Ascorbic Acid Monoglu coside as Antioxidant and Radioprotector

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Ascorbic acid monoglu coside (AsAG), a glucoside derivative of ascorbic acid, has been examined for its antioxidant and radioprotective abilities. AsAG neutralized 1, 1 diphenyl -2-picryl-hydrazyl (DPPH), a stable free radical in a concentration dependent manner thus indicating its antioxidant ability. AsAG protected mice liver tissues in vitro from peroxidative damage in lipids (measured as TBARS) resulting from 25Gy γ irradiation. It also protected plasmid pBR322 DNA from gamma-radiation induced strand breaks as evidenced from studies on agarose gel electrophoresis of the plasmid DNA after radiation exposure. Oral administration of AsAG to mice prior to whole body gamma radiation exposure (4Gy) resulted in a reduction of radiation induced lipid peroxides in the liver tissue indicating in vivo radiation protection of membranes. Pulse radiolysis studies indicated that AsAG offered radioprotection by scavenging free radicals. The rate constants for the reactions OH and N₂ radicals with AsAG were determined to be 6.4 × 10⁹ dm³ mol⁻¹ s⁻¹ and 2.3 × 10⁸ dm³ mol⁻¹ s⁻¹, respectively at pH 7. It was observed that AsAG radicals undergo conjugation as the pH of the solution is raised to 11 in the case of a one-electron oxidation reaction. As the OH⁻ radical adds to the ring, the conjugation effect starts appearing at pH 10.

INTRODUCTION

The deleterious effects of radiation to living cells are mediated by the generation of free radicals and reactive oxygen species that damages vital cellular targets such as DNA and membranes. Many compounds with antioxidant activities (ability to neutralize free radicals or their reactions) are proved to be effective radioprotectors.¹ Conversely in several novel approaches, the antioxidant effect has been utilized to demonstrate radioprotective properties. Radiation protecting drugs and compounds are of great importance owing to their potential application during planned radiation exposures such as radiotherapy, diagnostic scanning, undertaking cleanup operations in nuclear accidents, space expeditions etc and unplanned radiation exposures such as accidents in nuclear industry, nuclear terrorism, natural background radiation etc.²,³ Although a large number of compounds showed good radioprotection in in vitro studies, most of them failed in vivo application due to acute toxicity and side effects. Radioprotective compounds are important in clinical radiotherapy where normal tissues should be protected against radiation injury while cancers are exposed to higher doses of radiation to obtain better cancer control. There have been many attempts to find an ideal radioprotector that can preferentially protect normal tissues from radiation damage without affecting the sensitivity of cells of the tumour.⁴

Vitamin C (ascorbic acid) has been reported to be an effective antioxidant and free radical scavenger. Both under in vitro and in vivo conditions, it reduces oxidative and free radical induced damages to DNA and membranes in biological systems.⁵ It functions as a free radical scavenger of active and stable oxygen radicals. Vitamin C has been shown to protect several biological systems against ionizing radiations. The radioprotective effect of ascorbate seems to be due to its interactions with radiation induced free radicals.⁵ Ascorbic acid pre-treatment inhibited the radiation induced elevation in lipid peroxidation.⁶ It protected mice against radiation induced sickness, mortality and improves the healing of wounds after exposure to whole body γ radiation.⁷ In this paper we report the antioxidant and radioprotective properties of ascorbic acid-2-glucoside (AsAG), a glucoside derivative of ascorbic acid.
was incubated at ambient temperature with aqueous AsAG solutions of various concentrations and of Gadov was scavenging activity of AsAG was determined by the method prepared methanolic solution of DPPH (6.34 × 10−3 mol dm−3) was incubated with aqueous AsAG. Briefly, the reaction mixture contained 100 μl of liver homogenate (25%), 0.375% thiobarbituric acid, 0.025 N HCl, 15% trichloroacetic acid and 6.0 × 10−3 mol dm−3 EDTA. The reaction mixture was heated at 90°C for 30 minutes, cooled and centrifuged at 10,000 xg for 10 minutes. The amount of TBARS in the supernatant was estimated by measuring the absorption at 532 nm. The lipid peroxidation values are expressed as n moles of MDA per mg protein. 1, 1, 3, 3-tetraethoxypropane was used as the standard. Proteins were estimated with Lowry’s method.

In vivo studies
The animals were divided into three groups and treated as follows:
1. Sham irradiation
2. 4 Gy radiation + 0.1 ml water
3. 4 Gy radiation +100 mg/Kg AsAG in water

Irradiation: The animals were exposed to 4Gy whole body gamma-radiation at a dose rate of 1.88 Gy/min. AsAG was dissolved in water and administered per oral (p.o.) to mice 100 mg per kg body weight one hour before irradiation. Two hours after irradiation, the animals were sacrificed by cervical dislocation and the livers were excised. 25% liver homogenate was prepared and analyzed for the presence of TBARS following the method of Buege and Aust as described earlier.

Estimation of DNA damage
Radiation induced damage in DNA was determined by irradiating plasmid pBR322 (300 ng) at 25Gy in the presence and absence of AsAG. After irradiation, the supercoiled (ccc) and open circular (oc) forms of DNA were separated by agarose gel electrophoresis using 1% agarose gels in TBE buffer consisting of 89 × 10−3 mol dm−3 Tris Borate/ 2 × 10−3 mol dm−3 EDTA , pH 8.3. The gels were stained with ethidium bromide and DNA bands were photographed and analyzed. Radiation induced DNA damage was estimated in terms of decrease in the ccc form of DNA.

Pulse radiolysis studies
The pulse radiolysis set up consists of an electron linear accelerator (Forward Industries, England) capable of giving single pulses of 50 ns, 500 ns or 2 μs of 7 MeV electrons. The pulse irradiates the sample contained in a 1 cm × 1 cm
suprasil quartz cuvette kept at a distance of approximately 12 cm from the electron beam window, where the beam diameter is approximately 1 cm. The transient changes in the absorbance of the solution caused by the electron pulse are monitored with the help of a collimated light beam from a 450 W xenon arc lamp. The output from the PMT is fed through a DC offset circuit to the Y input of an L & T storage scope which can transfer 400 mega samples/sec on each input channel at 250 ns/div time base range with sensitivity of 5 mV/div and having a bandwidth of 100 MHz. Further details of the LINAC can be seen elsewhere.\(^{13}\) An aerated 10\(^{-2}\) mol dm\(^{-3}\) KSCN solution was used for dosimetry and (SCN)\(^{2-}\) radical was monitored at 475 nm. The absorbed dose per pulse was calculated\(^ {14}\) assuming \(G_\varepsilon [(SCN)_{2}^{2-}] = 2.6 \times 10^{-4}\) m\(^2\) J\(^{-1}\) at 475 nm. Pulses employed were of either 50 ns or 2 \(\mu\)s. The dose employed in the present study, unless otherwise stated, was typically 16 Gy per pulse.

**RESULTS**

**DPPH radical scavenging assay**

DPPH (1, 1 diphenyl-2-picryl-hydrazyl) is a stable free radical available commercially and having a characteristic absorption at 515 nm due to an unpaired electron. The DPPH radical reacts with compounds having antioxidant activity and gets reduced. The reduced form of DPPH is colorless. Hence the decrease in \(A_{515}\) is considered directly relate to the electron scavenging capacity of the antioxidant compounds. As can be seen in Fig. 1, AsAG could reduce the DPPH radical. Maximum electron scavenging activity of AsAG was observed at concentration of 0.5 \(\times\) \(10^{-3}\) mol dm\(^{-3}\) giving up to 64.59% inhibition.

**Analysis of in vitro membrane damage**

Exposure of the liver tissues to 25 Gy \(\gamma\)-irradiation resulted in the formation of 4.26 nano moles of TBARS/mg protein. Presence AsAG inhibited the radiation induced lipid peroxidation in the liver in a concentration dependent manner as illustrated in Fig. 2. At 1 \(\times\) \(10^{-3}\) mol dm\(^{-3}\) of AsAG, there was inhibition of lipid peroxidation up to 72%.

**Analysis of in vivo membrane damage**

The membrane damage was measured in terms of TBARS. As can be seen in Fig. 3 exposure of mice to whole body gamma radiation resulted in elevated levels of TBARS in liver tissue. Administration of AsAG (100 mg/kg body weight) prior to radiation exposure could inhibit this peroxidative damage in liver tissue. When calculated using the formula;
The effect of ionizing radiation on the water solvent is illustrated in equation 1. Radiolysis of water produces free radicals according to the stochiometry\(^\text{15,16}\):

\[
\text{H}_2\text{O} \rightarrow \text{H}^+ + \cdot \text{OH} + \cdot \text{H}_2\text{O} + \text{H}_2\text{O}^+ + \text{H}_3\text{O}^- + \text{e}_{\text{aq}}^- + \cdot \text{N}_2\text{O} + \cdot \text{H}_2\text{O} \rightarrow \text{N}_2 + \cdot \text{OH} + \text{OH}^- \quad (2)
\]

\[
k_2 = 9.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}
\]

\[
\text{H}^+ + \cdot \text{N}_2\text{O} \rightarrow \text{N}_2 + \cdot \text{OH} \quad (3)
\]

\[
k_3 = 2.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}
\]

Due to slow reaction for H\(^+\) scavenging, N\(_2\)O saturation ultimately doubles the amount of hydroxyl radicals by scavenging electrons.

When electron transfer conditions were desired, the azide radical was formed according to reaction (4)\(^\text{17}\):

\[
\text{N}_3^- + \cdot \text{OH} \rightarrow \cdot \text{N}_3 + \text{OH}^- \quad (4)
\]

\[
k_4 = 1.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}
\]

It is reported that ascorbic acid has pK\(_a\) at 4.19.\(^\text{18}\) To check that whether presence of glucose moiety has any affect on it we have determined the pK\(_a\) of AsAG. Fig. 5 shows the variation in optical absorbance at 250 nm with the change of pH.

| pH | O.D. at max |
|----|------------|
| 3  | 1.94       |
| 4  | 2.08       |
| 5  | 2.08       |
| 6  | 2.08       |
| 7  | 2.08       |
| 8  | 2.08       |
| 9  | 2.08       |
| 10 | 2.08       |
| 11 | 2.08       |
| 12 | 2.08       |

Fig. 4. Agarose gel electrophoresis pattern of pBR322 DNA exposed to gamma-radiation in presence and absence of 5 mol dm\(^{-3}\) AsAG. Lane 1: 25 Gy; Lane 2: 25 Gy, 5 mol dm\(^{-3}\) AsAG; Lane 3: 0 Gy, 5 mol dm\(^{-3}\) AsAG; Lane 4: 0 Gy.

Fig. 5. Variation in the optical absorbance at 250 nm with increase of pH for a solution containing 1.65 \(\times\) 10\(^{-4}\) mol dm\(^{-3}\) ASAG.

It can be noticed that above pH 4 optical absorbance increases drastically and then it levels at above pH 6. The pK\(_a\) calculated from the inflection point was found to be 4.05. Thus, it seems that presence of glucose moiety does not affect ascorbic acid pK\(_a\) drastically. All the work reported in this paper was carried out at pH higher than 6 to make sure that the parent compound exist in one form only.

Pulse radiolysis experiments were conducted at pH 7, 10 and 11 and the solutions were saturated with nitrous oxide gas. As the reaction between \'OH radical and AsAG was preceded, we observed an increase in the absorbance of the transient. The maximum absorbance of the transient formed on the microseconds time scale, is shown in Fig. 6.
It can be seen that the transient absorption spectrum shows two bands having $\lambda_{\text{max}}$ at 290 nm and 360 nm. The kinetics of the growth of the transients at different concentrations of the AsAG at 290 nm and 360 nm is shown in Fig 7.

Decay kinetics of the transient is shown in the inset of Fig. 7. It can be noticed that the rate for the formation kinetics at 290 and 360 nm increases with increase in the concentration of AsAG. The bimolecular rate constant for the formation of the transients was calculated by plotting the pseudo first order rate constant against the concentration of AsAG. The bimolecular rate constants obtained from the slope of the linear line are compiled in Table 1.

There was no change in the decay kinetics of the transient with the concentration of the concentration of the AsAG. The transients decay by second order kinetics and the rate constants are compiled in Table 2.

Hydroxyl radicals are electrophilic in nature. Therefore, they can add or cause one-electron oxidation reaction of the compounds. To confirm that the observed transient is due to addition or oxidation of the AsAG we carried out experiments with specific one-electron oxidants. Fig. 8 shows the transient absorption spectrum obtained on pulse irradiation of N$_2$O-saturated solution containing $2 \times 10^{-4}$ mol dm$^{-3}$ AsAG, $1 \times 10^{-2}$ mol dm$^{-3}$ N$_3^-$ at pH 7. The observed transient spectrum shows only one band with $\lambda_{\text{max}}$ at 290 nm. The kinetic profiles at different concentrations of AsAG are shown in the inset of Fig. 8.

**Table 1.** Kinetics and Absorption Characteristics of the Transient Produced by the Reactions of Various Free Radicals with AsAG

| Radicals | pH | Rate constant at 290 nm (dm$^3$ mol$^{-1}$ s$^{-1}$) | Rate constant at 360 nm (dm$^3$ mol$^{-1}$ s$^{-1}$) |
|----------|----|-------------------------------------------------|-------------------------------------------------|
| 'OH      | 7  | $6.4 \times 10^9$                               | $5.6 \times 10^9$                               |
| 'OH      | 10 | $4.5 \times 10^9$                               | Could not be analyzed                           |
| $O^-$    | 11 | $2.8 \times 10^9$                               | $5.3 \times 10^9$                               |
| N$_3^+$  | 7  | $2.3 \times 10^9$                               | $6.3 \times 10^9$                               |
| N$_3^+$  | 11 | $1.8 \times 10^9$                               | $6.3 \times 10^9$                               |

**Table 2.** Decay Rate Constant of the transients and Produced by the Reactions of Various Free Radicals with AsAG

| Radicals | pH | Second order Rate (2k/dl) at 290 nm (s$^{-1}$) | Rate constant at 360 nm (dm$^3$ mol$^{-1}$ s$^{-1}$) |
|----------|----|---------------------------------------------|-------------------------------------------------|
| 'OH      | 7  | $7.5 \times 10^4$                           | $1.0 \times 10^5$                               |
| 'OH      | 10 | Neither first nor second due to intramolecular transfer reaction | $1.0 \times 10^5$ |
| N$_3^+$  | 7  | $6 \times 10^4$                             | $6 \times 10^4$                                 |
| N$_3^+$  | 11 | $6 \times 10^4$                             | $6 \times 10^4$                                 |
The bimolecular rate constant and other spectral properties of the transients are compiled in Table 1. On comparing the transient absorption spectrum with that obtained on 'OH reaction (Fig. 6) it appears that N$_3^*$ radical produces distinctly one band. As N$_3^*$ radical is a specific one-electron oxidant it can be presume that the transient observed is due to the oxidation of AsAG. Thus, it seems that 'OH radical produces two different transients on reaction with ASAG presumably adduct and one-electron oxidized product of AsAG.

On comparing the results reported in literature for one-electron oxidation of ascorbic acid in aqueous solution it was noticed that the absorption spectrum obtained is significantly different. This could be due to the fact that in AsAG there is no conjugation as was possible in ascorbic acid due to the presence of two –OH groups at α-position.

To study the effect of conjugation on the transient absorption spectrum experiments were carried out at different pH’s 10.1 and 11. Typical case for pH 10.1 is shown in Fig. 9.

It can be seen that the time resolved spectrum shows different behavior as compared to that obtained at pH 7 (Fig. 6). As the radiolytic yield of the 'OH radical remains almost similar at pH 7 and 10, the increase in the yield of the transient having $\lambda_{\text{max}}$ at 360 nm band could be due to the intramolecular transformation due to the pK$_a$ of the cation radical. The bimolecular rate constant obtained at different pH’s for the reaction of 'OH radical with AsAG are compiled in Table 2.

To further confirm the pH effect one-electron oxidation reactions were carried out with N$_3^*$ radical at pH’s 11. It was observed that under identical conditions at pH 11 there was no significant change in the yield of the radical absorbing at 290 nm. This shows that the one-electron oxidized product is not having any pK$_a$ in this pH range. However, a marginal increase in absorbance at 360 nm was observed. This could be due to deprotonation at other site which might be leading some conjugation in the ring. The interesting observation is that 'OH radical due to the addition to the ring leads to conjugation at pH much lower than that observed for one-electron oxidation reaction. Therefore, a detailed study on the effect of pH on the absorbance yield of the transient formed due to the adduct formation by reaction of 'OH radical in the ring was carried out. The results are shown in Fig. 10.
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It can be seen that above pH 9 the yield of the transient starts increasing. For comparison the yield of the one-electron oxidized product obtained after reaction with N2\textsuperscript{+} radical is also plotted in the same Fig.. It is clear that the oxidized product does not show any significant variation in the yield up to pH 11.

**DISCUSSION**

Radiation-induced damages to cells and tissues involve generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which in turn cause alterations in DNA, membrane-lipids and proteins eventually leading to cellular dysfunction or cell death.\(^{22}\) It has been suggested that DNA damage by ROS leads to alteration and elimination of bases, formation of single and double stranded breaks resulting in cell cycle arrest and recruitment of DNA repair enzymes to rescue cells from the DNA damage.\(^{23}\) The radiation-induced cellular damage may be manifested as clonogenic cell death or alterations in cell- signaling cascades resulting in activation of responsive genes inducing apoptosis. Apart from DNA, another major target of radiation inactivation is the membranes of cytoplasmic organelles and plasma membrane.\(^{3}\) Oxidative damage to membrane is generally mediated by the degradation of phospholipids, which are the major constituents of membrane. Membrane lipids are easily peroxidised by ROS produced by ionizing radiation, causing structural and functional impairment of the membrane.\(^{21}\) Oxidative damage of membrane causes alterations in both lipid bilayer fluidity and permeability properties. Contributions of radiation induced oxidative damage to membranes as well as to DNA damage via ROS are complex and ultimately result in cytotoxicity and development of radiation induced deleterious cellular effects.

AsAG is a glucoside derivative of vitamin C. It has been shown that ascorbic acid is a potent antioxidant. In the present paper, we have shown that AsAG, a glucoside derivative of ascorbic acid is having antioxidant and free radical scavenging properties. AsAG inhibited stable DPPH free radical. The results of pulse radiolysis studies on reaction of AsAG with free radicals show that the reactivity of AsAG with free radicals is high and the results corroborates our findings done with DPPH radical. It also protected the membrane lipids from peroxidative damage induced by radiation. AsAG also protected pBR322 DNA from gamma-radiation induced strand breaks. The cellular membrane and DNA are the two main targets of radiation induced lethality and mutagenicity. The formation of lipid peroxides in the tissues is one of the markers of membrane damage due to γ-radiation exposure. The oral administration of AsAG protected the animals from undergoing the radiation induced lipid peroxidation in liver.

Ascorbic acid has been reported to have protective effect against radiation-induced mortality and cytogenetic damage.\(^{24}\) The radioprotective effect of ascorbic acid has been ascribed to its interactions with radiation-induced free radicals.\(^{2}\) The similar protective effect of AsAG and vitamin C against radiation induced mortality and cytogenetic damage indicate that addition of a glucose moiety to ascorbic acid does not alter its radioprotective ability. Instead, the structural modification may make the molecule readily available at the crucial sites. Further investigations are required to examine whether addition of glucose moiety has any effect on the bioavailability of ascorbic acid.

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