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Folic Acid as a Fenton-modulator: Possible Physiological Implication

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Abstract: Acting as a redox switch, folic acid (1) might be a promising iron modulator to protect cellular machinery against oxidative stress and iron overload. The vitamin 1 can directly control the iron concentration by oxidizing it even if present in chelated forms. In addition, during its role as a reducing agent for the biologically relevant reactive oxygen species (ROS), it furnishes 6-formyl pterin. This folate-derived intermediate possesses a stronger Fe^{2+}-oxidizing capacity than 1. Thus, compound 1 can reduce the iron toxicity in two ways. Although, the Fe^{2+}-oxidizing capacity is nullified in the presence of a strong biological reductant like ascorbic acid, this property may play a predominant role during pathogenesis when the cellular ascorbic acid levels deplete significantly. The iron-modulatory property of 1 was also confirmed with the L929 mouse fibroblast cell line.

INTRODUCTION

Folic acid (**1**) and its derivatives are essential cofactors for *de novo* synthesis of amino acids and nucleic acids and are also important for cell division. Folate deficiency is known to cause numerous genetic abnormalities, promote cardiovascular disease [1] and neural defects [2] *via* hyper-homocysteinemia and lead to carcinogenesis and apoptosis [3] due to the increased level of uracil. Its supplementation has been suggested as a preventive measure against these ailments [4,5]. Given that oxidative stress has been implicated for many of the diseases, we recently studied the antioxidant activity of **1** and found it to scavenge a diverse array of reactive oxygen species (ROS) efficiently [6]. This, in part, might account for its therapeutic potential.

It is well recognized that the essential element, iron potentiates oxygen toxicity causing enhancement of hepatocarcinoma occurrence [7], repurfusion injury [8], liver damage [9] *etc*. Proper regulation of iron assimilation would prevent oxidative stress and diseases related to iron overload. Evolution of new strategies for control of iron assimilation and metabolism, thus, has assumed great importance. To this end, exploration of natural dietary antioxidants as iron modulators is appealing because of their possible non-toxicity. The present study was specifically aimed at assessing the potential of **1** in controlling the iron-mediated oxidative reactions. The results, as discussed in this paper, clearly support for the first time, its positive role as an effective iron modulator *via* a mechanism involving oxidation of Fe^{2+} by **1** itself, or one of its oxidized metabolites.

RESULTS AND DISCUSSION

The Fenton mediated oxidation of 2-deoxyribose is a reliable method for assessing the capacity of a test compound in modulating cellular iron even at a very low concentration [10] and was extensively used in the present study. Fig. (**1**) shows the comparative protective activities of **1** and mannitol against Fe^{2+}-H_2O_2 mediated oxidation of 2-deoxyribose. Compound **1** could prevent the 2-deoxyribose

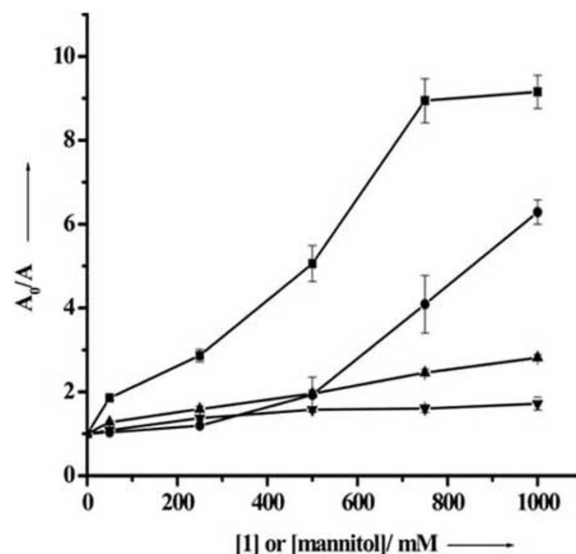

Fig. (1). Concentration dependent protective activities of **1** and mannitol against Fe^{2+} (80 μM)-H_2O_2 (200 μM) induced oxidation of 2-deoxyribose (2.8 mM) in KH_2PO_4-KOH (100 mM) buffer at pH 7.4 in the absence and presence of EDTA (100 μM). The reactions were conducted for 60 min. The concentrations of **1** and mannitol were x 10^{-3} and x 10^{-2} respectively. ■-**1** (with EDTA), ●-**1** (without EDTA), ▲-mannitol (with EDTA) and ▼-mannitol (without EDTA). Values are mean±S.E (n = 4).

oxidation concentration dependently, both in the presence and absence of a strong iron chelator, EDTA. The protective activity of **1** was better in the presence of EDTA than its absence. In the absence of EDTA, Fe^{2+} gets complexed with 2-deoxyribose, leading to the generation of

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·OH radicals in the vicinity of the detector molecule (2-deoxyribose). This causes extensive site-specific damage to 2-deoxyribose, as the reactive radicals are not efficiently intercepted by **1**. However, in Fe^{2+}-EDTA system, the ferrous ions are in the bulk of the medium and thus, the damage to 2-deoxyribose can only be caused by the diffusible ·OH radicals which are scavenged by **1**. This explains the better 2-deoxyribose protecting efficiency of **1** in the presence of EDTA. However, more significantly as shown in Fig. (**1**), compound **1** could inhibit the oxidative degradation of 2-deoxyribose at a fairly low concentration compared to those found with cellular water-soluble antioxidants [11,12]. Even at a ten-fold less concentration, the protective activity of **1** was much higher than that of mannitol, a pure ·OH radicals scavenger, irrespective of whether the iron was free or complexed. This was also apparent from the non-linear concentration dependence of its protective activity *vis-à-vis* that of mannitol. These results could not be explained by considering the ·OH radicals scavenging property of **1** alone, and revealed its direct role in modulating the ·OH radicals generating Fenton system. In principle, compound **1** can modulate the Fenton process *via* H_2O_2 scavenging, Fe^{2+} chelation and/ or Fe^{2+} oxidation. However, no iron chelation or H_2O_2 scavenging by **1** was evident from absorption spectroscopy and enzymatic assay [13] respectively. From this, a possible Fe^{2+} oxidizing role of **1** was envisaged.

This was also indicated in the time dependent study on the protective activity (Fig. (**2**)) of compound **1** against 2-deoxyribose oxidation by Fe^{3+}-EDTA-H_2O_2. The Fenton

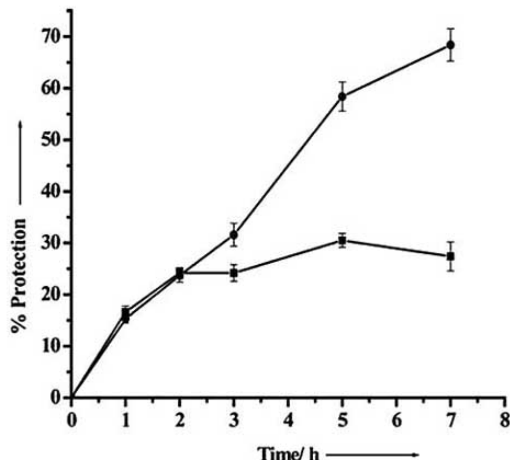


Fig. (2). Time dependent protective activities of **1** (250 μM) against $FeCl_3$ (20 μM)-EDTA (100 μM)-H_2O_2 (200 μM) induced oxidation of 2-deoxyribose (2.8 mM) in KH_2PO_4-KOH (10 mM) buffer at pH 7.4 in the absence and presence of ascorbic acid (300 μM). ●-without ascorbic acid, ■-in presence of ascorbic acid. Values are mean±S.E (n = 4).

system is known to generate Fe^{2+}-EDTA spontaneously *via* the intermediacy of a perferryl EDTA complex [14]. In this case when ascorbic acid was absent, the protective activity of **1** sharply increased to 16.6% in just 1 h and continued to increase over 2-7 h, reaching 68.4% in 7 h. The time dependent increase in the protective activity of **1** against Fenton-mediated 2-deoxyribose oxidation can only be explained by considering its role as an iron oxidant. It appears that compound **1** oxidizes the small amount of Fe^{2+} that is produced by the chosen Fenton system. This reduces the concentration of ·OH radicals, causing less oxidation of 2-deoxyribose. Based on the above argument, presence of a strong reductant such as ascorbic acid would overwhelm the iron oxidizing power of **1** resulting in its lesser protective efficacy. It was indeed so, although the amount of protection was still appreciable and remained constant (~27%) over the entire period of study (up to 7 h). Addition of ascorbic acid 5 h after the incubation also provided the same amount of protection up to 7 h (data not shown). The 2-deoxyribose protective activity of **1** in the presence of ascorbic acid might be primarily by radical scavenging. The potential of **1** to oxidize Fe^{2+} was confirmed by carrying out the time dependent ferrozine assay [15] using three different concentrations (100, 250 and 500 μM) of **1**. The assay results are shown in Fig. (**3**). It was found that the oxidation of Fe^{2+} was manifold faster in the presence of **1** (250 μM) than in its absence, and was accelerated more with a higher concentration of **1** (500 μM).

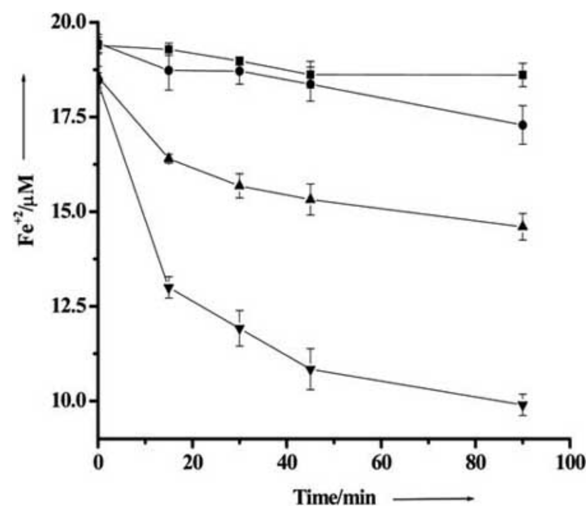


Fig. (3). Time course of Fe^{2+} (20 μM) oxidation in presence and absence of varying amounts of **1** as measured by ferrozine (600 μM) complexation assay. ■-without **1**, ●-in presence of **1** (100 μM), ▲-in presence of **1** (250 μM), ▼-in presence of **1** (500 μM). Values are mean±S.E (n = 4).

It is well known that the pterin moiety of **1** is susceptible to reduction by various reducing agents [16]. Thus, its role as a Fe^{2+}-oxidant is anticipated. This will produce a reduced folate derivative, which was envisaged to be 7,8-dihydrofolic acid (**2**). For its identification, the compound was prepared by reducing **1** with Fe^{2+} as well as with e_{aq}^- generated by γ-radiolysis of H_2O, both furnishing the same product. Comparison of its NMR spectral data with those of an authentic sample of **2** synthesized by a known procedure [17] confirmed its identity

Thus, overall, the Fe^{2+} oxidizing power of **1** coupled with its ·OH radicals scavenging activity appeared to partly explain the results obtained so far. However, given that the

cellular Fe^{2+} concentration might be quite high (especially in case of iron overload), the efficacy of the Fe^{2+}-oxidizing potential of **1** was assessed as a function of Fe^{2+} concentration. The results are shown in Fig. (**4**).

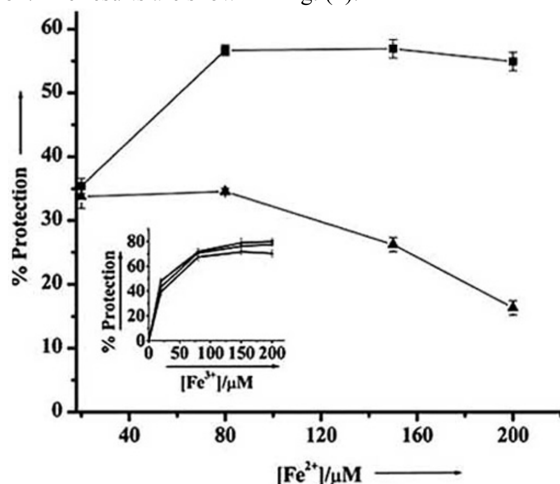


Fig. (4). Dependence of Fe^{2+} concentration on oxidation of 2-deoxyribose (2.8 mM) induced by Fenton reagent [Fe^{2+} (20-200 μM)-EDTA (250 μM)-H_2O_2 (200 μM)] in KH_2PO_4-KOH (100 mM) buffer at pH 7.4 in the presence of **1** (150 μM) after 60 min incubation. ■-in absence of ascorbic acid, ▲-in presence of ascorbic acid (300 μM). Inset: Dependence of Fe^{3+} concentration (20-200 μM) on 2-deoxyribose (2.8 mM) oxidation in absence of ascorbic acid after 150 min incubation, other conditions as above. ■-250 μM **1**, ●-150 μM **1**, ▲-50 μM **1**. Values are mean$\pm$S.E (n = 5).

Under normal circumstances, increasing the Fe^{2+} concentration would result in the formation of higher amounts of the ·OH radicals, which, in turn, should fuel up the 2-deoxyribose oxidation. However, as shown in Fig. (**4**), the opposite happened in the present case. In the absence of ascorbic acid, the protective activity of **1** (150 μM) increased sharply with the increase in concentration of Fe^{2+} up to 80 μM and thereafter, remained steady. Even considering its role as an ·OH scavenger and a Fe^{2+}-oxidant, the 2-deoxyribose protecting activity of a fixed concentration of **1** is anticipated to decrease gradually or at best, remain constant under the experimental conditions. Thus, the result in Fig. (**4**) suggested the involvement of another intermediate as an iron oxidant, generated from **1** possibly by an oxidative process.

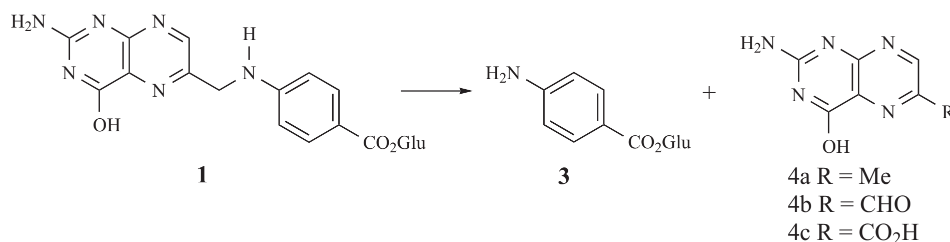
A careful analysis of the data of Figs. (**2**) and (**3**) also pointed to this. The time dependent protection offered by **1** against Fenton-mediated 2-deoxyribose oxidation was much higher than that accountable by the rate of **1**-induced oxidation of Fe^{2+}. The results also indicated that the oxidized metabolite of **1** might be even a better oxidant for Fe^{2+} than **1** itself.

Oxidative degradation of **1** in biological systems is well documented and is known to involve superoxide radicals ($O_2^{\cdot-}$) [18,19] or a ferryl-hydroxo (Fe^{4+}-OH) complex [20,21] as the oxidant. The reaction proceeds *via* the cleavage of its C9-N10 bond furnishing the amine **3** and 6-substituted pterins **4a-c** (predominantly **4b**) as the principal products (Scheme **1**). Very recently, we have demonstrated [22] that the ·OH radicals, generated by the Fenton reaction or an iron-independent radiolytic process, can also cause oxidative cleavage of **1** leading to the formations of the amine **3** and the aldehyde **4b**. Further, the ·OH radicals-induced cleavage was more efficient than that caused by the $O_2^{\cdot-}$ radicals. Although the important biochemical process has been known since long, the physiological implication of the process is still not clear.

Between the oxidized products of **1**, compound **4b** was envisaged to show the redox effect. A progressive increase in Fe^{2+} concentration would trigger the generation of ·OH radicals, which, in turn, would gradually build up the concentration of **4b**. Compound **4b**, thus generated, can oxidize the pool Fe^{2+}, retarding the 2-deoxyribose oxidation. This might explain the results shown in Fig. (**4**). The positive influence of increasing the concentration of Fe^{2+} was, however, offset by ascorbic acid as its reducing capacity possibly outweighed the oxidizing capacity of **4b**.

The effect of varying concentrations of Fe^{3+} on the protective activity of **1** (150 μM) was also similar and increased from 48.06-79.9% on increasing the Fe^{3+} concentration from 20 to 200 μM (Fig. (**4**) inset). Evidently, in this case, the ·OH radicals generated *via* the perferryl intermediate [14] furnished **4b** from **1**, accounting for the above effect. The studies with both Fe^{2+} and Fe^{3+} were carried out using three different concentrations of **1** with similar results (full data not shown). Given that the concentrations of iron used in this study were higher than those at cellular level, we used a higher concentration of **1**. However, at a lower iron concentration, compound **1** should play a similar role at a physiologically relevant concentration.

All these experiments were carried out with Fe^{3+}/Fe^{2+}-EDTA to mimic biological systems where iron is present as its complexes. The ferrozine assay [15] used for establishing



Scheme (1). Oxidative degradation reaction of **1**.

the role of **1** as a Fe^{2+}-oxidant was unsuitable under the experimental conditions, where a strong iron chelator such as EDTA is present. Hence, it was necessary to ensure the oxidizing capacity of **1** and **4b** for the chelated Fe^{2+}. Considering the Fenton reaction, gradual addition of Fe^{2+}-EDTA to a fixed concentration of H_2O_2 would normally deplete the level of the latter. However, if **1** acts as an oxidant even for chelated Fe^{2+}, the extent of H_2O_2 depletion should be less in its presence. Thus, the oxidizing capacity of **1** for chelated Fe^{2+} could be assessed by estimating the H_2O_2 concentration [13].

Measurement of the concentrations H_2O_2 as a function of added Fe^{2+} in the presence and absence of **1** revealed (Fig. (**5**) and inset) that compound **1** not only prevented the consumption of H_2O_2 but in fact, protected it better with increasing concentrations of Fe^{2+} (>20 μM). Given that **1** is unreactive to H_2O_2, the result conclusively established the oxidizing power of **1** even for chelated Fe^{2+}. The amount of protection was maximum (86.94%) with 200 μM Fe^{2+} and almost negligible (6.24%) when the Fe^{2+} concentration was 20 μM. The better H_2O_2 protective capacity of **1** with increasing concentrations of Fe^{2+} also revealed the intermediacy of the superior Fe^{2+}-oxidant **4b** as proposed earlier. As mentioned earlier, increasing concentration of Fe^{2+} would produce higher amount of **4b**, which would oxidize the pool Fe^{2+}, thereby protecting H_2O_2.

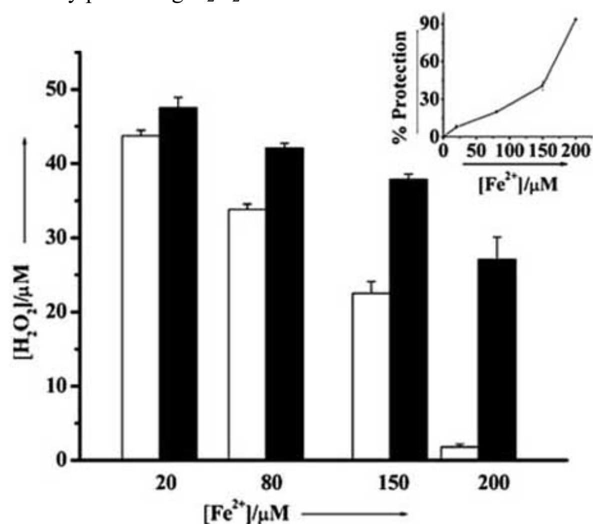


Fig. (5). Dependence of the status of H_2O_2 on Fe^{2+} concentration in the Fenton reagent in KH_2PO_4-KOH (10 mM) buffer at pH 7.4. □-without **1**, ■-in presence of **1** (250 μM). Inset: Replot of the above data showing the % protection of H_2O_2 by **1**. Values are mean±S.E (n = 5).

Finally, the direct evidence of the oxidative role of **4b** was obtained as follows. The product mixture obtained at different time intervals after γ-radiolysis of **1** in N_2O-saturated aqueous solution (up to 8 h) was added to a stock solution of Fe^{2+} and its concentrations were estimated by the ferrozine assay. As seen in the Fig. (**6**), with increasing irradiation period, a sharp decrease in the concentrations of Fe^{2+} was evident indicating that the product **4b** acted as an efficient oxidant for the ion. A replot of the data (Fig. (**6**), inset) for the percentage of oxidized Fe^{2+} revealed that in an unirradiated sample (when **1** alone was the Fe^{2+}-oxidant), the concentration of Fe^{3+} was merely ~5%. However, within 0.5 h of irradiation, when a small amount of **4b** was built up, the Fe^{3+} concentration shot up sharply to 20%. In 5 h, most (90%) of the Fe^{2+} was oxidized indicating a stronger iron oxidizing power of **4b** than that of **1**. The presence of an additional reducible function (CHO) in **4b** may partly explain its enhanced oxidizing capacity.

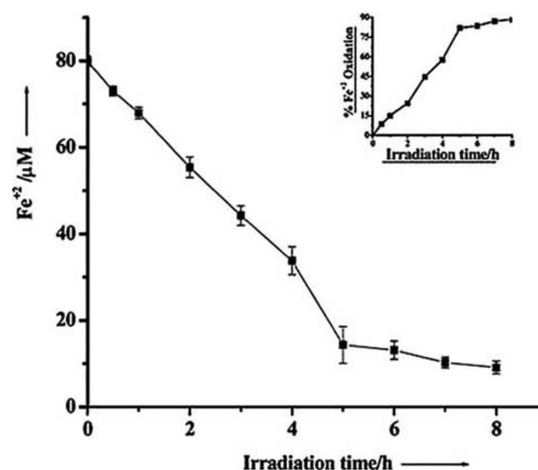
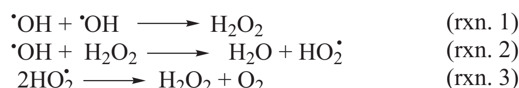


Fig. (6). Residual concentrations of Fe^{2+} (80 μM, final concentration) in the presence of increasing amounts of **4b** produced during oxidation of **1** (250 μM, final concentration) by radiolytically generated $^{\bullet}OH$. The incubation was carried out up to 5 min. Inset: Replot of the above data showing the % oxidation of Fe^{2+} by **4b**. Values are mean±S.E (n = 4).

The above radiolysis experiment is expected to produce a large amount of H_2O_2 which can also oxidize the Fe^{2+}. Hence, it was essential to assess the contribution of H_2O_2 (if any) in the Fe^{2+}-oxidation. The formation and decomposition of H_2O_2 in a system involving γ-radiolysis of N_2O-saturated (2.5 mM) aqueous solution can be represented by the reactions shown in Scheme **2**. Amongst these, the contribution of reaction 1 would be maximum due to its very high rate constant (10^{10} M^{-1} s^{-1}), while those of the reactions 2 and 3 with considerably low rate constants would be much less. From practical purpose, these may be safely ignored.



Scheme (2). Formation and depletion of H_2O_2 during γ-radiolysis of N_2O-saturated aqueous solution.

After 2 h, the γ-radiolysis at a dose rate of 560 Gy/h would theoretically generate a maximum of 82 µM of H_2O_2. However, during this period, the depletion of the N_2O level would result in lesser production of the $^{\cdot}OH$ radicals. Hence the actual concentration of H_2O_2 would be much less than the theoretical value. Further, H_2O_2 is also known [23] to decompose to O_2 and H_2O ($G(O_2) \propto [H_2O_2]^{0.5}$ (dose rate)$^{-0.5}$)

and this reaction will also be significant with the increase in the H_2O_2 concentration. Overall, in absence of any additional $^{\cdot}OH$ radicals scavenger, there would be a marginal build-up of H_2O_2. We also observed the same in a blank time dependent experiment carried out with the buffer in the absence of compound **1**. The radiolysis led to a gradual increase in the concentration (~15 μM) of H_2O_2 upto 2 h (data not shown).

Recently, we have reported [6] that like its dimerization (reaction 1), the $^{\cdot}OH$ radicals also react with compound **1** at a diffusion controlled rate. Hence, in presence of a large excess of compound **1**, its reaction with the vitamin would be almost exclusive. This was also confirmed in the present study. The radiolysis of an aqueous solution of **1** resulted in an insignificant amount of H_2O_2. This established direct oxidation of Fe^{2+} by compound **4b**. Initially, the radiolysis would result in an increased production of **4b**, which, in turn, would fuel-up Fe^{2+} oxidation. However, with the progress of time, the concentration of the $^{\cdot}OH$ radicals would reduce due to the depletion of N_2O, attaining a limiting level. This would also restrict the build-up of **4b** and its concentration would reach a steady, but limiting value. This is also evident from our results in Fig. (**6**), which shows no significant depletion of the Fe^{2+} concentration above 5 h.

The Fe^{2+}-oxidizing role of compound **4b** was further confirmed by isolating the radiolyzed products of **1** and assessing its potential for Fe^{2+} oxidation. For this, samples of **4b** produced after 2 and 4 h of radiolysis were isolated and added to the stock solution of Fe^{2+}. The ferrozine assay revealed that 13.8 and 43.1% of the Fe^{2+} were oxidized by the above samples (data not shown).

Further, the efficacy of compound **1** as a Fenton modulator was tested in a cellular system. To this end, we have chosen H_2O_2 as an oxidant to kill L929 mouse fibroblast cell line, where the killing of cells are known to be induced by iron induced Fenton reactions [24]. The cytotoxic effect of H_2O_2 on the proliferation of L929 cells in the presence and absence of **1** was assessed by the MTT assay. Cells were grown in a folic acid-deficient medium or medium containing (3, 10 and 50 μM of compound **1**) for 24 h to prime the cells as folic acid deficient or enriched. The concentration of 3 and 10 μM of **1** were chosen as these are the concentrations used normally in cell culture media (RPMI and DMEM cell culture media), while a higher concentration (50 μM) was used for comparing the concentration dependent protection. The L929 cells grown under the above conditions were washed, challenged with H_2O_2 (50 μM) for 30 or 60 min in phosphate buffered saline (PBS) and subsequently grown using a normal medium containing 3 μM of **1**. After 48 h of incubation, the cell deaths, in each case were assessed. As shown in the Fig. (**7**), the addition of H_2O_2 (50 μM) reduced the cell viability in a time-dependent manner (81 and 71% survival after 30 and 60 min exposure to H_2O_2 respectively). However, the L929 cells enriched with 3, 10 and 50 μM of **1** for 24 h showed higher survival response (93-98%) towards H_2O_2 exposure. The increased cell survival due to the presence of **1** was independent of its concentrations. This indicated that the offered protection is attributable to a stable intracellular folate pool irrespective of extra cellular concentration of **1**. Although, **1** exists in several other forms in cells, a small pool of folate exists in the native form. This along with its oxidative degradation product **4b** can protect cellular system from oxidative stress by scavenging radicals and modulating iron induced Fenton system.

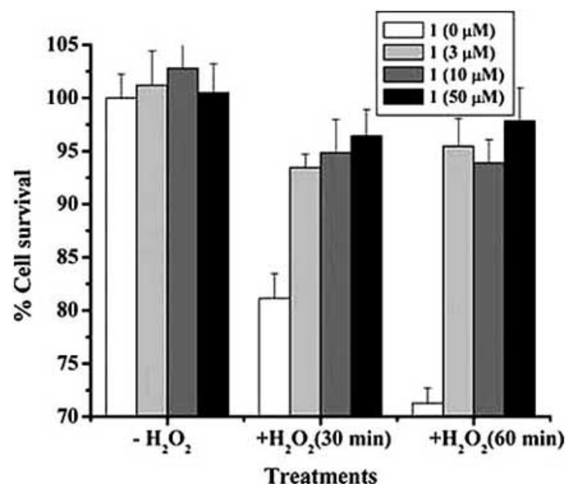
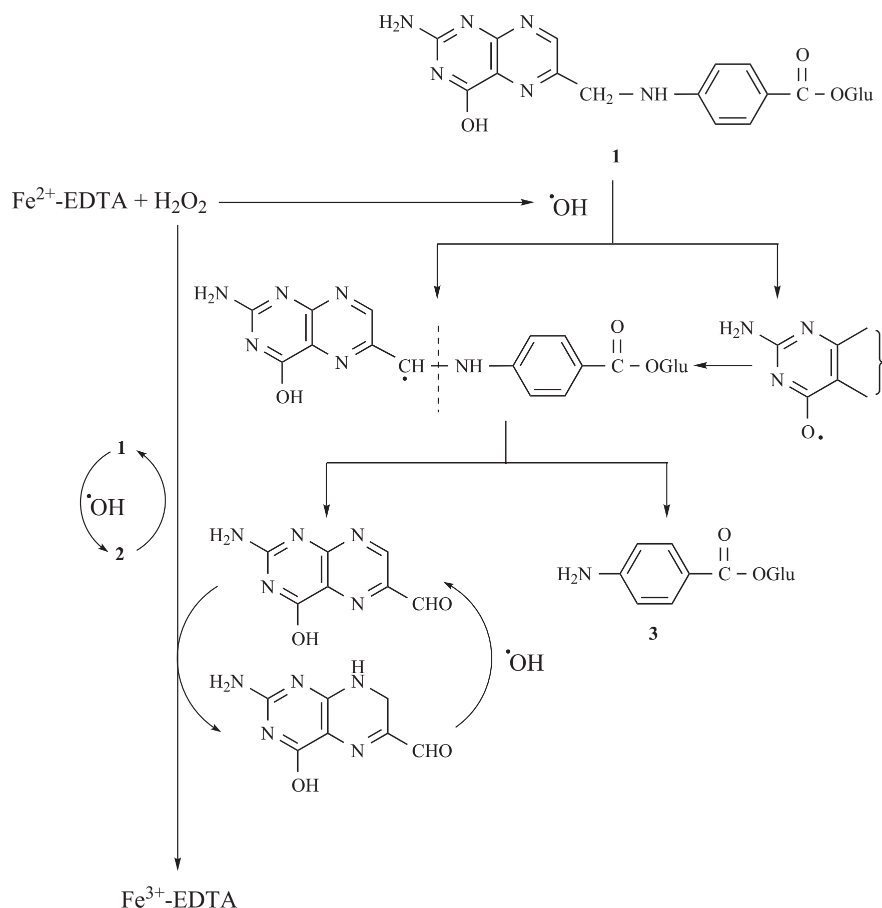


Fig. (7). H_2O_2 induced cytotoxicity of folic acid-deficient or enriched L929 mouse fibroblast cells. Cells were grown in folic acid deficient or enriched media for 24 h, challenged with H_2O_2 (50 μM) for 30 or 60 min and regrown in normal medium (RPMI and 10 % FCS containing 3 μM of **1**) for 48 h. Cell survival was measured by the MTT assay. Values are mean±S.E (n = 4).

CONCLUSION

The present study revealed a switchable redox action of **1**, which makes it a promising and effective biological iron-modulator. In a cyclic pathway, it can oxidize both free and chelated Fe^{2+}, and the reduced product **2** formed in this reaction can easily regenerate [17] **1**. On the other hand, its reducing action as a ROS scavenger generates a pterine metabolite **4b** which is a stronger oxidant for Fe^{2+} than its progenitor, **1** (Scheme **3**) and hence, can act as an efficient Fenton-modulator. The data obtained with the cell lines also confirmed the iron-modulatory property of **1**. Currently, we are carrying out further studies with the other forms (dihyrofolate, tetrahydrofolate, 5-methyl tetrahyrofolate etc) of **1** and its metabolites like **4b** to provide a conclusive evidence of antioxidative mechanism of folates. However, the biological implication of the present results is manifold. A key point to emerge is that **1** may serve a hitherto overlooked function as a strong agent to minimize $^{\cdot}OH$ induced damaging process, by acting more efficiently on the chelated form of iron pool. Dietary inclusion of **1** can, therefore, be envisaged as a part of defense machinery, which set into motion to counteract threatening consequences of oxidative stress and iron overload. Given that the concentrations of the cellular reductants including ascorbic acid deplete significantly during pathogenesis [25] the suggested Fe^{2+} oxidative action of **1** is justified under these conditions. The above findings may partly explain the increased need of folic acid supplementation in case of iron-overload diseases. Besides some established biochemical mechanisms, the iron oxidizing action of **1** and its oxidative metabolite **4b**, as revealed in the study, might also account for the reported beneficial action of **1** in iron overload diseases.



Scheme (3). Pathways of Fenton-modulatory properties of **1**.

MATERIALS AND METHODS

Materials

Ascorbic acid, ferrous ammonium sulfate ($(NH_4)_2Fe(SO_4)_2.6H_2O$), 2-thiobarbituric acid (TBA), 2-deoxyribose and phenol red were obtained from Himedia Lab. Pvt. Ltd., India. H_2O_2 (35%) was purchased from Lancaster, England, while horse radish peroxidase (HRPO, sp. Act. 350-500 U/mg protein) was from SRL, India. Other materials used were EDTA (Sarabhai Chemicals, India), $FeCl_3$, trichloroacetic acid (both from Thomas Baker, India), ferrozine, fetal calf serum, glutamine, penicillin, streptomycin sulfate, trypan blue, trypsin, RPMI 1640 cell culture medium (all from Sigma), KH_2PO_4 and KOH (both from BDH, UK). L929 (mouse fibroblasts) cell lines were obtained from National Centre for Cell Science (NCCS), Pune (India).

Stock solutions of ferrous ammonium sulfate and EDTA were freshly prepared in water. Stock solution of 1% (w/v) TBA were prepared in 50 mM NaOH and used within one week. All solutions were made in triply distilled water. High purity N_2O, from British Oxygen Corporation India Pvt. Ltd. was used for all radiolytic experiments.

Deoxyribose Assay with Fe^{3+}

The reaction mixture (1 mL) contained 2-deoxyribose (2.8 mM), $FeCl_3$ (20 μM), EDTA (100 μM) [EDTA and $FeCl_3$ were mixed prior to the addition of 2-deoxyribose], H_2O_2 (200 μM) and **1** (250 μM) in KH_2PO_4-KOH buffer (pH 7.4, 10 mM). After incubating the mixture at 37 ^{0}C for 1-7 h in absence or presence of ascorbic acid (300 μM), a solution of TBA in 50 mM NaOH (1 mL, 1% w/v) and trichloroacetic acid (1 mL, 2.8% w/v aqueous solution) was added. The reaction mixture was heated for 15 min on a boiling water bath and the amount of chromogen produced was measured [10] spectrophotometrically from the absorption at 532 nm.

For studying the dependence of Fe^{3+} concentration on the protection of 2-deoxyribose, the incubation was carried out for 150 min in the presence of $FeCl_3$ (20-200 μM).

Deoxyribose Assay with Fe^{2+}

The reaction was carried out as above, except for using $(NH_4)_2Fe(SO_4)_2.6H_2O$ (80 μM) in place of $FeCl_3$ and incubating the mixture for 60 min. For the concentration dependent studies, experiments were also carried out using varying

amounts of **1** and mannitol (0-1000 µM). In some experiments EDTA was omitted. For studying the dependence of Fe^{2+} concentration on the protection of 2-deoxyribose, the incubation was carried out in the presence of varying amounts of Fe^{2+} (20-200 µM).

H_20_2 Assay in Fenton System

The assay was carried out with minor modification of a reported procedure [13]. To a reaction mixture (0.8 mL) containing KH_2PO_4-KOH (10 mM) pH 7.4 buffer, **1** (250 µM) and H_2O_2 (60 µM), were added a premixed solution of $(NH_4)_2Fe(SO_4)_2.6H_2O$ (0-200 µM) and EDTA (250 µM). After incubating the mixture at 37 ^{0}C for 30 min, a solution (0.2 mL) containing NaCl (140 mM), dextrose (5.5 mM), phenol red (0.28 mM) and HRPO (8.5 U/mL) was added and the mixture incubated for 5 min at room temperature. Subsequently aqueous NaOH (10 µL, 1N) was added to the mixture and the absorbance of the chromogen was read at 610 nm against the appropriate blank. The concentration of H_2O_2 stock solution was calculated from its absorbance at 230 nm, using an extinction coefficient of 81 $M^{-1}cm^{-1}$.

Ferrozine Assay for Oxidation of Fe^{2+} by 1 or 4b

The reaction mixture (1.0 mL) containing $(NH_4)_2Fe(SO_4)_2$. $6H_2O$ (20 µM) and **1** (0-500 µM) was incubated at room temperature for 30 min. After addition of ferrozine (600 µM), formation of Fe^{2+}-ferrozine complex was measured at 562 nm [15]. The Fe^{+2} concentrations at various time intervals were quantified from a standard curve of the absorbancce (562 nm) of the Fe^{+2}-ferrozine complex.

In a similar manner, the oxidation of Fe^{2+} by **4b** was studied by mixing equal volumes of $(NH_4)_2Fe(SO_4)_2.6H_2O$ and **4b** [obtained by irradiation of **1** (500 *m*M) for 0-8 h in N_2O purged aqueous solution]. The final concentrations of Fe^{2+} and the irradiated folate mixture in the solution were 80 and 250 µM respectively. In this case, the incubation was carried out at room temperature for 5 min.

Iron Chelation Study

An aqueous solution of **1** (100 µM) and varying amounts of ferrous ammonium sulfate (0-200 µM) or $FeCl_3$ (0-200 µM) was incubated at room temperature for 10 min and the absorption spectra (190-600 nm) were recorded.

Cell Culture Study

The L929 cells were routinely seeded at a density of 0.1-3 x 10^6 cells/mL and grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 mU/mL penicillin, and 100 mg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37 ^{o}C. Cells were passaged every 3-4 d to maintain the cell density below 0.4 x 10^6 cells/mL. The cell density and viability were determined by the trypan blue dye exclusion assay. Subcultures were obtained by trypsinization (0.25% trypsin in PBS).

Assay of H_2O_2 Induced Cytotoxicity in Presence and Absence of Compound 1

The cell viability was determined by the conventional MTT dye reduction assay [26]. The L929 cells (2x10^4 cells per well) were grown in 96-well microtiter plates for 24 h in a folic acid-deficient RPMI solution containing 10% dialyzed fetal calf serum and folic acid (0, 3, 10 or 50 µM). After 24 h, the medium was removed, the cells were washed with PBS and 200 µL of PBS containing folic acid (0, 3, 10 or 50 µM) was added followed by 20 µL of H_2O_2 (0 or 50 µM in PBS). The plates were incubated at 37 ^{o}C for 30 or 60 min. After removing PBS solution, RPMI solution (200 µL) containing dialyzed fetal calf serum (10%) and folic acid (3 µM) was added to all the wells. After incubation for 48 h, the medium was removed by aspiration, the cells were washed twice with PBS, and MTT solution (0.5 mg/mL, 100 µL) in PBS was added to each well. After 4 h, SDS solution (10% SDS in 0.01 M HCl, 100 µL) was added and kept at 37 ^{o}C for another 12 h. The absorbance at 560 nm was read with a spectrophotometric plate reader.

REFERENCES

[1] Doshi, S. N.; McDowell, I. F.; Moat, S. J.; Lang, D.; Newcombe, R. G.; Kredan, M. B.; Lewis, M. J.; Goodfellow, J. *Arterioscler Thromb Vasc. Biol*., **2001**, *21*, 1196.

[2] MRC Vitamin Study Research Group. *Lancet*, **1991**, *338*, 131.

[3] Pogribny, I. P.; Muskhelishvili, L.; Miller, B. J.; James, S. J. *Carcinogenesis*, **1997**, *18*, 2071.

[4] Lynn, B. B. ed. *Folate in health and disease*. series volume 1, Mercel Dekker: New York, 1995.

[5] Kim, Y. -I. *Nutr. Rev*., **1999**, *57*, 314.

[6] Joshi, R.; Adhikari, S.; Patro, B. S.; Chattopadhyay, S.; Mukherjee, T. *Free Rad. Biol. Med*., **2001**, *30*, 1390.

[7] Britton, R. S.; Bacon, B. R.; Tavill, A. S. In *Iron metabolism in health and disease*, J. H. Brock; J. W. Halliday; M. J. Pippard; L. W. Powell, Eds.; W. B. Saunders: London, **1994**, pp. 311-351.

[8] Hershko, C. In *Iron metabolism in health and disease*, J. H. Brock; J. W. Halliday; M. J. Pippard; L. W. Powell, Eds.; W. B. Saunders: London, **1994**, pp. 391- 426.

[9] Britton, R. S.; Ferrali, M.; Magiera, C. J.; Recknagel, R. O.; Bacon, B. R. *Hepatology*, **1990**, *11*, 1038.

[10] Halliwell, B.; Gutteridge, J. M. C.; Aruoma, O. I. *Anal. Biochem*., **1987**, *165*, 215.

[11] Klein, S. M.; Cohen, G.; Coderbaum, A. I. *Biochem*., **1981**, *20*, 6006.

[12] Schulman, H. M.; Hermes-Lima, M.; Wang, E. M.; Ponka, P. *Redox Rep*., **1995**, *1*, 373.

[13] Pick, E.; Keisari, Y. *J. Immunological Meth*., **1980**, *38*, 161.

[14] Gutteridge, J. M. C.; Bannister, J. V. *Biochem. J*., **1986**, *234*, 225.

[15] Carter, P. *Anal. Biochem*., **1971**, *40*, 450.

[16] Moorthy, P. N.; Hayon, E. *J. Org. Chem*., **1976**, *41*, 1607.

[17] O'Dell, B. L.; Vandenbelt, J. M.; Bloom, E. S.; Pfiffner, J. J. *J. Am. Chem. Soc*., **1947**, *69*, 250.

[18] Shaw, S.; Jayatilleke, E.; Herbert, V.; Colman, N. *Biochem*., **1989**, *257*, 277.

[19] Vasquez-Vivar, J.; Whitsett, J.; Martasek, P.; Hogg, N.; Kalyanaraman, B. *Free Radic. Biol. Med*., **2001**, *31*, 975.

[20] Taher, M. M.; Lakshmaiah, N. *Arch. Biochem. Biophys*., **1987**, *257*, 100.

[21] Innocentini, L. H.; Duran, N. *Braz. J. Med. Biol. Res*., **1982**, *15*, 11.

[22] Patro, B. S.; Adhikari, S.; Mukherjee, T. Chattopadhyay, S. *Bioorg. Med. Chem. Lett*., **2005**, *15*, 67.

[23] Bansal, K. M.; Freeman, G. R. *Rad. Res. Rev*., **1971**, *3*, 209.

[24] Lomonosova, E. E.; Kirsch, M.; De Groot, H. *Free Radic Biol Med.,* **1998**, *25*, 493.

[25] Youdim, K. A.; Joseph, J. A. *Free Radic. Biol. Med*., **2001**, *30*, 583.

[26] Mosmann, T. *J. Immunol. Meth.,* **1983**, *65*, 55.

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