mM) and NADH: as (-0.1; -0.2; -0.8 and -1.6 mM respectively)
Figure 8.4  Decay of the photoinduced EPR signal of Q• dependence of the reciprocal of the radical concentration at the time of the dark incubation

Note: The experimental points correspond to the kinetic runs in Figure 2a and b respectively.
8.3.2 Reactions in Aerated Solutions

The absence of an EPR spectrum of $Q^\cdot$ in aerated solutions is suggesting the complete oxidation of the radical (Wilson 1970). Therefore, it is of interest to determine if production of $Q^\cdot$ radicals can be observed upon photochemical activation of MPTQ in aerated solutions in the presence of NADH. These nicotinamide adenine dinucleotides are considered as suitable electron donors because in aerated solutions their radical forms are rapidly oxidized generating $Q^\cdot$ with the high rate constant of $1.9 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$.

Illumination of MPTQ, NADH and DMPO in aerated DMF / buffer mixture generates an EPR spectrum of the DMPO-O$_2^{-\bullet}$ radicals adduct I, with hfcc’s as $a_N = 13.35 \text{ G}$, $a_H^{\beta} = 10.65 \text{ G}$ and $a_H^{\gamma} = 1.25 \text{ G}$ (Figure 8.5B). The values of the splitting constants are between those found in aqueous ($a_N = 14.3 \text{ G}$, $a_H^{\beta} = 11.7 \text{ G}$, (Harbour and Bolton 1975) and in DMF solutions ($a_N = 12.8 \text{ G}$, $a_H^{\beta} = 9.9 \text{ G}$, (Harbour and Hair 1978). The spectrum is not generated if either MPTQ or NADH is omitted. Addition of SOD (40 $\mu$g/ml) prior to irradiation prevents formation of adduct I (Figure 8.5C). Thermally denatured SOD is without any effect on the EPR spectrum of adduct I. This confirms the formation of radical and thus assigned adduct I for radical formation at this stage. The addition of catalase (80 $\mu$g/ml) in the sample slows down the production of adduct I by catalase 30%, suggesting the role for hydrogen peroxide in the formation of the superoxide anion radical.

An EPR spectrum of adduct I (Figure 8.5B) is recorded with a similar response to added SOD in other studies with dauxorubicin where NADH was replaced by NADPH by Land et al (1983). Illumination of the MPTQ and DMPO in DMF / buffer mixture without NADH produces the EPR spectrum (Figure 8.5D). This spectrum contains lines from DMPO-O$_2^{-\bullet}$; or a related species, the formation of which is mediated by O$_2^{-\bullet}$, since
exposed to optical dose of the same sample with SOD (40 μg/ml) gives only
the four line spectrum (Figure 8.5E).

Figure 8.5  EPR spectra from aerated DMF/buffer mixtures containing
MPTQ (0.67 mM), NADH (1 mM), DETAPAC (1 mM) and
DMPO (74 mM)

Note: Relative gain is indicated on the right side of the spectra in brackets.
Spectrometer settings; microwave power 20 mW; modulation amplitude
5.4G; time constant 0.5 s; scan time 500 s
Sodium azide (20 mM and 100 mM) has an insignificant effect on the formation of adduct I. Therefore it is tentatively concluded that singlet oxygen is not involved in the photosensitized production of $\text{O}_2^{-\bullet}$. In the presence of sodium azide, the azidyl radical is produced during irradiation as is evidenced by the recorded EPR spectrum of the DMPO-N$_3$, adduct II ($a_{\text{N}_1} = 13.8 \text{G}$, $a_{\text{N}_2} = 3.0 \text{G}$, $a_{\text{H}}^{\beta} = 13.9 \text{G}$). The splitting constants (hfcc’s) for the same adduct in aqueous solution are $a_{\text{N}_1}=14.9\text{G}$, $a_{\text{N}_2}=3.0\text{G}$, $a_{\text{H}}^{\beta} =14.9$ (Bucttner and Oberly 1980). Adduct II production is particularly apparent when SOD (40 $\mu$g/ml) is introduced into the sample (Figure 8.6C) which eliminates the DMPO-$\text{O}_2^{-\bullet}$ and EPR components are not having any effect on DMPO-N$_3$ adduct formation. The presence of catalases (80 mg/ml) does not eliminate the spectrum of DMPO-N$_3$ (Figure 8.6D). Photoirradiation of MPTQ, Na$_2$N$_3$ and DMPO with absence of NADH also generate the EPR spectrum of DMPO-N$_3$ adduct species (Figure 8.6E). The central components in each of the EPR triplets is conspicuous with higher amplitudes (as is the EPR spectra shown in Figure 8.6F suggesting the formation of DMPO-·OH adduct III during photoirradiation of the samples.
Figure 8.6  EPR spectra from aerated DMF/buffer (pH 7.4) mixture containing MPTQ (0.67 mM); DMPO (74 mM); Na₂N₃ (0.1M) and NADH

Illumination of the MPTQ, NADH and DMPO in aerated 100% aqueous buffer (pH 7.4) has produced EPR spectra of both DMPO-O₂⁻ (\(a_N = 13.8\) G, \(a_H^\beta = 11.3\) G and \(a_H^\gamma = 1.25\) G) and DMPO-•OH (\(a_N = a_H^\beta = 15.0\) G) radicals (Figure 8.7B). The addition of SOD (40 \(\mu\)g/ml) has eliminated the spectrum of the DMPO-O₂⁻ (Figure 8.7C). However, the thermally denatured enzyme is without effect. Photoirradiation of the MPTQ
and DMPO, without NADH, also give the EPR spectrum of the DMPO-$^\bullet$OH radical for adduct III (Figure 8.7D) and in accord with earlier reported observations in other radical studies (Finkelstein et al 1980; Lown and Chen 1981b). Comparison of the EPR spectra of DMPO-$^\bullet$OH recorded in this studies has led to infer that MPTQ photosensitized production of requires higher sensitizer concentration in 100% aqueous buffer than in DMF / buffer mixture to obtain comparable EPR spectra (Figures 8.5B and 8.7B).

Figure 8.7 EPR spectra from aerated buffer pH 7.4 (aqueous) containing: MPTQ (1.3 mM), DMPO (74 mM), NADH (1 mM)
8.4 DISCUSSION

This study has demonstrated that the MPTQ undergoes sensitized photooxidation in the presence of the biologically relevant electron donor as NADH. The reaction parallels with the formation of the free radical form of the sensitizer, Q− and its EPR spectrum is observed upon illuminating the sample in deaerated solutions. Because MPTQ is the only light-absorbing species in present sample, the semiquinoline Q− could be produced according to the following reactions:

\[ Q \xrightarrow{hv} Q^- \quad (8.1) \]

\[ Q^- + Q \rightarrow Q^- + Q^+ \quad (8.2) \]

\[ Q^- + \text{NADH} \rightarrow Q^- \dagger \text{NAD}^+ + H^+ \quad (8.3) \]

\[ Q + \text{NAD}^+ \rightarrow Q^- \text{NAD}^+ \quad (8.4) \]

Equation (8.2) represents a concentration quenching of the excited state of the MPTQ is a electron transfer involving another molecule of Q in the ground state. This may account for radical formation in the absence of other electron donors and is particularly pronounced for [MPTQ] ≥ 1mM. The semioxidized state, Q^++ is not observed, presumably due to its instability, and this perhaps be responsible for an irreversible photo-destruction of the sensitizer. Equation (8.4) is considered because the known values of the redox potentials of NAD^+ radical (-0.94 V vs. NHE at pH 7.0, (Land et al 1983) and MPTQ (-0.399 V vs. NHE in CH_3CN buffer, 1:1 v/v, pH 7.1), (Nohi and Jordin 1983) which make this reaction highly probable, especially under anaerobic conditions. This type of reduction by NAD radical was observed earlier for viologen which also has an one-electron reduction
potential close to or more negative than MPTQ, daunorubicin, dauxorubicin etc. (Land et al 1983; Anderson 1980; Forni et al 1986).

It is inferred that the kinetics decrease of the EPR signal of $Q^\cdot$ in the dark cannot be described either by a second order or a first order reaction over the whole period of observation (Svingen and Powis 1981; Houee-Levin et al 1985). However, the decay curve in the initial portion of the curve may be described by second order kinetics (Figure 8.4a). The steady-state concentration of $Q^\cdot$ radicals has been estimated using the known concentration of a stable nitroxide radicals as a standard. This shall permit the radical recombination (Equation 8.5) which is believed to be a primary mode of their reactions.

$$Q^- + Q^- + 2H+ \rightarrow Q + QH_2$$  \hspace{1cm} (8.5)

The rate constant is estimated to be $K_5 = 5.15 \times 10^2 \text{ M}^{-1}\text{s}^{-1}$ in DMF/buffer (1:1) mixture and $5.8 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ in buffer. The value found for the reaction 5 in aqueous solution is of the same order as was reported earlier by Kalyanranman et al (1984) where an enzymatic system was used to generate semiquinone.

The photosensitized reaction is particularly efficient in DMF/aqueous buffer mixtures. The DMF apparently stabilizes the radical anion $Q^-$. Recombination of the anion radicals (Equation 8.5) is much slower in DMF/buffer than in aqueous buffer which is in accordance with the values of the rate constants that have been obtained for these two solvent systems in the present work.
The role of the composition of the reaction medium on the EPR spectra of derived radicals has been described recently for daunorubicin (Schreiber et al 1989). The xanthine-xanthine oxidase enzymatic systems have used to produce the radicals in aqueous buffer and in buffer containing varying concentrations of DMSO or ethanol. Transformation of the EPR spectrum from low-resolution to high-resolution by radicals can be achieved by changing the medium from aqueous buffer to DMSO / buffer (1:1 v/v). It has been suggested that this is due to the increased solubility of the free radicals in such a solvent mixture. Results described here are in corroboration with the interpretation of Schreiber et al (1989). In aerated media, the predominant mode of decomposition of the semiquinones is their reaction with oxygen during which they undergo reoxidation by the characteristic redox cycling process, to the parent molecule Q, yielding the superoxide ion radical (Equation 8.6).

\[
Q^\cdot + O_2 \longrightarrow Q + O_2^\cdot \quad (8.6)
\]

It is also found in this study that the rate constant for this reaction \((k_6 \sim 10^8 \text{ M}^{-1} \text{s}^{-1})\) is several orders of higher magnitude (Farrington et al 1980; Anderson 1980) than for recombination of the radicals (Equation 8.5). Direct reaction between the excited sensitizer drug molecule, \(Q^\cdot\) and oxygen has also reported to contribute to superoxide (Equation 8.7).

\[
Q^\cdot + O_2 \longrightarrow Q^+ + O_2^\cdot \quad (8.7)
\]

As a result of the facile photosensitized production of \(Q^\cdot\) and \(\text{NAD}^\cdot\), efficient generation of \(O_2^\cdot\) is observed, particularly in aerated DMF/buffer mixture. Because the superoxide radicals mediate formation of \(H_2O_2\), the reaction concerted with reduction of Fe(III) complexes (by Fenton
reaction) yield highly reactive hydroxyl radicals (Lown and Chen 1981; Lown et al 1982) which are very likely to behave powerful photokilling redical species activated by PDA of MPTQ with MCF-7 cell line. Thus MPTQ seems to be efficient photosensitizer candidate and exerting maximum photocytocidal activity. Cytocidal property is correlated to the cumulative effect of chemical processes during the differential OFR generation.

8.5 CONCLUSION

Taking together, all chemical processes, the efficient cause of cell death (MCF-7 cell lines) with MPTQ in this work is the consequence of involving various mode and differential quantum of OFR (O$_2^\bullet$, H$_2$O$_2^\bullet$,OH) generation along with azidyl radicals (N$_3^-$) production (in case of sodium azide medium). This property attributes to the deleterious effect towards cell systems via bioreductive activation of the drug quinoline moiety and subsequent re-oxidation redox cycling (Kalyanaraman et al 1980).

Singlet oxygen is not involved in the photooxidation reactions since sodium azide does not affect superoxide formation. However, azide actively participates in the photosensitized reactions as have been demonstrated by detection of EPR spectrum of DMPO-N$_3$. Since neither SOD nor catalase hindered the production of the azidyl radical in the present study, it is inferred that O$_2^\bullet$ and H$_2$O$_2$ are not involved in the oxidation of N$_3$. Compiling all these observations imply that the N$_3^-$ radical can be produced in a direct reaction between Q$^\bullet$ (or Q$^+$) and N$_3^-$ process via Type I mechanism (electron transfer process).